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On the Cover

Grant Wood (1891–1942), *Spring in the Country,* 1941.
Oil on Masonite, 24 in x 22 1/8 in / 60.9 cm x 55.9 cm. Courtesy of the Cedar Rapids Museum of Art, Cedar Rapids, Iowa; Museum Purchase. 93.12

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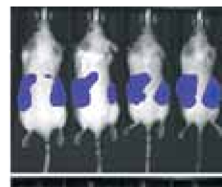

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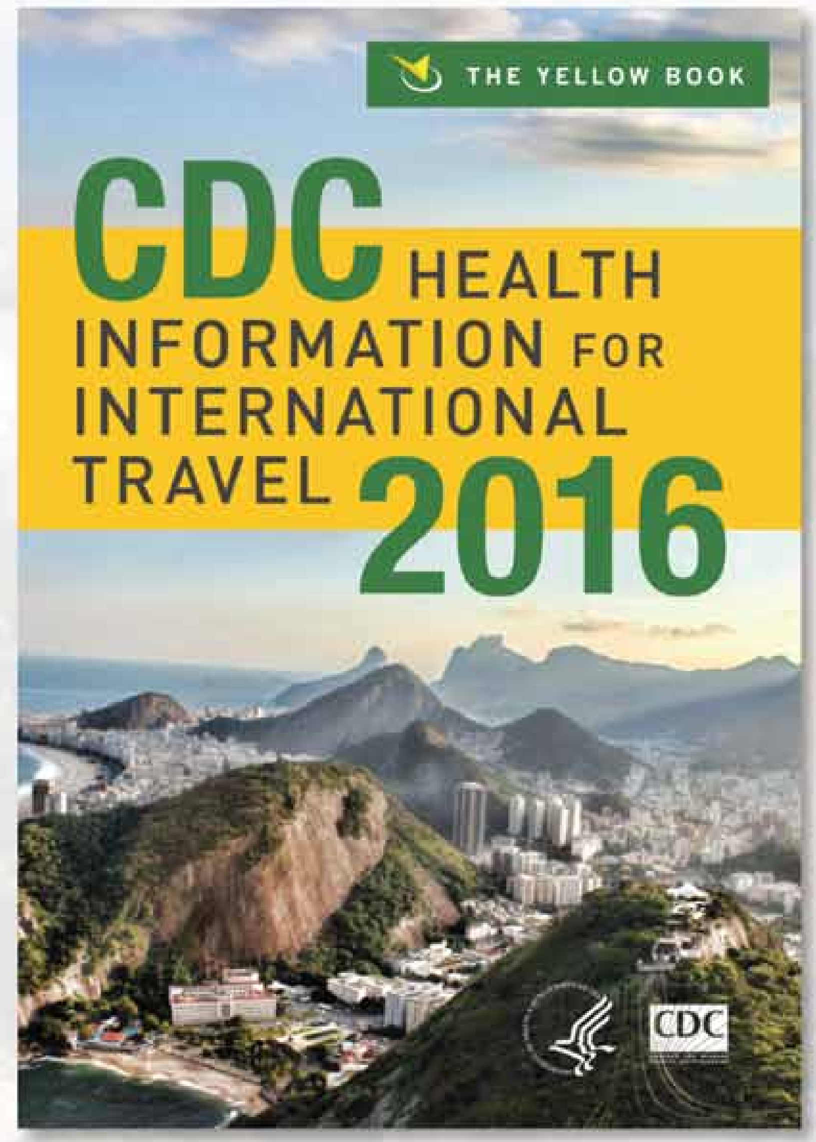

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PERSPECTIVE

Determinants and Drivers of Infectious Disease Threat Events in Europe

Jan C. Semenza, Elisabet Lindgren, Laszlo Balkanyi, Laura Espinosa, My S. Almqvist, Pasi Penttinen, Joacim Rocklöv

Infectious disease threat events (IDTEs) are increasing in frequency worldwide. We analyzed underlying drivers of 116 IDTEs detected in Europe during 2008–2013 by epidemic intelligence at the European Centre of Disease Prevention and Control. Seventeen drivers were identified and categorized into 3 groups: globalization and environment, sociodemographic, and public health systems. A combination of ≥2 drivers was responsible for most IDTEs. The driver category globalization and environment contributed to 61% of individual IDTEs, and the top 5 individual drivers of all IDTEs were travel and tourism, food and water quality, natural environment, global trade, and climate. Hierarchical cluster analysis of all drivers identified travel and tourism as a distinctly separate driver. Monitoring and modeling such disease drivers can help anticipate future IDTEs and strengthen control measures. More important, intervening directly on these underlying drivers can diminish the likelihood of the occurrence of an IDTE and reduce the associated human and economic costs.

The Middle East respiratory syndrome coronavirus (MERS-CoV) outbreak and the large Ebola outbreak in West Africa are striking examples of how emerging and reemerging infectious diseases can threaten international public health and strain governmental resources (*1*,*2*). Historically, novel pathogens have emerged and reemerged repeatedly in human populations and affected public health; similarly, pathogens that have been present in a population at low levels have increased rapidly in incidence or geographic range with equally grave consequences (*3*). The context of infectious disease emergence has changed over the centuries, but Europe has remained and even intensified as a hot spot for emerging infectious diseases over recent decades (*4*). Many of the fundamental and basic determinants of emerging infectious diseases have persisted over time, but dynamic global trends provide more opportunities for emerging infectious diseases to occur and expand swiftly (*5*,*6*).

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A 2008 study, which was conducted by the European Centre for Disease Prevention and Control (ECDC) and based on expert consultation and literature review, projecting how the risk of emerging infectious diseases in Europe will be shaped in the future determined that drivers can be categorized into 3 main groups: globalization and environment, sociodemographic, and public health systems (Table 1) (*7*). Although the 3 groups are somewhat artificial and not entirely mutually exclusive, they can serve as a framework for the interpretation of infectious disease threat events (IDTEs) in Europe.

We conducted this study to identify, differentiate, and rank drivers of observed IDTEs in Europe detected through ECDC epidemic intelligence activities. By doing this, the disparate drivers that act on different dimensions and different scales can be disaggregated. A ranking of the relative importance of these drivers can help prioritize risk-based surveillance to anticipate disease emergence and spread (*8*,*9*). The effect of IDTEs on public health can be attenuated by strengthening the detection of and early response to the threats. However, more important, the likelihood of IDTEs originating in the first place can be reduced by intervening directly on their underlying drivers. Mitigation strategies to reduce the causes rather than the effects of IDTEs can be more cost effective (*10*).

Event-Based Surveillance

Persons, services, goods, capital, and microbes are free to move across borders of the European Union (EU), which currently has 28 member states and an estimated population of 508.2 million. The ECDC is an EU agency with a mission to identify, assess, and communicate current and emerging threats to human health posed by infectious diseases. This charge is accomplished through epidemic intelligence, a process to detect, verify, analyze, assess, and investigate events that may represent a threat to public health. These activities are conducted by a team of >10 epidemiologists in the Emergency Operation Center at ECDC. The daily activity of epidemic intelligence at ECDC involves active or automated web searches from confidential and official

Table 1. Determinants and drivers of infectious disease threat events, Europe, 2008–2013

Drivers, by group	Examples*
Globalization and environment	
Climate	Temperature, humidity, wind, rainfall. Can have an effect on exposure pathways of foodborne and waterborne diseases or the distribution of vectorborne diseases.
Natural environment	Land cover, vegetation, water ways, oceans, coastlines, water resources, land use, habitats, biodiversity. Can shift the distribution range and influence abundance of vectors (e.g., rodents, mosquitoes, ticks) as well as of host and reservoir animals.
Human-made environment	Urbanization, built environment, infrastructure, industries, intensive agriculture. Can enable propagation and dissemination of pathogens.
Travel and tourism	Movement of populations by automobile, train, ship, airplane. Can enable the importation of vectors, pathogens and infected persons into Europe and their dispersion within Europe.
Migration	Immigrant, emigrant, asylum seeker, settler. Can be vulnerable to or contribute to spread of infectious diseases in origin country, in transit, or in destination country.
Global trade	Import and export of goods and services across international boundaries via ship, airplane, rail, truck. Can result in the exportation or importation (on purpose or involuntarily) of host animals, disease vectors, or pathogens.
Sociodemographic	
Demographic	Population composition with regards to age, income, education. Can be associated with greater health vulnerabilities.
Social inequality	Uneven distribution of resources in society, including income, wealth, rights, privileges, social power, education. Disadvantaged groups can suffer disproportionately from infectious diseases.
Vulnerable groups	Children, premature infants, pregnant women, elderly persons, men who have sex with men, immunocompromised persons. Vulnerability can increase exposure and susceptibility to infectious diseases or decrease access to care and recovery.
Prevention	Childhood vaccination programs, adherence to treatment regimes, appropriate prescription practices. Distrust in prevention efforts can undermine control efforts (e.g., childhood vaccination programs. Neglect of prevention when traveling
Lifestyle	High-risk behavior, such as intravenous drug use or unprotected sex with multiple partners. Can increase exposure and infection rates.
Occupational	Healthcare workers, veterinary and animal care personnel, butchers, farmers, cleaners. Lapses in infection control practices can put healthcare workers at risk.
Terrorism	Intentional release or dissemination of biologic agents. Intentional contamination of drinking water can result in community outbreaks.
Public health systems	
Healthcare system	European healthcare structure for the delivery of health services, including general practitioners, hospitals, clinics. Access to care, medicines, diagnostics, insurance coverage, for example, can affect health outcomes. Healthcare systems contribute to nosocomial infections.
Animal Health	Veterinary services, animal health and welfare measures, intensive livestock practices. High animal densities can promote infectious disease transmission. Infected animals close to human settlements can increase the risk for zoonotic epidemics.
Food and water quality	Agriculture, husbandry, farming, processing, handling, preparation and storage of food, man-made water systems (e.g., cooling towers, hot and cold water systems, spa pools, humidifiers), water treatment and distribution systems. Contamination of drinking and irrigation water sources and water distribution systems can result in both localized and community outbreaks. Contamination of foodstuff along the chain from farm to fork can result in multistate epidemics
Surveillance and reporting failure	Systematic ongoing collection, collation, analysis, and dissemination of infectious disease data. Lapses in surveillance can impede a rapid response to infectious disease outbreaks. In contrast, increased surveillance will contribute to increased awareness and thus result in increased reporting of cases

*Examples are purposely not exhaustive and should be considered illustrative.

sources (e.g., EWRS [Early Warning and Response System], ProMED [Program for Monitoring Emerging Diseases], MediSys [Medical Information System], and GPHIN [Global Public Health Intelligence Network]), as well as individual reports from the EU and European Economic Area (i.e., EU countries plus Iceland, Liechtenstein, and Norway) member states. The sources of epidemic intelligence information include several websites and a large number of webpages retrieved through specialized search engines. Additional information is gathered through direct contact with epidemiologists and health authorities in the EU and abroad.

Data collection for epidemic intelligence at ECDC was standardized in 2008; thus, we analyzed the epidemiologic characteristics of each IDTE in Europe from July 1, 2008, through December 31, 2013. For each IDTE the following data are routinely collected by ECDC: type of disease or pathogen, geographic location of source of infection, source of infection (e.g., contaminated bean sprouts), duration of the epidemic or of surveillance activities, number of countries affected by the event, number of cases, and number of deaths. IDTEs included in this study were restricted to outbreaks affecting >5 persons in the EU (excluding Croatia, which was not yet an EU member). Persons infected abroad and returning to the EU were included in our analysis. The IDTEs were sorted into 10 categories (Table 2).

Table 2. Infectious disease threat events detected in Europe, 2008–2013

Threat event category	Definition and examples*
Foodborne and waterborne	All types of diseases caused by the transmission of organisms through food or water (e.g., drinking water, recreational water): salmonellosis, hepatitis A, *Escherichia coli* infection, norovirus infection, shigellosis.
Vectorborne and rodentborne	All vectorborne and rodentborne diseases (epidemics or first autochthonous cases): West Nile fever, malaria, dengue fever, Hantavirus infection.
Other zoonoses	Diseases caused by transmission of organisms through contact with animals or animal discharges: Q fever, cowpox disease, psittacosis.
Vaccine preventable	Main vaccine-preventable diseases that are normally part of the public health system's vaccination programs: measles, pertussis, mumps (boys), rubella (girls).
Multidrug resistance associated	Emerging multidrug-resistant infections of public health concern: carbapenemase-producing *Enterobacteriaceae*, *Klebsiella pneumoniae*.
Healthcare associated	Infections contracted while hospitalized or transmitted through healthcare practices: meningococcal meningitis.
Injection drug use associated	Infections caused by injection drug use: botulism, HIV, anthrax.
Sexually transmitted	Emerging sexually transmitted diseases and increases in incidence of serious complications: meningococcal infections.
Influenza	Seasonal influenza and other pandemic influenzas.
Airborne	Respiratory diseases acquired through transmission of pathogens through air (e.g., particles, droplets): for example, legionellosis. Includes respiratory infections that can be transmitted through air or other pathways, including infections transmitted through aerosols, fomites, or direct contact: Middle East respiratory syndrome coronavirus.

*Examples are purposely not exhaustive and should be considered illustrative.

Information about the underlying drivers of these IDTEs was extracted from several sources: the Communicable Disease Threats Report (a weekly bulletin generated by the epidemic intelligence team at ECDC), epidemiologic reports and communications, rapid risk assessments, threat assessments, mission reports, and associated peer-reviewed publications retrieved from PubMed. The IDTE drivers were organized into 3 categories: globalization and environment, sociodemographic, and public health systems (Table 1) (*7*). Expert assessment, performed by the authors, was used to evaluate the quality and validity of the information regarding the drivers. Discordant assessments were resolved by consensus.

Drivers were subjected to descriptive analyses (individually or in combinations), including frequency rates and ranking of the drivers, in relation to different types of IDTEs. Euclidian distances (based on how the IDTE types occurred with the driver category in the empirical data) were also calculated between each 1 driver pair (*11*). The calculation of the distance between 2 drivers was derived from the multidimensional driver space, based on whether the drivers were present (1) or absent (0) during the emergence of an IDTE. We then used a set of dissimilarities for the distances between drivers to perform a hierarchical cluster analysis (*11*). Dissimilarity distances between clusters were recomputed by the Lance–Williams updated formula, according to the average clustering method, by using the statistical computing program R and the algorithms in the R Stats Package (*11*). The driver terrorism was not included here because only 1 threat event was linked to the driver. Dissimilarity between clusters of IDTE drivers were described graphically by using tree diagrams (dendrograms) to visualize the similarity and dissimilarity of drivers in the occurrence of the IDTE. The clustering algorithm applied to binary data has been reported to perform well (*12*). The distance between clusters measure how similar, or dissimilar, different drivers are in their co-occurrences in outbreaks.

Determinants and Drivers of IDTEs

Of 274 IDTEs that occurred within the EU during July 2008–December 2013, a total of 116 met the study inclusion criteria. Foodborne and waterborne IDTEs were the most frequently occurring events (n = 48), followed by vectorborne and rodentborne IDTEs (n = 27), airborne IDTEs (n = 10) vaccine preventable IDTEs (n = 10), other zoonotic IDTEs (n = 7), injection drug use–associated IDTEs (n = 4), influenza IDTEs (n = 4), healthcare-associated IDTEs (n = 3), multidrug resistance–associated IDTEs (n = 2), and sexually transmitted IDTE (n = 1). The driver category that was by far the most frequently involved in single IDTEs was globalization and environment (61%), followed by the public health system failure (21%) and sociodemographic (18%) groups. The individual driver travel and tourism was linked to 9 of the 10 IDTE categories, and the vulnerable groups and lifestyle driver categories were linked to 7 and 6 IDTE categories, respectively. Most IDTEs had a combination of drivers: 51% had 2 drivers, and 25% had 3 drivers (Figure 1). Foodborne and waterborne diseases and vectorborne diseases were the most commonly occurring drivers in combinations of 2 and 3 drivers. The most common driver combinations were travel and tourism in combination with food and water quality and global trade in combination with food quality, both of which caused foodborne and waterborne diseases (Figure 2).

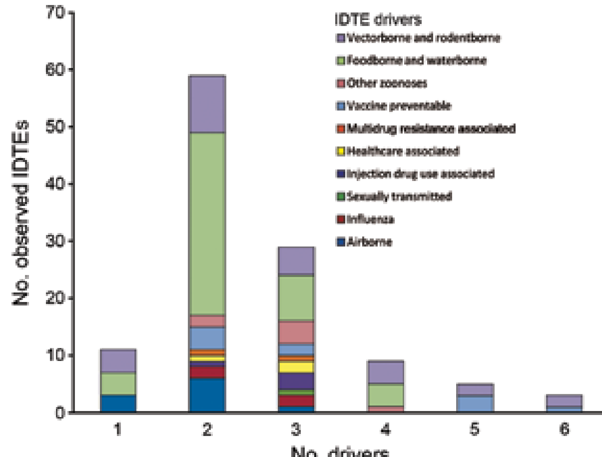

Figure 1. Number of observed infectious disease threat events (IDTEs) in relation to number of drivers for each IDTE group, Europe, 2008–2013.

Foodborne and Waterborne IDTEs

The foodborne and waterborne category included all types of diseases transmitted through food or water (Table 1) and was responsible for the most IDTEs. The global burden of foodborne diseases is considered to be substantial, although no current estimates exist (*13*). The most common cause of the observed foodborne and waterborne outbreaks in Europe was norovirus, followed by hepatitis A and *Escherichia coli* (hemolytic uremic syndrome and Shiga-like toxin–producing *E. coli* infections) (*14*,*15*). The strongest driver in this IDTE group was food and water quality, implicating the food industry and water treatment infrastructure, often in combination with the travel and tourism or global trade drivers (Figure 2, panel A). An example was the norovirus epidemic that affected >11,000 schoolchildren in 6 countries; the origin of the epidemic was traced to contaminated frozen strawberries (*16*).

Vectorborne and Rodentborne IDTEs

Nearly half of the 27 IDTEs within the vectorborne and rodentborne IDTE category were caused by West Nile virus (WNV) infections. Four of these IDTEs consisted of the first autochthonous WNV cases in 4 different European countries and 1 large outbreak in southeastern Europe with >260 cases (*16*). WNV infection and malaria are notifiable diseases in the EU and, thus, subject to indicator surveillance; however, special threat events are picked up by event-based surveillance as well. The natural environment driver was present in all WNV infection events; in half of those events, it was present with the climate driver, and in 6 events, it was present with the surveillance and reporting failure driver (Figure 2, panel B). This finding is consistent with other findings that show environmental and climatic determinants play contributing roles in WNV infection outbreaks (*17*). Of 7 malaria threat events, 5 included autochthonous cases (in Spain, Greece, and Belgium). Our data also included the large dengue outbreak in Madeira, Portugal, with >2,000 cases (*18*) driven by climate, natural environment, and travel and tourism (Figure 2, panel B). A large outbreak of hantavirus infections in Germany in 2010 was attributed to bank vole (*Clethrionomys glareolus*) populations, which had increased substantially due to excessive seed production the previous year (*19*); human behavior (e.g., outdoor activities in summer); dust contaminated with rodent excreta following dry and warm weather; and heightened awareness, with better diagnosis and reporting.

Other Zoonoses IDTEs

Q fever, psittacosis, and diseases caused by cowpox virus and *E. coli* (with an unusual transmission pathway, e.g., contaminated farm soil and petting of contaminated animals) were included in the other zoonoses IDTE category. Outbreaks often occurred among farm and animal workers. However, the 2007–2009 Q fever outbreak in the Netherlands affected >3,000 persons in densely populated areas (demographic driver) situated in close proximity to commercial dairy goat farms (climate and animal health drivers) (Figure 2, panel C) (*20*). Contaminated dust particles from ruminant farms probably caused airborne transmission of *Coxiella burnetii*, the causative agent of Q fever. Vaccination, hygiene measures, and culling of pregnant animals on affected farms eventually ended the outbreak (*21*). Due to persistence of *C. burnetii* in the environment, continued surveillance for Q fever is warranted.

In autumn 2009, an outbreak of 93 cases of *E. coli* O157 (verotoxin-producing *E. coli*) infection in southern England was related to environmental and animal exposure on a petting farm visited by families and children (tourism, vulnerable groups, animal health, and natural environment drivers) (Figure 2, panel C) (*22*). Horizontal integration of the human, animal, and environmental health sectors, according to the One Health approach, can tackle some of these public health predicaments (*23*).

Vaccine-Preventable IDTEs

Ten IDTEs, including measles, mumps, rubella, and pertussis outbreaks, were reported for the vaccine-preventable IDTE category. A measles outbreak in Bulgaria in 2009–2010, which affected predominantly migrant and hard-to-reach Roma populations, resulted in >24,000 cases and 24 deaths in 1 year (*24*). The drivers responsible for this outbreak were a combination of prevention, lifestyle, migration, social inequality, and healthcare system (Figure 2, panel D). Measles is still endemic in many European countries because of low vaccination coverage among migrants and hard-to-reach populations and vaccine hesitancy. Specific vaccination strategies are often

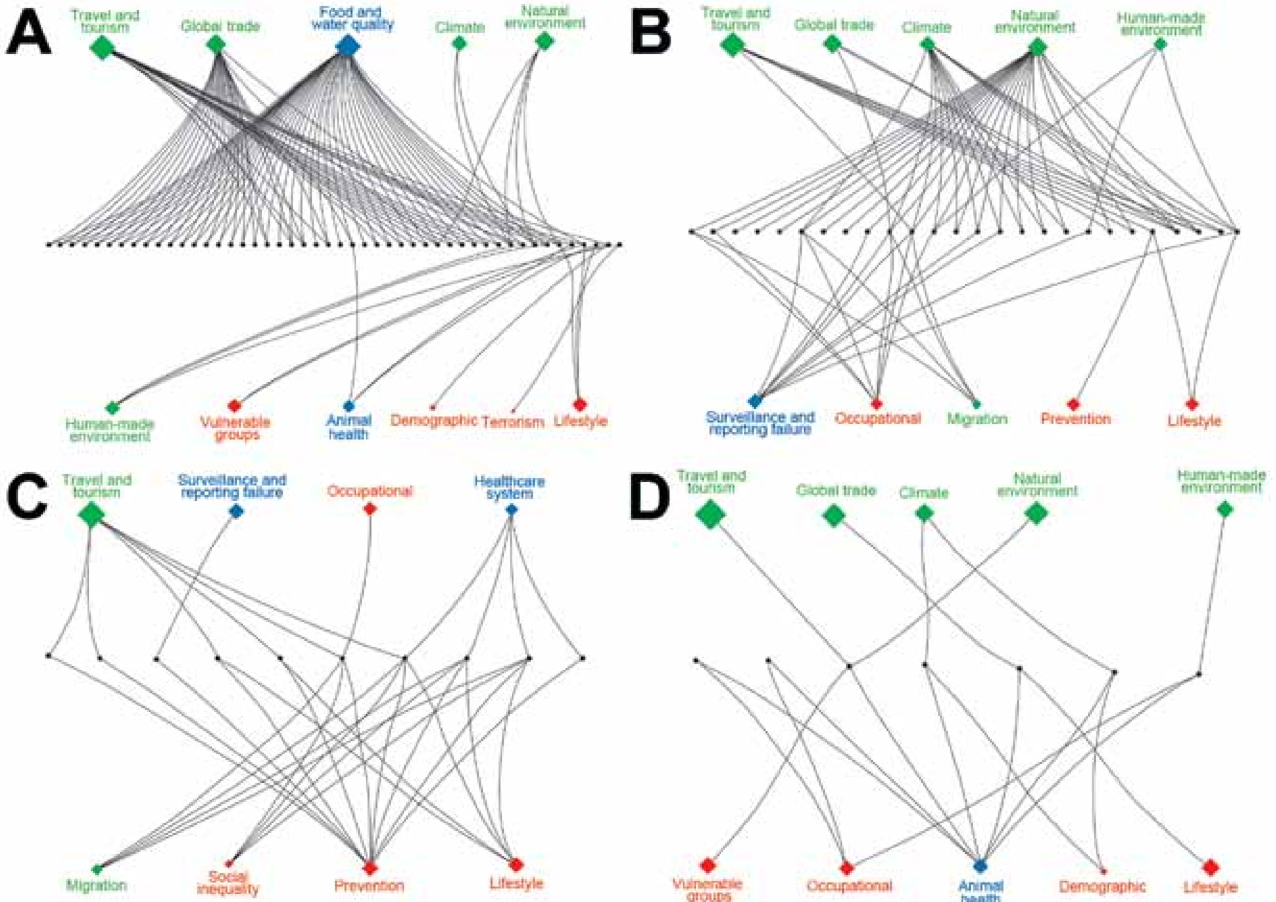

Figure 2. Infectious disease threat events (IDTEs), by contributing drivers, observed in Europe, 2008–2013. The 3 IDTE categories are represented by green (globalization and environment), red (sociodemographic), and blue (public health systems) symbols, the sizes of which are proportional to the overall frequency of the driver. A) Foodborne and waterborne IDTEs. B) Vectorborne and rodentborne IDTEs. C) Other zoonoses IDTEs. D) Vaccine preventable IDTEs.

necessary for these populations to protect children and adults from infectious diseases, prevent spread of infection due to crowded living conditions, and ensure continuity of childhood immunization schedules. Failure to vaccinate susceptible populations diminishes herd immunity and may trigger outbreaks (*24*). There was no evidence of an increased risk of infectious disease transmission in the host population in Europe during the 2015 influx of migrants or asylum seekers; however, ECDC advocated for the implementation of basic public health measures, health assessments, and vaccination to address the health needs of migrants (*25*). This strategy is supported by the findings from this analysis, in which migration was a comparatively infrequent driver of IDTEs, relative to travel and tourism (Figure 3).

Multidrug Resistance– and Healthcare-Associated IDTEs

Multidrug-resistant tuberculosis cases were identified as single case events and therefore did not meet our inclusion criteria in this study. The events in the multidrug resistance–associated IDTE group consisted of a nosocomial outbreak of multidrug-resistant carbapenemase-producing *Klebsiella pneumoniae* infection in 2 hospitals in Ireland and 69 infections with New Delhi metallo-β-lactamase-1 carbapenemase-producing *Enterobacteriaceae* in persons in the United Kingdom with a travel history to India or Pakistan (*26*).

The relatively few healthcare-associated IDTEs picked up by ECDC's epidemic intelligence represent only a fraction of the expected number in Europe. Seven deaths occurred among 49 detected cases; 6 deaths were in newborns who had been infected while in hospitals (healthcare system, vulnerable groups, human-made environment drivers). The number of events and deaths for other event categories was far below the actual number expected for Europe. An ECDC point-prevalence survey of healthcare-associated infections and antimicrobial use in long-term care facilities in Europe showed that ≈4.1 million patients contract a healthcare-associated infection in the EU each year, and

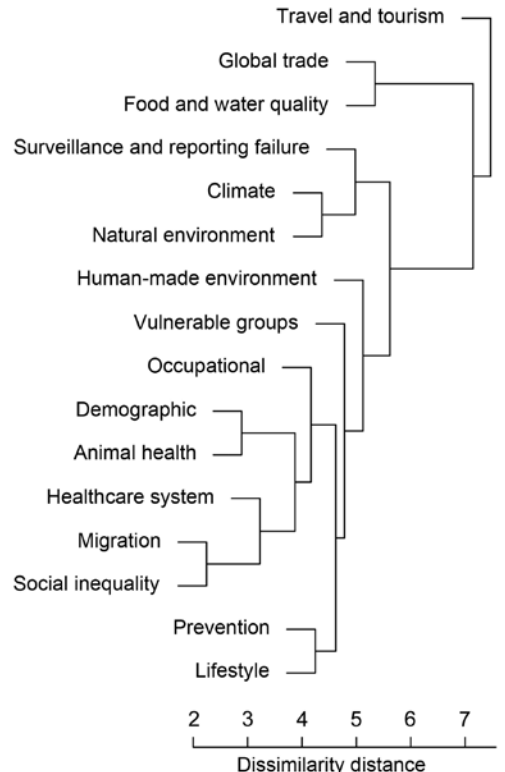

Figure 3. Cluster dendrogram from hierarchical cluster analysis of drivers contributing to observed infectious disease threat events (IDTEs), Europe, 2008–2013. Individual segments (leaves) on the lower part of the tree are more related to each other, as indicated by distances between the branches. Drivers below travel and tourism also occurred less often as underlying drivers of IDTEs and tended to be more contextual in nature. Scale bar indicates dissimilarity distance for drivers, as measured by frequency of pairwise co-occurrence in clusters. Similar drivers (e.g., that co-occurred in outbreaks) are at a close distance, and those that were more independent of other drivers show higher dissimilarity.

≈37,000 deaths occur annually as a direct consequence of these infections (*27*). However, these types of infections were not captured by epidemic intelligence due to reporting disincentives (e.g., legal and financial).

Injecting Drug Use–Associated IDTE

Reported infections caused by injection drug use were due to botulism, HIV, and anthrax and caused 69 illnesses and 8 deaths (*28*,*29*). As an example, a contaminated batch of heroin (global trade driver) caused 31 anthrax infections among heroin users in Scotland (vulnerable groups, lifestyle, sociodemographic drivers) (*30*).

Sexually Transmitted IDTE

Only 1 sexually transmitted IDTE was identified by epidemic intelligence. The event was reported from 3 countries and consisted of invasive meningococcal disease among men who have sex with men; the men had been infected while traveling or through contacts from abroad. Of note, however, many sexually transmitted infections tend to be silent and reach endemic levels that are not captured by epidemic intelligence.

Influenza IDTEs

Rather than registering as recurrent IDTEs, influenza precipitated several influenza outbreaks that were recorded as Public Health Events at ECDC; examples are the avian influenza A(H5N1) outbreaks and the influenza A(H1N1) pdm09 pandemic. Thus, the number of IDTEs attributed to influenza is underestimated. The drivers for the influenza IDTE group of both seasonal and pandemic influenza were travel and tourism, vulnerable groups, social and demographic, and surveillance and reporting.

Airborne IDTEs

Ten IDTEs were reported for the airborne IDTE category. Most events were due to legionellosis, but the IDTEs also encompassed the emergence of MERS-CoV infections in 2012–2013; a total of 9 MERS cases were reported from the EU (*2*). Our analysis identified human-made environments, in particular contaminated cooling towers or spa pools, to be the overarching driver of *Legionella* infection events (*31*). Proper maintenance of the physical infrastructure can prevent these IDTEs. Two drivers, travel and tourism and healthcare system, were identified for the emergence and spread of MERS-CoV into Europe (*2*).

Driver Ranking

An overall frequency ranking of all events ranked the individual contribution of the top 5 drivers in the following order: travel and tourism, food and water quality, natural environment, global trade, and climate. The hierarchical cluster analysis revealed travel and tourism to be separate from all the other drivers; thus, this driver can be considered distinct, indicating that the distribution of IDTEs within travel and tourism is significantly different from the distribution in the remaining clusters (Figure 3). The hierarchical cluster analysis revealed several similarly clustered segments, such as climate and natural environment and migration and social inequality, indicating that these drivers are more related to each other than to the other drivers.

Limitations

Although sociodemographic and public health system drivers were less frequent in our analysis of IDTEs, they are nevertheless key contributors to the disease burden from infectious diseases in Europe (*32*). They may also be more directly amenable to interventions. However, epidemic intelligence detects IDTEs, not endemic infectious diseases,

to which these drivers contribute substantially. Epidemic intelligence is heavily influenced by media coverage, geographic focus, length of the epidemic intelligence monitoring cycle, diagnostic procedures, and sensitivity of surveillance systems, among many other factors. The captured events are then filtered and verified before they are assessed and investigated. One event during the study period was categorized as bioterrorism, because sabotage was suspected due to coliform contamination of drinking water tanks at a hotel. If need be, a Public Health Event is declared to initiate control measures.

However, the ECDC screening of IDTEs is not designed to capture infectious diseases that do not reach outbreak levels or are not picked up by event monitoring (e.g., healthcare-associated or sexually transmitted infections). Therefore, our analysis pertains only to the drivers of IDTEs rather than endemic infectious diseases that are not recorded by epidemic intelligence. Long-term monitoring of the incidence, prevalence, or both of notifiable diseases is performed on a national level and reported through a different reporting system, the European Surveillance System at ECDC (*33*).

Discussion

We found globalization and environment to be the most noteworthy driver category for IDTEs in Europe. More specifically, travel and tourism, food and water quality, natural environment, global trade, and climate were the top 5 drivers of all IDTEs identified through epidemic intelligence at ECDC. Among these, travel and tourism proved to be significantly distinct in the hierarchical cluster analysis and cluster dendrogram (Figure 3). In this analysis of epidemic intelligence data, travel and tourism was not only the most distinct but also the most recurrent driver implicated in the emergence of IDTEs. The volume of international travelers on commercial flights with a final destination in Europe has increased steadily over the years; >103 million travelers entered Europe in 2010 alone, and this number will probably continue to grow (*34*). International travel from areas with epidemic and endemic diseases has resulted in continuous importation of infected persons into Europe who can, for example, trigger outbreaks of airborne diseases. Similarly, pathogen introduction into competent vector populations can result in local transmission and threaten the safety of the blood supply (*35*). Restricting international travel in a globalized world to reduce the likelihood of IDTEs is both unrealistic and undesirable; however, monitoring and modeling air traffic patterns for pathogen importation risk can potentially accelerate early case detection and rapid response and effective control of IDTEs (*36*).

Food and water quality was the second most frequent driver of IDTEs in Europe. Suboptimal food safety systems, even if they are distant to the outbreak, become an international public health issue in an interconnected world in which food and humans move freely (Figure 2) (*37*). The occurrence of an IDTE can potentially be mitigated by addressing this driver. Fostering multisectorial collaboration between the food industry, public health, and environmental agencies can prevent IDTEs. High-density agricultural practices need to be subjected to stringent farm biosecurity and sanitary practices to prevent multinational outbreaks (*23*). Upgrading water treatment and distribution systems can prevent communitywide outbreaks (*38*).

Changes in the natural environment are increasing on a nonlinear scale with habitat destruction and loss of ecosystem services (http://www.esa.org). Monitoring and modeling environmental precursors of IDTEs can help to anticipate, or even forecast, an upsurge of IDTEs (*39*). The utility of such predictive models has been documented on several occasions: environmental drivers of IDTE with prediction tools have been made available by ECDC through the E3 (European Environment and Epidemiology) Geoportal (https://e3geoportal.ecdc.europa.eu/SitePages/Home.aspx) (*40*).

In summary, we have taken a systematic approach to categorize and rank the underlying drivers of observed IDTEs in Europe to help anticipate, respond to, and recover from probable, imminent, or current impacts of these events. Drivers of IDTEs can arise as epidemic precursors of IDTEs. Monitoring and modeling these drivers can serve as early warning systems of IDTEs and accelerate responses (*39*,*40*). However, it is desirable to proactively prevent possible public health emergencies rather than respond to IDTE after they have occurred. Thus, the most cost-effective strategy would be to directly tackle the underlying drivers of an IDTE rather than deal with the actual IDTE after the fact (*10*). Intervening directly on drivers may prevent the occurrence of IDTEs and reduce the human and economic cost associated with IDTEs.

Acknowledgments

This paper is dedicated to the memory of Dr. James Mendlein (1950–2015), whose commitment to global health and passion for social justice was an inspiration to all of us (http://www.tephinet.org/announcement/remembering-jim-mendlein).

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July 2015: **Malaria**

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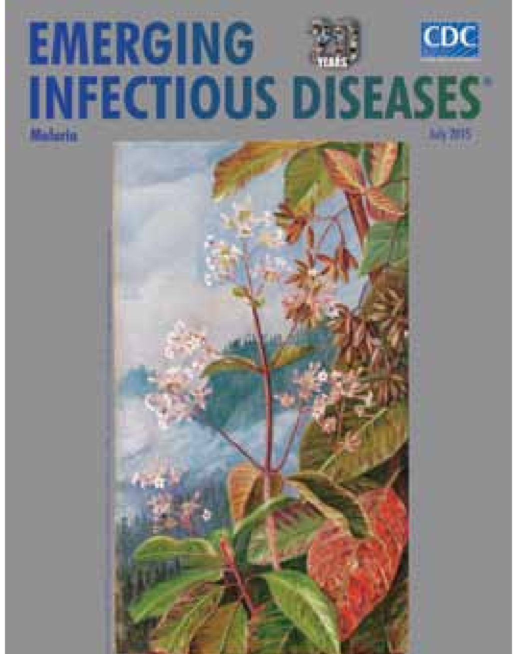

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SYNOPSIS

Shiga Toxin–Producing *Escherichia coli* O157, England and Wales, 1983–2012

Natalie L. Adams, Lisa Byrne, Geraldine A. Smith, Richard Elson, John P. Harris, Roland Salmon, Robert Smith, Sarah J. O'Brien, Goutam K. Adak, Claire Jenkins

We evaluated clinical Shiga toxin–producing *Escherichia coli* O157 infections in England and Wales during 1983–2012 to describe changes in microbiological and surveillance methods. A strain replacement event was captured; phage type (PT) 2 decreased to account for just 3% of cases by 2012, whereas PT8 and PT21/28 strains concurrently emerged, constituting almost two thirds of cases by 2012. Despite interventions to control and reduce transmission, incidence remained constant. However, sources of infection changed over time; outbreaks caused by contaminated meat and milk declined, suggesting that interventions aimed at reducing meat cross-contamination were effective. Petting farm and school and nursery outbreaks increased, suggesting the emergence of other modes of transmission and potentially contributing to the sustained incidence over time. Studies assessing interventions and consideration of policies and guidance should be undertaken to reduce Shiga toxin–producing *E. coli* O157 infections in England and Wales in line with the latest epidemiologic findings.

Shiga toxin–producing *Escherichia coli* (STEC) serogroup O157 emerged as a pathogen of public health concern during the early 1980s and was first isolated in the United Kingdom in July 1983 (Figure 1) from 3 cases linked to an outbreak of hemolytic uremic syndrome (HUS) (*1*). After this emergence, the Gastrointestinal Bacteria Reference Unit (GBRU), Public Health England (PHE) (then the Public Health Laboratory Service), reviewed a large archive of isolates and concluded that, before 1983, STEC O157 was not a major cause of gastrointestinal disease in England and Wales (*2*).

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Despite being relatively rare in comparison with other gastrointestinal infections, STEC O157 is of public health concern because of its potential for severity. Symptoms of infection include abdominal cramps, nausea, and bloody diarrhea. In 5%–14% of cases, infection leads to HUS, a severe and potentially fatal systemic condition primarily affecting the kidneys (*3*). The primary STEC virulence factor is Shiga toxin (Stx), which targets cells expressing the glycolipid globotriaosylceramide, disrupting host protein synthesis and causing apoptotic cell death (*4*). Children and elderly persons are most susceptible to severe illness, and HUS is recognized as the most common cause of acute renal failure among children in the United Kingdom (*5*).

Cattle and other ruminants are natural reservoirs for STEC O157, and transmission to humans occurs through direct or indirect contact with the animals or their feces or through ingestion of contaminated food or water. A low infectious dose and propensity for person-to-person spread means transmission in households and closed settings such as schools is common (*6*,*7*), as is the potential for large outbreaks (*8*–*12*). We describe changes in the epidemiology of STEC O157 in England and Wales during a 30-year period (1983–2012) against a background of changing microbiological and surveillance methods over time.

Methods

Case Ascertainment, 1983–2012

Beginning in 1983, only fecal specimens from patients with HUS or hemorrhagic colitis were referred for STEC O157 testing; before 1989, few specimens were referred for testing. Beginning in 1997 in England and Wales, referral for testing was extended to all patients with symptoms of gastrointestinal infection, including vomiting, diarrhea, or bloody feces.

Microbiology Methods, 1983–2012

GBRU provides the national reference service for STEC in England and Wales. Beginning in 1983, individual colonies were tested for toxin production by using the verocytotoxin cell assay, and positive colonies were identified biochemically and serotyped (*13*). In 1987 at GBRU, the verocytotoxin

cell assay was replaced with a molecular probe assay to detect the *stx* gene (*14*) and sorbitol MacConkey culture medium. Later, sorbitol MacConkey culture medium containing cefixime and tellurite was developed, facilitating isolation of STEC O157 from fecal specimens, but testing was not implemented for all patients with symptoms of gastrointestinal infection in all local hospital laboratories until 1997 (*15*). Isolates of *E. coli* O157 identified locally are sent for confirmation and typing at GBRU.

Detection and confirmation of STEC at GBRU includes biochemical identification and serotyping of bacterial isolates. Since 1989, strains belonging to *E. coli* O157 have been further differentiated by using a phage typing scheme developed in Canada (*16*). Retrospective phage typing was undertaken for all viable strains collected before 1989. During 1994–2011, detection of *stx1* or *stx2* used a block-based PCR (*17*), which was replaced in 2012 with real-time PCR targeting *stx1* or *stx2* and the intimin (*eae*) gene, associated with intimate attachment of the bacteria to the host gut mucosa (*18*).

Data Collection Methods, 1983–2012

The amount of epidemiologic and microbiological data increased during the study period. During 1983–2003, a

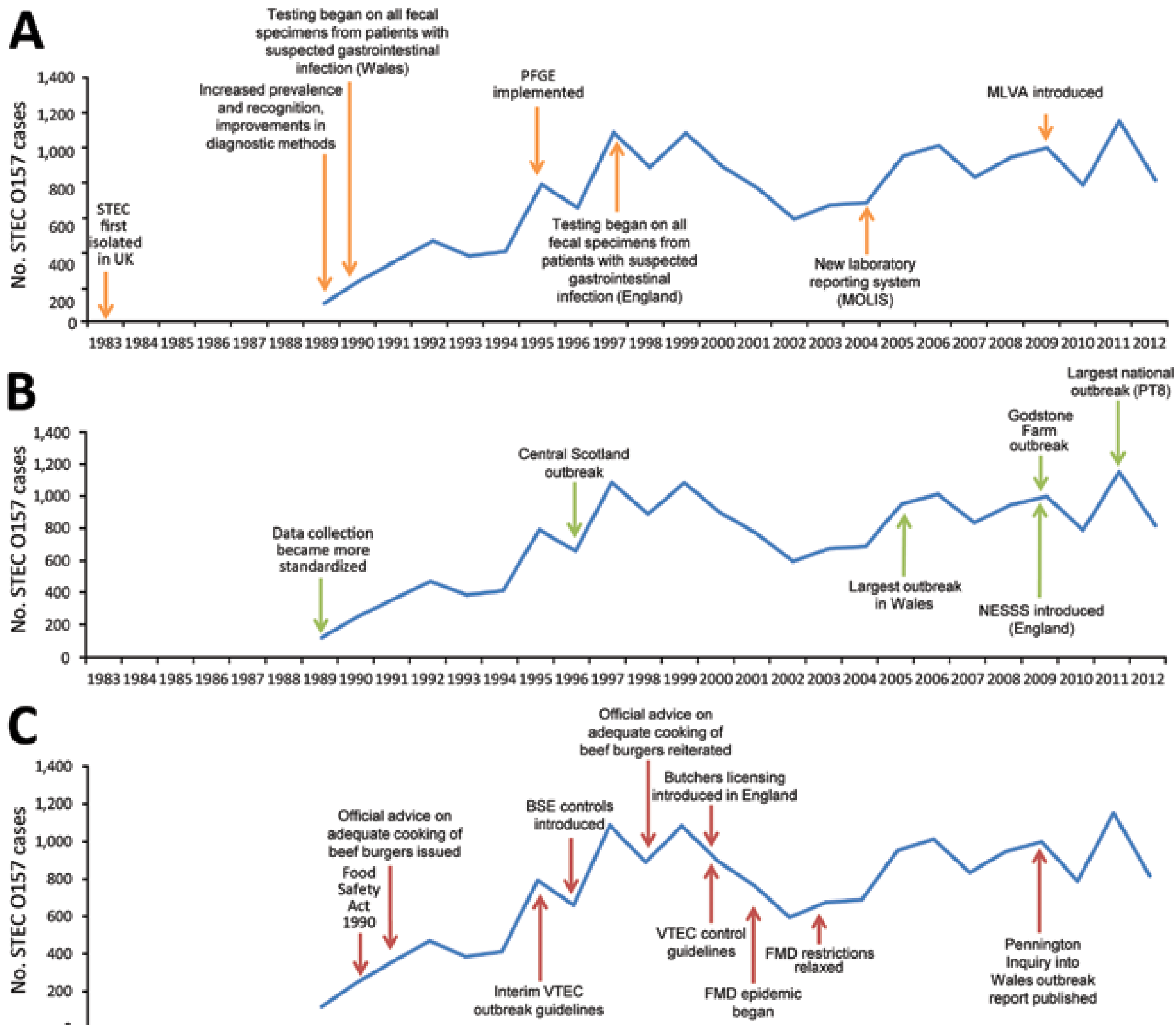

Figure 1. Timeline of key events influencing the epidemiology (A), microbiology (B), and guidance and control (C) of STEC O157, England and Wales, 1983–2012. Numbers before 1989 are available only as an aggregate for that period and therefore cannot be presented by year. BSE, bovine spongiform encephalopathy; FMD, foot and mouth disease; MLVA, multilocus variable-number tandem-repeat analysis; MOLIS, Modular Open Laboratory Information System; NESSS, National Enhanced Surveillance Scheme for STEC; PFGE, pulsed-field gel electrophoresis; PT, phage type; STEC, Shiga toxin–producing *Escherichia coli*; VTEC, verocytotoxin–producing *E. coli*.

dedicated laboratory database was used to record patient and microbiological data. In 2004, a new laboratory reporting system, Modular Open Laboratory Information System, was implemented (Figure 1). These laboratory databases captured microbiological results and demographic details of cases, as well as limited epidemiologic data (HUS diagnosis, outbreak association, recent history of foreign travel).

In January 2009, PHE introduced the National Enhanced Surveillance Scheme for STEC (Figure 1) (*19*). This scheme captured epidemiologic information through standardized questionnaires administered to all persons with STEC and linked to microbiological data in the Modular Open Laboratory Information System.

Detection of outbreaks relied on detecting unusual increases in STEC activity or reporting of shared exposures among cases of the same phage type (PT). Outbreaks were recorded on paper before 1992. In 1992, PHE began standardized surveillance of outbreaks of gastrointestinal disease where ≥2 persons with the same infection are linked, or probably linked, to the same source. In brief, local PHE units report standardized epidemiologic data on all outbreaks of gastrointestinal diseases, including source of infection and microbiological data.

Data Analyses

Data on STEC O157 patients in England and Wales were analyzed in 3 time periods, 1983–1988, 1989–1996, and 1997–2012, to account for periods of differing case ascertainment and data collection. Case numbers for 1989–1996 were small and represent biased sampling toward severe STEC O157 infections therefore calculation of incidence and interpretation of trends would be meaningless, and these were calculated only for 1997–2012.

We performed descriptive analyses in Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). Crude incidence rates were calculated by using the Office of National Statistics mid-year population estimates (*20*). Crude incidence rate ratios (RR) and 95% CIs were calculated in Stata version 13.0 (StataCorp LP, College Station, TX, USA) for comparison among groups.

Results

Microbiology of STEC O157, 1983–2012

In England and Wales during 1983–1988, a total of 279 patients were infected with STEC O157, including 110 from 3 outbreaks. Of the 169 non–outbreak-related isolates, 155 were retrospectively phage typed; the most common types were PT2 (49 [31.6%] cases), PT1 (38 [24.5%]), and PT49 (22 [14.2%]).

During 1989–1996, the number of cases increased (3,448 total cases), and the proportions of common PTs changed annually (Figure 2). In 1996, a new PT was designated PT21/28 after reexamination of the lysis profiles of PT21 and PT28 isolates (*21*). By 1996, PT2 (244 [37%] isolates), PT8 (85 [12.9%] isolates), and PT21/28 (92 [13.9%] isolates) were the most common PTs, and the proportion of PT1 (28 [4.2%] isolates) and PT49 (42 [6.4%] isolates) had declined.

During 1997–2012, the decline in these PTs continued, and PT1 and PT49 were rarely observed. PT2 also declined to just 28 (3.4%) isolates by 2012 from a peak of 430 (54.3%) isolates in 1995, a significant decrease

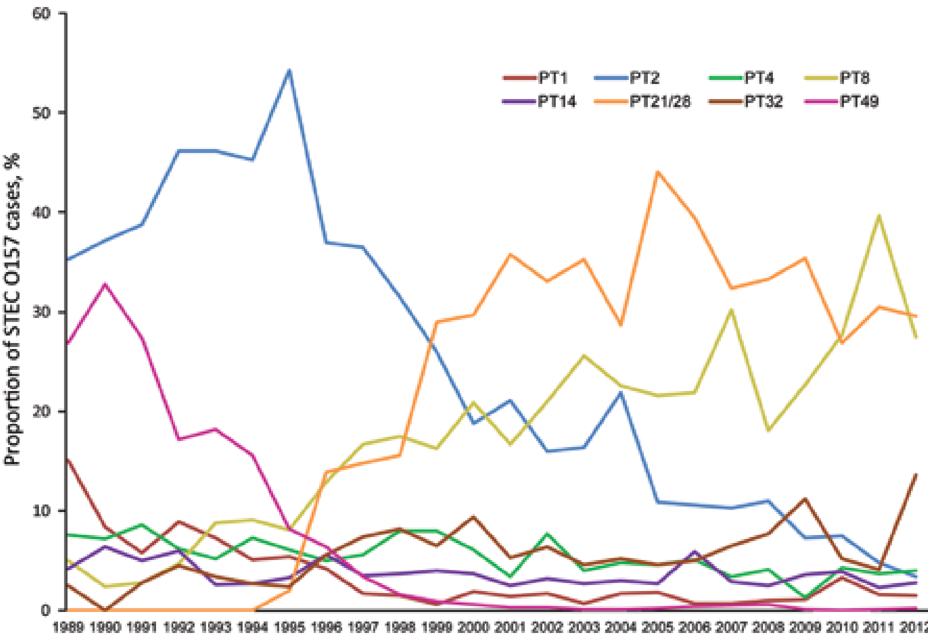

Figure 2. Proportions of common phage types (PTs) of Shiga toxin–producing *Escherichia coli* O157 identified, England and Wales, 1989–2012.

for this period (p<0.001) (Figure 2). Concurrently, numbers of PT21/28 rapidly increased, accounting for 420 (44.1%) cases by 2005, a significant increase for the period (p<0.001), and thereafter remaining the most frequently detected PT (Figure 2). PT8 also increased significantly, from 182 (16.7%) to 225 (27.5%) of cases by 2012 (p<0.001).

Strains encoding Stx1 only were rare (81 [0.6%] isolates), and most (45 [55.6%] isolates) were PT8. Strains encoding Stx2 only were most frequent (10,182 [71.8%] isolates), followed by Stx1+2 (3,921 [27.6%] isolates). Stx type and PT are interrelated; most PT8 strains (3,040 [93.2%] isolates) possessed *stx1+2*, whereas PT2 and PT21/28 usually possessed *stx2* only (2,100 [92.8%] and 4,340 [99.7%] isolates, respectively).

Epidemiology of STEC O157, 1997–2012

Case Numbers and Crude Incidence

A total of 14,184 laboratory-confirmed STEC O157 cases were identified in England and Wales; the mean was 887 (95% CI 802–972) cases per year. Crude incidence was 1.65 (95% CI 1.49–1.81) cases/100,000 person-years but varied by year, geography, and patient age and sex (Figure 3). Identifiable peaks in case numbers corresponded to reported outbreaks (Figure 1; Table, http://wwwnc.cdc.gov/EID/article/22/4/15-1485-T1.htm). Crude incidence decreased from 1999, reaching its lowest in 2002 (1.1 cases/100,000 person-years [595 cases]), but returned to previous levels in 2005 and was sustained thereafter.

STEC O157 infections demonstrated a distinct seasonality. Cases began to increase in April and declined beginning in September (data not shown).

Patient Age and Sex

Patient age was reported for 13,015 (91.8%) cases. Children <15 years of age constituted 5,867 (45.1%) cases; the greatest proportion (2,970 [22.8%] cases) occurred among those 1–4 years of age. Crude incidence decreased with increasing age; incidence was lowest for persons >60 years of age (0.98 [95% CI 0.82–1.12] cases/100,000 person-years) (Figure 3). Crude incidence was significantly higher for children 1–4 years of age (7.21 [95% CI 6.34–8.04] cases/100,000 person-years) than for those 20–59 years of age (RR 7.16, p<0.001) and >60 years of age (RR 7.36, p<0.001).

Sex was reported for 13,947 (98.3%) patients. Female patients accounted for 7,717 (55.3%) cases, and crude incidence was significantly higher for female than for male patients (RR 1.19, p<0.001; 1.76 [95% CI 1.59–1.93] cases/100,000 person-years , vs. 1.48 [95% CI 1.34–1.62] cases/100,000 person-years). Age and sex were reported for 12,848 (90.6%) patients. Sex disparity was highest for those 20–59 years of age (RR 1.60 for women vs. men; p<0.001).

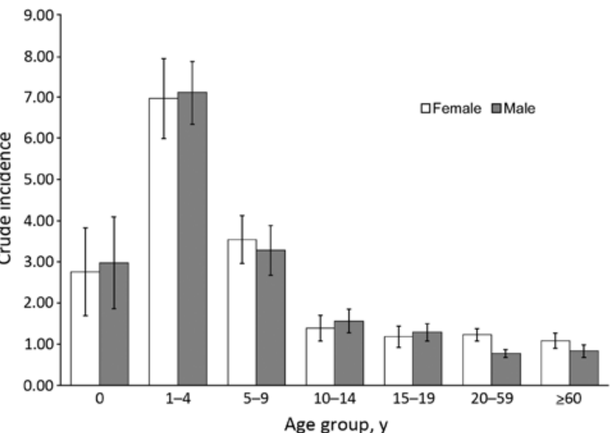

Figure 3. Crude incidence (cases per 100,000 person-years) of Shiga toxin–producing *Escherichia coli* O157, by patient age group and sex, England and Wales, 1997–2012. Error bars indicate 95% CIs.

The proportion of Stx2-only strains decreased with increasing age. Most (288 [81.8%]) children 1–4 years of age were infected with strains carrying Stx2 only, compared with 1,352 (65.4%) of persons ≥60 years of age (p<0.001). In parallel, the proportion of Stx1+2 profiles increased with age; 548 (16.1%) 1–4-year-olds were reported to have Stx1+2, compared with 698 (33.7%) of persons ≥60 years of age (p<0.001). We found no differences in sex by PT or Stx.

Geography

Annual crude incidence was highest in Cumbria and Lancashire (North West England) (3.70 [95% CI 2.70–4.70] cases/100,000 person-years), followed by Yorkshire and Humber (North East England, 2.75 [95% CI 2.37–3.13] cases/100,000 person-years) and Devon, Cornwall, and Somerset (South West England, 2.71 [95% CI 2.35–3.07] cases/100,000 person-years), whereas annual crude incidence was lowest in London (0.99 [95% CI 0.83–1.14] cases/100,000 person-years). Cases were almost 4 times more likely to be reported in Cumbria and Lancashire (RR 3.72) than in London (p<0.001). Within areas, crude incidence remained stable over time, other than peaks associated with outbreaks. We found no notable differences by geography in age, sex, PT, or seasonality.

Outbreaks, 1983–2012

During 1983–2012, a total of 335 outbreaks were reported, ranging from 0 to 25 outbreaks annually (Table). These outbreaks constituted 3,107 (17.4%) cases (median 5 cases, range 2–257 cases).

Large outbreaks caused peaks in annual crude incidence (Figure 1). For example, in 1995, eleven outbreaks comprising 141 cases occurred (Table), including a large nursery outbreak in Wales affecting 49 children (*6*). In

1999, nineteen outbreaks (236 cases) occurred, causing incidence to peak. Nine were attributed to contaminated food vehicles, including 3 caused by milk pasteurization failures, 1 affecting 88 persons (*22*). Outbreaks caused by postpasteurization contamination of milk also occurred in 2000 and 2002, as did 2 outbreaks associated with drinking raw milk in 2000, but no milk-related outbreaks were observed during the remainder of the study period.

Food vehicles contributed the highest number of outbreaks (101 [30.3%]) and outbreak cases (1,418 [45.9%]) (Table). These outbreaks included 38 attributed to eating contaminated meat; 16 to eating undercooked meat, such as burgers at barbecues; and 22 to cross-contamination of cooked meats. The cross-contamination outbreaks were larger; the largest meat-related outbreak occurred in Wales when meat from a butcher supplied to institutions infected 118 persons with STEC O157 in 2005 (*12*). After that, meat-related outbreaks were infrequent; 7 meat-related outbreaks (compared with 31 before this outbreak) were reported in the subsequent 7 years.

The first implicated food vehicle in this study was raw potatoes in a 1985 outbreak, and outbreaks associated with eating vegetables were reported throughout the years. The largest national outbreak in Great Britain (252 cases) caused by STEC O157 PT8, linked to handling raw leeks and potatoes, was reported in 2011 and led to the highest incidence during the period (Figure 1) (*23*).

Person-to-person spread in institutional settings accounted for 29.1% of outbreaks and more than one quarter of outbreak cases (825 cases). Twenty-six outbreaks occurred in institutional settings: care homes (16 outbreaks), prisons (4 outbreaks), and hospitals (6 outbreaks). No outbreaks in these settings occurred after 2007. Seventy-two outbreaks, which resulted in 808 cases, occurred in child-care facilities. Each year, 1–7 outbreaks in child-care facilities occurred, but outbreaks increased in frequency in later years; during 1983–2003, a total of 25 outbreaks (313 cases) were reported, whereas 47 outbreaks (495 cases) were reported in the subsequent 9 years.

Direct or indirect contact with animals through the environment accounted for 22.4% of outbreaks and 17.3% of patients linked to outbreaks. The number of petting farm outbreaks increased during the study period. During 1983–2002, a total of 12 petting farm outbreaks were reported; during 2003–2012, a total of 31 outbreaks on petting farms were reported, including, in September 2009, the largest reported farm outbreak, which affected 93 persons (*9*).

Most outbreaks were caused by the most frequently detected STEC O157 PTs, including PT21/28 (117 [34.9%] outbreaks), PT2 (79 [23.6%] outbreaks), and PT8 (42 [12.5%] outbreaks). In accordance with the general trends in PT, PT2 outbreaks declined over time, whereas PT8 and PT21/28 outbreaks increased. For outbreaks attributed to contact with animals or their environments, almost half (28 [47.5%] outbreaks) were caused by PT21/28 strains, a further 16 (27.1%) by PT2 strains, and only 4 (6.8%) by PT8 strains. Ten outbreaks attributed to contaminated water were caused by PT2 (5 outbreaks), PT21/28 (4 outbreaks), and PT4 (1 outbreaks); none were caused by PT8. In foodborne outbreaks, 25 (28.1%) were caused by PT2, 32 (36.1%) by PT21/28, and 20 (22.5%) by PT8.

Discussion

Our review provides a historical perspective contributing to the evidence of the evolving epidemiology of STEC O157. The data capture a strain replacement event showing the dramatic decline in PT2 and the increase and dominance of PT8 and PT21/28. Outbreak settings and vehicles also changed during the study period; prison, hospital, and care-home outbreaks decreased, and outbreaks in childcare facilities increased. Additionally, outbreaks associated with meat and milk decreased, and outbreaks attributed to petting farms increased. These data support previous reports that PT21/28 is indigenous to Great Britain and PT8 is largely imported, because most PT8 outbreaks were foodborne and a greater proportion of PT21/28 were attributed to environmental or animal contact (*19*,*21*).

The reasons for the decline in STEC incidence during 2000–2004 are unknown and cannot be attributed to any particular event or intervention, although several possible explanations exist. After a large STEC O157 outbreak in central Scotland in 1996 (*8*), specific interventions were implemented throughout the entire United Kingdom in catering, retail, and meat hygiene sectors to reduce the risk for infection. These included butchers' licensing, legislation, and enforcement of Hazard Analysis and Critical Control Point systems; amendment of the Food Standards Agency Code of Practice; and introduction of the Clean Livestock Policy, which aimed to reduce contamination by feces or mud on the coats and fleeces of animals for slaughter (*24*). The effectiveness of these policies was apparent through the shift in causes of outbreaks presented in this study; after their implementation, outbreaks caused by cross-contamination from raw meat clearly declined.

Why the decline in STEC incidence was not sustained beyond 2004 is unclear; however, declining numbers were observed in the United States in 2003 and 2004, followed by increases beginning in 2005. The decline coincided with industry measures aimed at reducing contamination of ground beef; however, as in the United Kingdom, the reason for the subsequent increase is unknown (*25*). Apparent changes in sources and outbreak settings might indicate changes in food vehicles or transmission routes among all cases, and although earlier interventions successfully controlled transmission of STEC infection, other effective transmission routes have taken hold in more recent years.

Also, in this study, outbreak detection relied on the classic epidemiologic triad of person, place, and time, along with PT. Any outbreaks dispersed over time, or of a common PT, might have gone undetected. Data collected on outbreaks and sources—and therefore trends—will be incomplete.

Farming methods and destruction of animal populations changed considerably during the study period after concerns about bovine spongiform encephalopathy in 1996 and foot and mouth disease in 2001. The decline of PT1, PT2, and PT49, and the corresponding emergence of PT21/28, was mirrored in Scotland (*26*) and suggests a strain replacement event. The destruction and restocking of UK cattle herds after concerns about bovine spongiform encephalopathy and foot and mouth disease might have been a causative factor. In Ireland, PT32 is the most commonly reported PT (*27*); PT21/28 is rarely detected outside the British Isles (*19*).

Improvements in data collection during our study led to increased ascertainment of epidemiologic data during the 30-year period alongside important developments in microbiological methods. Thus, the sustained incidence of infection could be a surveillance artifact, masking the success of interventions through increasing case ascertainment, a potential bias when datasets spanning many years, such as this one, are analyzed. In England and Wales, although surveillance of clinical STEC infections is routine, no surveillance programs are ongoing to monitor the prevalence of STEC in cattle or other animals. Efforts by the agricultural, veterinary, and food industries to monitor STEC incidence and strain types would inform the success of interventions and provide insight into the ecology of the pathogen. However, STEC rarely causes disease in animals, and funding is limited for such programs in England and Wales. In Europe, current monitoring information is generated from outbreak investigations and ad hoc studies skewed toward foodborne transmission of STEC O157 and might be limited in assessing the role of environmental transmission.

As described previously, infection is highest in children and females (*5*,*19*,*28*). Children 1–4 years of age had 7 times the risk of persons ≥60 years of age, probably because of a complex interplay of various factors, such as host immunity or reporting artifacts, with children more likely to seek care at healthcare settings (*29*). Additionally, the propensity for household transmission of STEC O157 (*30*) might be exacerbated by children having poorer hygiene practices that increase exposure to STEC O157 from the environment (*19*), and the potential for prolonged excretion in children (*7*). Children were more often infected with STEC O157 Stx2-only strains, associated with more severe disease (*4*,*5*,*21*,*31*), which might in part explain why cases occurred more often in children, because they were more likely to require care at healthcare settings. The higher crude incidence rates for female than for male patients has been reported previously (*19*,*28*); the reasons are unknown but might reflect biologic host factors, differences in health-seeking behavior, or other behaviors placing women at increased risk for infection, such as having contact with children or being primary household food handlers (*19*,*32*).

In our review, crude incidence was higher in the north and southwest than in the central and southeastern areas of England. Crude incidence in Scotland is consistently higher still (*19*). Previous studies have described such geographic variation and demonstrated that differences reflect differences in weather, land use, or environmental exposure between persons living in or visiting rural areas and those in urban areas, fitting with environmental transmission of STEC O157 (*19*,*33*).

Our 30-year review captures the emergence of a clinically significant zoonotic pathogen in a well-characterized population sample and documents the effectiveness of improvements in epidemiologic and microbiological methods on ascertaining STEC O157. However, despite interventions that successfully shifted outbreak settings, these organisms persist in causing illness in England and Wales, and the crude incidence of STEC O157 has remained relatively stable. Robust studies are required to assess the effectiveness of interventions, which currently remain unclear, and to consider future policies and guidance to reduce STEC O157 infection in England and Wales in the context of the complex interaction between the organism, reservoir, food chain, and transmission pathway.

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August 2015: **Surveillance**

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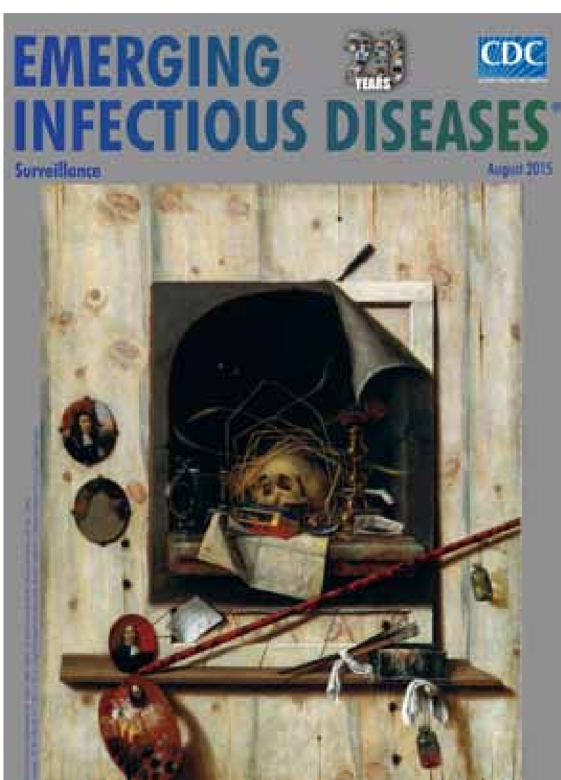

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Nosocomial Co-Transmission of Avian Influenza A(H7N9) and A(H1N1)pdm09 Viruses between 2 Patients with Hematologic Disorders

Huazhong Chen,[1] Shelan Liu,[1] Jun Liu,[1] Chengliang Chai, Haiyan Mao, Zhao Yu, Yuming Tang, Geqin Zhu, Haixiao X. Chen, Chengchu Zhu, Hui Shao, Shuguang Tan, Qianli Wang, Yuhai Bi, Zhen Zou, Guang Liu, Tao Jin, Chengyu Jiang, George F. Gao, Malik Peiris,[2] Hongjie Yu,[2] Enfu Chen[2]

A nosocomial cluster induced by co-infections with avian influenza A(H7N9) and A(H1N1)pdm09 (pH1N1) viruses occurred in 2 patients at a hospital in Zhejiang Province, China, in January 2014. The index case-patient was a 57-year-old man with chronic lymphocytic leukemia who had been occupationally exposed to poultry. He had co-infection with H7N9 and pH1N1 viruses. A 71-year-old man with polycythemia vera who was in the same ward as the index case-patient for 6 days acquired infection with H7N9 and pH1N1 viruses. The incubation period for the second case-patient was estimated to be ≤4 days. Both case-patients died of multiple organ failure. Virus genetic sequences from the 2 case-patients were identical. Of 103 close contacts, none had acute respiratory symptoms; all were negative for H7N9 virus. Serum samples from both case-patients demonstrated strong proinflammatory cytokine secretion but incompetent protective immune responses. These findings strongly suggest limited nosocomial co-transmission of H7N9 and pH1N1 viruses from 1 immunocompromised patient to another.

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As of January 4, 2016, a novel avian influenza A virus, A(H7N9), first identified in China in March 2013 (*1*), had caused 676 laboratory-confirmed cases of influenza in humans and 275 influenza-associated deaths in mainland China (Chinese Center for Disease Control and Prevention, unpub. data). Most H7N9 virus infections have been acquired through exposure to live poultry markets (LPMs) in urban settings (*2*) and have been sporadic, but a few have occurred in clusters of >2 epidemiologically linked cases (*3*). Human-to-human transmission is difficult to prove because exposure to a common source often cannot be excluded. Nosocomial transmission of H7N9 virus has recently been reported in China's Zhejiang Province (*4*). We investigated possible nosocomial co-transmission of H7N9 and influenza A(H1N1)pdm09 (pH1N1) viruses between 2 immunocompromised patients in Zhejiang Province.

Methods

Patients and Samples Collection

China's national surveillance system for influenza-like illness (ILI), severe acute respiratory illness, and pneumonia of unexplained origin indicated a cluster of 2 H7N9 virus infections occurring in the same ward at Taizhou Hospital in Zhejiang Province, China, during January 10–15, 2014. We collected throat swabs and serum from the 2 affected case-patients for virologic, serologic, and cytokine studies. We collected specimens from the upper respiratory tract by using pharyngeal swabs or from the lower tract by using bronchoalveolar lavage. These specimens were collected on January 19 and 21 from the index case-patient and the second case-patient, respectively.

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[2]These authors contributed equally to this article.

Controls
We retrieved stored serum samples from 21 other H7N9 virus–infected patients (mean age 60 years, range 44–76 years) from Zhejiang Province (mean number of days from onset 6 days, range 2–10 days). Serum samples from 6 healthy volunteers were collected for use as controls.

Investigation of Contacts
Close contacts were defined as described (*1*); all health-care workers, patients sharing the same ward, and those patients' family members were included. Close contacts were placed under daily active surveillance for 7 days. After written informed consent was obtained, a structured questionnaire was used to gather information on demographic characteristics, exposure history, and clinical outcome. Throat swab samples for H7N9 virus testing were taken from all close contacts during the observation period, and convalescent-phase serum samples were collected 3–4 weeks after the last exposure to a patient with H7N9 virus infection.

Laboratory Methods
Specific real-time reverse transcription PCR (rRT-PCR) assays for seasonal influenza viruses (H1, H3, and B) and avian influenza viruses (H5N1 and H7N9) were conducted as described (*5*). All specimens found to be positive for virus RNA were inoculated into MDCK cell cultures for virus isolation (*2*). Virus genetic sequences were obtained directly from clinical specimens or from virus isolates by using an MiSeq desktop sequencer (Illumina, Inc., San Diego, CA, USA) as described (*6*–*8*). We designed 3 different controls to verify results: the H7N9 RNA negative and positive controls and an MDCK cell control.

Microneutralization (MN) and hemagglutination inhibition (HI) assays were performed by using A/Anhui/1/2013(H7N9) virus antigen in accordance with World Health Organization protocols (*9*). A titer of >1:40 was defined as seropositive.

Concentrations of cytokines and chemokines in serum were measured by using the Bio-PlexPro Human Cytokine Array 27-Plex Group I and 21-Plex Group II (Bio-Rad, Hercules, CA, USA). Raw data were analyzed by using xPONENT (Merck Millipore, Darmstadt, Germany).

Statistical Analyses
A value of 0.1 pg/mL for cytokine level was assumed for statistical purposes in cases in which the concentration was undetectable. The 95% CIs for the cytokine levels of healthy controls and the control patients with H7N9 virus infection were generated by using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA).

Ethics
This research was determined by the China's National Health and Family Planning Commission to be part of a continuing public health outbreak investigation and therefore exempt from institutional review board assessment. The protocol for collection of epidemiologic data and serologic testing of close contacts was approved by the institutional review board of the Zhejiang Provincial Center for Disease Control and Prevention. Written informed consent was obtained from all contacts and controls who participated.

Results

Clinical Features of the 2 Confirmed Cases
The index case-patient was a 57-year-old man with untreated, Rai stage IIIA chronic lymphocytic leukemia, which manifested itself as absolute lymphocytosis, anemia, and cervical lymphadenopathy but was otherwise asymptomatic. On January 8, 2014, the patient experienced onset of fever, chills, and mild cough with sputum (Figure 1). He had not received a seasonal influenza vaccination. Two days later, he was admitted to the hematology ward at Taizhou Hospital (bed 26) because of continued fever (Figure 2; online Technical Appendix Figures 1 and 2, http://wwwnc.cdc.gov/EID/article/22/4/15-1561-Techapp.pdf). Chest radiography performed 4 days after disease onset indicated consolidation of the left lower lung (online Technical Appendix Figure 3). On day 7 of illness, the patient experienced rapid respiratory deterioration that required mechanical ventilation. He was transferred to the intensive care unit (ICU) of the respiratory ward (bed 3) (online Technical Appendix Figure 4). Oral oseltamivir (75 mg twice a day) was started on day 10 of illness. A throat swab sample collected on day 11 of illness was positive for H7N9 and pH1N1 viruses. The patient was then moved to the department of infectious diseases (bed 10) for isolation (online Technical Appendix Figure 5). On day 10 of illness, the geometric mean titer of the H7-specific antibody from MN was 63.5. On day 15 of illness, the patient died of complications, including acute respiratory distress syndrome, septic shock, and multiorgan failure (Figure 1; Table 1).

The second case-patient was a 71-year-old man with diabetes who had been undergoing insulin therapy for 20 years. He had received no vaccination against seasonal influenza. He had polycythemia vera that had been diagnosed 5 years before and was undergoing cytoreductive therapy with interferon and hydroxyurea. On December 30, 2013, he was admitted to a hospital in Jiaojiang District (also located in Taizhou) for mild fever (37.8°C) that was compatible with adverse effects from interferon therapy (Figure 1). The fever resolved by January 5, 2014. On January 8, the patient was transferred to the hematology ward (bed 27,

next to the index case-patient) in Taizhou Hospital to optimize clinical management of his polycythemia vera (Figure 2). He remained afebrile until January 14, when he experienced a high fever (39.7°C) and productive cough. One day later, the patient received inhalation and parenteral methylprednisolone therapy for asthma. On January 19, shortness of breath developed, and he was moved to the hospital ICU, where tracheal intubation and mechanical ventilation

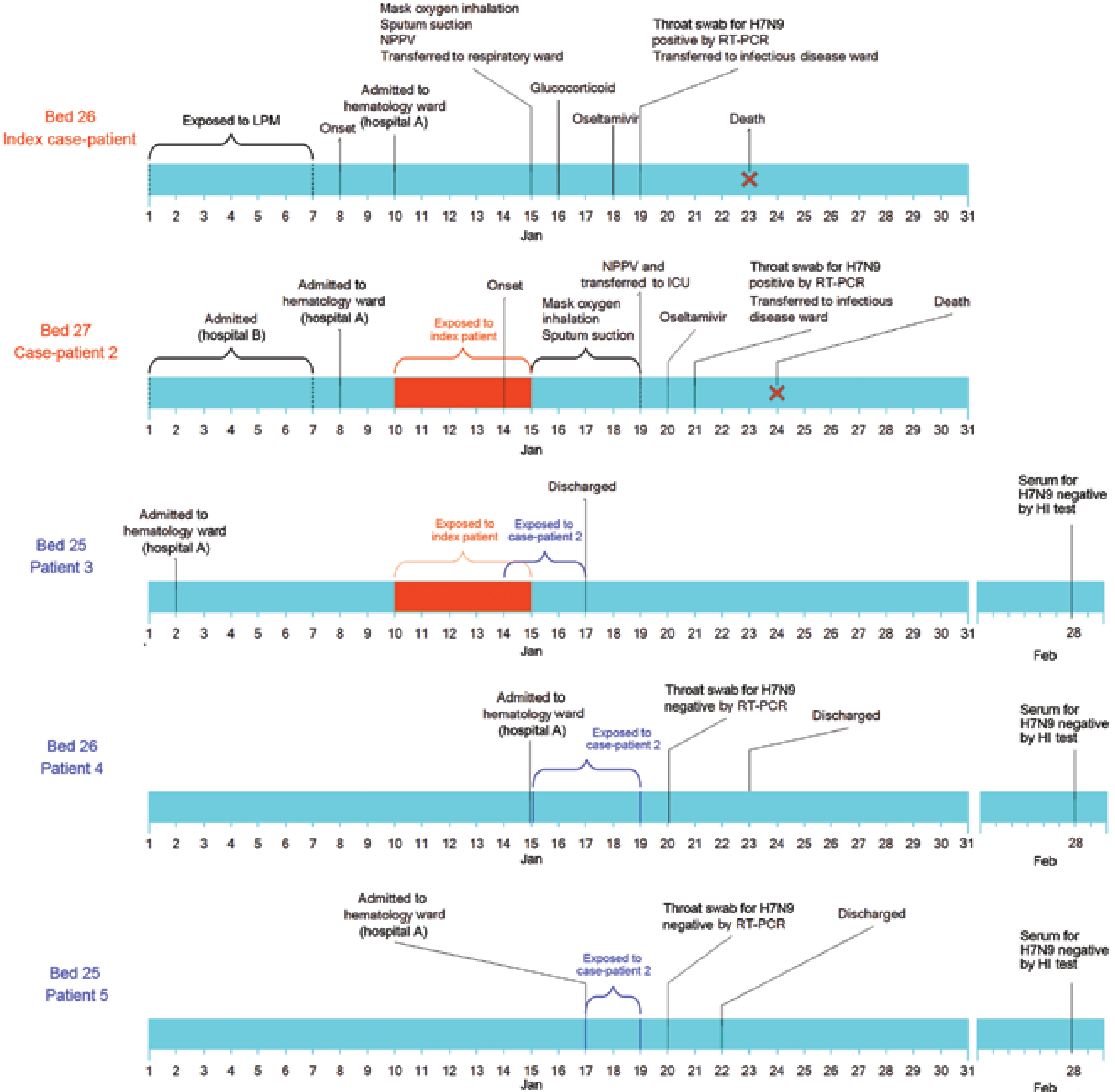

Figure 1. Timeline of pertinent exposures, dates of illness onset, and virologic findings for 2 patients (index case-patient and case-patient 2) who were co-infected with avian influenza A(H7N9) and A(H1N1)pdm09, and 3 non–H7N9-infected patients who shared the same hematology ward, Taizhou Hospital (hospital A), Zhejiang Province, China, January 10–15, 2014. Orange box indicates the period when patients 2–5 were exposed to the index case-patient. Blue line indicates that the period when the 3 non–H7N9-infected patients (3–5) were exposed to case-patient 2. Index case-patient was a 57-year-old man with confirmed co-infection with H7N9 and pH1N1 viruses. Case-patient 2 was a 71-year-old-man also with confirmed co-infection with H7N9 and pH1N1 viruses. Patient 3 was a 78-year-old man with chronic B lymphoma cell leukemia. Patient 4 was a 50-year-old man with acute myeloid leukemia. Patient 5 was a 61-year-old man with macroglobulinemia. H7N9, avian influenza A(H7N9) virus; HI, hemagglutination inhibition; ICU, intensive care unit; LPM, live bird market; NPPV, noninvasive positive pressure ventilation; pH1N1, influenza A(H1N1)pdm09 virus; rRT-PCR, real-time reverse transcription PCR.

were initiated (online Technical Appendix Figure 6). The next day, oseltamivir treatment (75 mg 2×/d) was initiated, and throat swab samples collected for virology testing were positive for influenza H7N9 and pH1N1 viruses. The patient was transferred to the Infectious Disease Department on January 21 (online Technical Appendix Figure 7). The geometric mean titers obtained through H7N9 virus MN on day 9 of illness was 113.1. The patient died on day 10 of acute respiratory distress syndrome and multiple organ failure (Figure 1; Table 1).

Epidemiologic Links and Exposure History

The index case-patient lived with 4 family members in a 3-floor house in Linhai District. The second case-patient lived with 2 family members in an apartment in Jiaojiang District and had not come into contact with the index case-patient before being hospitalized. No pets, domestic animals, or birds were present in the immediate vicinity of the residences of either patient. However, the index case-patient worked daily as a butcher in an LPM near his residence during the 2 weeks before illness onset; the last known exposure in the LMP occurred on January 7 (online Technical Appendix Figures 8, 9). The second case-patient had no history of exposure to live birds or LPMs in the 2 weeks before his hospital admission. The 2 patients had no history of eating poultry or known contact with a person with febrile illness in the 2 weeks before onset.

The second case-patient (in bed 27) shared the same room and was in the adjacent bed to the index case-patient (in bed 26) during January 10–15 (Figure 2). During this period, the index case-patient remained mostly confined to his bed, whereas the second patient was ambulatory. Aerosol-generating procedures were performed for the index case-patient on January 14–15, including continuous oxygen mask inhalation and negative pressure suction. Endotracheal intubation and bag valve mask ventilation was used for 2 hours and mechanical ventilation for 5 hours until he was transferred to the ICU on January 15. During January 10–15, the index case-patient had continued high fever with sweating and was washed

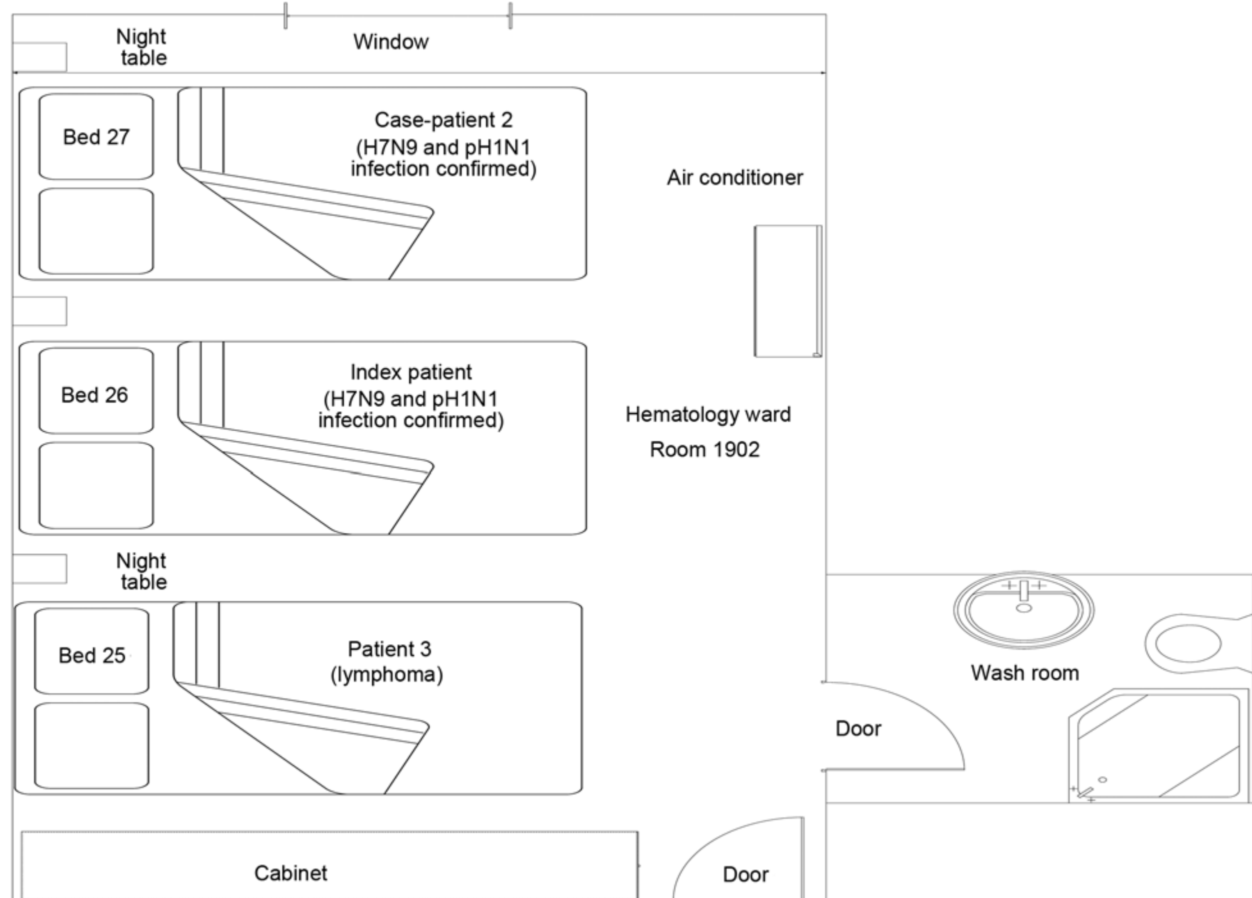

Figure 2. Schematic floor plan of the hematology ward where 2 case-patients with confirmed avian influenza A(H7N9) and A(H1N1) pdm09 virus co-infection and 1 non–H7N9-infected patient stayed, Taizhou Hospital, Zhejiang Province, China, January 10–15, 2014. The room was 22.4 m^2 (6.4 m × 3.5 m) in floor area, with 1 door (30 cm × 40 cm) and 1 window (105 cm × 20 cm), and 0.6 m of space separated the beds of the patients. H7N9, avian influenza A(H7N9) virus; pH1N1, influenza A(H1N1)pdm09 virus.

Table 1. Selected characteristics of 5 patients who shared the same hematology ward at Taizhou Hospital, Zhejiang Province, China, January 2014*

Characteristic	Index case-patient	Case-patient 2	Patient 3	Patient 4	Patient 5
Demographics and medical history					
Age, y	57	71	78	50	61
Sex	M	M	M	M	M
Type of residence	Rural	Urban	Urban	Rural	Rural
Occupation	Farmer	Retiree	Retiree	Farmer	Farmer
History of smoking	No	Yes	No	No	No
History of alcohol use	No	No	No	No	No
Obesity	No	No	No	No	No
Underlying conditions	Chronic lymphocytic leukemia; hernia operation	Polycythemia vera; cerebral infarction; diabetes	B cell lymphocytic leukemia; hypertension	Acute myeloid leukemia (M2a)	Macroglobulinemia; gout; kidney stone
Chronic drug use	No	Yes	Yes	Yes	Yes
No. family members	5	3	6	4	5
Exposure history					
Birds, other animals at home	Yes	No	No	Yes	No
Visited an LPM	Yes	No	No	Yes	No
Exposure to a febrile person	No	Yes	Yes	Yes	Yes
Duration of exposure, d (source)	—	5 (index patient)	5 (index patient); 11 (patient 2)	4 (patient 2)	3 (patient 2)
Specimen collection date (type)	Jan 19 (throat swab and serum)	Jan 21 (throat swab and serum)	Jan 26 (Serum)	Jan 20 (throat swab); Jan 26 (serum)	Jan 20 (throat swab); Jan 26 (serum)
Diagnostic method	Virus isolation; rRT-PCR; HI	Virus isolation; rRT-PCR;HI	HI	rRT-PCR;HI	rRT-PCR;HI
Date of confirmation	Jan 19	Jan 21	NA (negative)	NA (negative)	NA (negative)
Laboratory results	H7N9 and pH1N1 positive	H7N9 and pH1N1 positive	Negative	Negative	Negative
Clinical features					
Exposure to onset, d	1	4	NA	NA	NA
Onset to admission, d	2	6	NA	NA	NA
Onset to ICU, d	7	5	NA	NA	NA
Onset to antiviral drugs, d	10	6	NA	NA	NA
Onset to death, d	15	10	NA	NA	NA
Days in hospital	13	16	23	9	5
Date of outcome	Jan 23	Jan 24	Jan 17	Jan 23	Jan 22
Outcome	Died	Died	Survived	Survived	Survived

*H7N9, avian influenza A(H7N9) virus; HI, hemagglutination inhibition; ICU, intensive care unit; LPM, live poultry market; NA, not available; pH1N1, influenza A(H1N1)pdm09 virus; rRT-PCR, real-time reverse transcription PCR.

and had clothes changed by his wife. Neither patient had vomiting or diarrhea. The ward floor and surfaces were disinfected by using domiphenbromide (1:400 concentration) twice a day.

Three other patients shared the same ward during this period (Figure 2; Table 1). Bed 25 was occupied during January 2–17 by patient 3, a 78-year-old man with chronic B cell leukemia, and during January 17–22 by patient 5, a 61-year-old man with macroglobulinemia. After the index case-patient was transferred to the ICU, during January 15–23, bed 26 was occupied by patient 4, a 50-year-old man with acute myeloid leukemia (Figure 1). Patient 3 was H7N9 virus antibody–negative by HI test 42 days and 44 days after his most recent exposures to the second case-patient and index case-patient, respectively. Patients 4 and 5 were negative by rRT-PCR for influenza A viruses 1 day after their most recent exposures and were negative for H7N9 virus antibody by HI test 40 days after their most recent exposure to the second case-patient. Because the diagnosis of H7N9 virus infection was not suspected during their stay in this room, neither the index case-patient nor the second case-patient wore a protective mask, and the 25 staff or visitors who visited the room did not use any personal protective equipment.

Investigation of Contacts

A total of 68 close contacts of the index case-patient were identified, including 2 patients in the hematology ward, 7 patients in the ICU of the respiratory ward, 51 healthcare workers, 4 household members, and 4 social contacts. The median duration of exposure to the index case-patient was 3.5 hours (interquartile range [IQR] 2.5–6.5 hours]. No close contacts reported taking oseltamivir chemoprophylaxis. A total of 64 close contacts of the second case-patient were identified, including 3 patients in the hematology ward, 6 patients in the ICU, 51 healthcare workers, 2 household members, and 2 social contacts. None of the contacts developed acute respiratory symptoms. The

median duration of exposure to at least 1 of the case-patients was 2 hours (IQR 1–3 hours) (Table 1).

Of the contacts of the index case-patient in the hematology ward, 31 close contacts (2 patients, 21 healthcare workers, 4 household members, and 4 social contacts) were traced. During the 7-day surveillance period, acute respiratory symptoms developed in 1 of 2 patients who shared the room in the hematology ward and in 2 of 4 social contacts. On January 23, a total of 8 throat swabs were collected from 2 of patients in the hematology ward, 4 household members, and 2 social contacts. Of these, only 1 (case-patient 2) was found to be infected with H7N9 virus. For patient 2, a total of 28 close contacts (3 patients, 21 healthcare workers, and 4 household members) were traced, and none of them had symptoms of disease. Three throat swab samples collected from the 3 patients were negative for H7N9 virus.

During January 15–19, a total of 27 close contacts of the index case-patient when he was in ICU (20 healthcare workers and 7 patients sharing the ICU ward) were traced; 6 of the patients and 4 of the healthcare workers had throat swab samples collected. One of symptomatic patients, a 71-year-old man who had been in the ICU during January 10–19 and had chronic obstructive pulmonary disease, pneumonia, and chronic renal failure, was positive for pH1N1 virus by rRT-PCR on throat swab samples collected on January 20. A total of 26 close contacts of the second case-patient (20 healthcare workers and 6 patients sharing the ICU ward) were traced. None of them none of them had signs or symptoms of disease. Throat swab samples were not collected.

Ten healthcare workers had contact with the index case-patient and 10 with the second case-patient for the periods these patients were in the infectious disease ward. All throat swab samples collected during January 20–24 from these 20 contacts were negative for H7N9 and pH1N1 viruses by rRT-PCR. Overall, serum samples from 84 contacts who consented to have serum collected for testing were negative for H7N9 virus antibodies by MN and HI assays.

On January 20, a total of 10 environmental samples from the LPM that the index case-patient worked in before his illness (online Technical Appendix Figure 8) were collected; 1 of these was positive for H7N9 and H9N2 viruses, and another was positive for H7N9 virus. Two throat swab samples from workers in this LPM were negative for influenza A virus. Furthermore, throughout January 2014, environment surveillance results in Linhai District showed that 17 (60.71%) of 28 LMPs were positive for H7N9 virus (online Technical Appendix Figure 9).

Viral Genetic Sequence Analysis of H7N9 and pH1N1 Viruses

No virus isolate was obtained from the index case-patient, but the partial genome of the virus was obtained by direct sequencing from his pharyngeal swab specimens. Full-length membrane protein and nonstructural gene sequences and partial hemagglutinin and neuraminidase sequences were obtained. A virus isolate was obtained from the second case-patient, and the full virus genome was ascertained. Alignment of the membrane protein and neuraminidase sequences from the index case-patient with the H7N9 virus sequences from the virus isolated from the second case-patient showed that these 2 sequences shared 99.5% and 100% nucleic acid sequence identity, respectively. Both case-patients had viruses that were oseltamivir-sensitive (E120V, H276Y and R294K in neuraminidase protein) but amantadine-resistant (S31N in matrix 2 protein). The pH1N1 virus neuraminidase gene segment from the index case-patient was 100% identical to that from the second case-patient.

Serum Cytokine Levels

Serum levels of cytokines and chemokines in the index case-patient (10 days after illness onset) and the second case-patient (9 days after illness onset) were compared with those of 21 other patients infected with H7N9 virus. Levels of 13 cytokines and chemokines (granulocyte colony–stimulating factor, growth-regulated oncogene-a, interleukin [IL] 10, IL-12p40, IL-16, IL-18, IL-1 receptor antagonist, IL-2 receptor antagonist, IL-3, IL-6, IL-8, leukemia inhibitory factor, monocyte chemoattractant protein-1, monocyte chemoattractant protein 3, and stromal cell–derived factors) from the index case-patient and the second case- patient were higher than the upper bound of the 95% CIs of the mean levels of other patients infected with H7N9 virus (Table 2; online Technical Appendix Figure 10). In contrast, levels of interferon-gamma (IFN-g), IL-2, IL-12, and IL-4, which are correlated with adaptive immune responses to influenza viruses, were comparable to those observed in healthy persons but much lower than those observed in nonimmunocompromised H7N9 virus–infected patients (online Technical Appendix Figure 11). The lack of these cytokines, which are associated with the development of T helper (Th) 1, Th2, and Th17 cells, might have led to an incapable adaptive immune response in these 2 case-patients and thereby enhanced the pathogenicity of the viruses.

Discussion

Although ≈600 cases caused by H7N9 virus have been reported, 41 clustered cases from 20 disease clusters have been reported. The clustered cases accounted for only 5.9% of the total cases, so there seems to be evidence of limited transmission (*10*). Our study found epidemiologic differences between 1 nosocomial cluster caused by co-infection with H7N9 and pH1N1 viruses and a previously documented family cluster induced by a single H7N9 virus.

Table 2. Cytokine and chemokine levels for controls compared with 2 patients with confirmed co-infection with avian influenza A(H7N9) and A(H1N1)pdm09 viruses, Taizhou Hospital, Zhejiang Province, China, January 2014*

Cytokine or chemokine	Healthy controls, n = 6		H7N9-infected controls. n = 21		Index case-patient	Case-patient 2	Change
	Mean	95% CI	Mean	95% CI			
G-CSF	27.6	21.9 to 33.3	47.5	32.1 to 62.9	3421.0	5,759.0	↑
GROa	260.2	79.6 to 440.8	164.8	89.4 to 240.2	320.8	757.4	↑
IL-10	0.8	0.4 to 1.2	17.8	12.9 to 22.7	42.2	234.1	↑
IL-12p40	719.8	59.5 to 1380.1	240.3	–28.2 to 508.8	5176.0	585.5	↑
IL-16	530.0	–71.7 to 1131.7	260.8	170.2 to 351.3	627.4	468.51	↑
IL-18	69.9	31.5 to 108.2	163.7	101.3 to 226.1	530.9	1,501.1	↑
IL-1Ra	111.8	89.5 to 134.1	218.3	164.3 to 272.2	506.7	1,340.1	↑
IL-2Ra	79.6	51.2 to 108.1	97.5	49.9 to 145.1	699.9	425.4	↑
IL-3	101.4	30.9 to 171.8	30.8	–0.8 to 62.4	815.3	210.4	↑
IL-6	4.1	2.8 to 5.4	91.5	50.7 to 132.3	819.6	22,009.0	↑
IL-8	7.0	2.7 to 11.4	62.2	45.5 to 79.0	1,228.3	2,075.3	↑
LIF	9.1	0.7 to 17.5	7.9	1.3 to 14.4	34.4	31.2	↑
MCP-1	7.0	2.9 to 11.0	238.4	91.8 to 384.9	569.7	757.4	↑
MCP-3	28.5	8.8 to 48.2	8.6	–1.4 to 18.7	28.8	39.5	↑
SDF-1a	83.0	43.4 to 122.6	72.6	37.3 to 107.9	641.8	273.7	↑
Extaxin	79.7	36.3 to 123.1	108.9	79.1 to 79.1	25.5	36.3	↓
GM-CSF	0.1	0.04 to 0.1	65.8	44.5 to 87.1	38.8	26.4	↓
IFN-γ	66.7	61.3 to 72.1	117.3	91.8 to 142.7	64.3	62.9	↓
IL-12 p70	8.0	5.3 to 10.7	32.7	25.7 to 39.7	18.0	6.0	↓
IL-2	3.9	1.9 to 5.8	17.8	13.2 to 22.4	5.3	7.9	↓
IL-4	2.9	2.2 to 3.5	8.4	6.5 to 10.4	1.2	1.7	↓
IL-5	1.7	1.1 to 2.2	7.2	4.5 to 10.0	2.1	1.8	↓
MIG	315.7	189.8 to 441.5	7,860.2	4245.5 to 11,474.9	2,246.4	429.8	↓
PDGF-bb	532.0	419.8 to 644.3	3,608.5	2,746.0 to 4,471.1	784.5	21.2	↓
RANTES	613.5	798.5 to 983.5	12,716.5	9,193.1 to 16,239.9	658.5	178.2	↓

*Levels are given in pg/mL. Arrows indicate cytokine and chemokine levels that were significantly higher (↑) or lower (↓) in the serum samples of the index patient and patient 2 compared with 95% CIs of the mean values of 21 other patients infected with the avian influenza A(H7N9) virus. G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony–stimulating factor; GROa, growth-regulated oncogene-α; IFN-γ, interferon-gamma; IL, interleukin; MCP, monocyte chemoattractant protein; MIG, macrophage-induced gene ; PDGF-bb, platelet-derived growth factor bb; Ra, receptor antagonist; RANTES, regulated on activation normal T cell expressed and secreted; SDF, stromal cell–derived factors.

The index case-patient had been exposed to an LPM that was retrospectively proven to be positive for influenza H7N9 virus. The second case-patient had no avian exposure but experienced onset of clinical disease within 4 days of close contact with the index case-patient. The second case-patient was co-infected with H7N9 and pH1N1 viruses, as was the index case-patient. Virus genetic sequences of H7N9 and pH1N1 viruses from the 2 case-patients were identical. These findings strongly suggest nosocomial human-to-human co-transmission of both influenza viruses in 2 non–blood-related patients with hematologic disorders. The findings also demonstrate a lack of transmission to other patients and healthcare workers, reinforcing the contention that H7N9 virus remains poorly transmissible from human to human.

In this particular event, the incubation period was 4 days, compatible with previous estimates of incubation period (*11*). This incubation period was more plausible than the incubation periods of 13 days, 7 days, and 10 days reported for the Jiangsu (*3*), Shanghai, and Guangdong clusters, respectively (*12,13*), where infection from a common source cannot be ruled out. In other family clusters, the index patient's illness was more severe, whereas most of the secondary cases were milder and rarely fatal (*14,15*). The greater disease severity in the second case-patient in our study might be attributable to the co-infection with influenza pH1N1 virus in combination with his underlying disease and the delayed initiation of antiviral therapy (*16*).

The exact route of transmission from the index case-patient to the second case-patient remains unclear. Transmission through respiratory droplets is possible because the index case-patient had repeated bouts of coughing, and he had several aerosol-generating procedures conducted during his hospitalization. Cross-infection through fomites, the unwashed hands of healthcare workers or visitors, or unsanitary medical equipment might also be possible. Before the 2 case-patients were confirmed to have H7N9 virus infection, no personal protective equipment was used.

Serologic methods rarely yield an early diagnosis of influenza virus. Apart from their retrospective diagnostic value, serologic methods are applied in epidemiologic and immunologic studies. In our study, we conducted HI and MN tests for all contacts of the 2 case-patients under negative and positive quality control. However, we did not detect any serologic evidence of secondary transmission in approximately 84 contacts. The basic reproduction number (R0), a measure of transmission potential of H7N9 virus compared with pH1N1 virus, was 0.1 versus 1.7, indicating that the transmission potential of H7N9 virus was much

lower than that of pH1N1 virus. Thus, prolonged exposure and the immunocompromised status of the 2 case-patients might have contributed to the transmission of infection. Co-infection of H7N9 and seasonal influenza A(H3N2) viruses has been reported in a patient from Jiangsu Province, China, but this patient recovered, possibly because of his young age (15 years) and early initiation of antiviral treatment; evidence of secondary transmission was not actively sought (*17*).

Patients with hematologic malignancy, such as chronic lymphocytic leukemia and polycythemia vera, have multiple immune defects (*18–21*), including defects in T-cell function and natural killer cell dysfunction, decreased antibody responses, and abnormal cytokine production (*21*). Patients with such disorders are thus more prone to new infections and prolonged virus shedding (*18,20,22–28*). For example, a patient with chronic lymphocytic leukemia who was not in an ICU was infected with pH1N1 virus and continued to shed virus for 59 days; antiviral resistance developed, consistent with the patient's immunocompromised condition (*28*). In our study, the index case-patient was confirmed as H7N9 virus–positive on day 11 after illness onset, which suggests prolonged viral shedding in older, immunocompromised patients. However, the consequences of H7N9 patients with hematologic malignancies are poorly understood in terms of clinical outcomes and transmission potential.

Innate immune dysregulation might contribute to the pathogenesis of severe avian influenza (*29*). In our study, the 2 H7N9 virus–infected case-patients who died had chronic lymphocytic leukemia and polycythemia vera, respectively. Chronic lymphocytic leukemia is associated with dysfunction of innate and adaptive immunity (*21,30,31*). Cytokines, such as IL-8, associated with acute respiratory distress syndrome (*32*), and IL-6, associated with severe cytokine-release syndrome, can occur in chronic lymphocytic leukemia patients after treatment with rituximab, an anti-CD20 monoclonal antibody (*33*). The underlying disease (polycythemia vera) in the second case-patient is associated with Janus kinase 2 mutation V617F, which leads to constitutive tyrosine phosphorylation activity that promotes cytokine hypersensitivity, including IL-3 and stem cell factor (*34,35*). The abnormal signaling pathway in cells of patients with polycythemia vera can also contribute to growth of fibroblasts and microvascular endothelial cells and induce the production of profibrogenic and angiogenic cytokines (*36*).

Hypercytokinemia has been reported in the peripheral blood of H7N9 virus–infected patients with substantially elevated levels of cytokines and chemokines (e.g., IL-1b, IL-6, IL-8, IL-10, IL-12, IL-17, monokine induced by IFN-g, macrophage inflammatory proteins 1a and 1b, monocyte chemotactic protein 1, interferon-induced protein of 10 kDa, and regulated on activation normal T cell expressed and secreted [RANTES]) (*5,29,37,38*). Despite underlying immune defects in the context of steroid therapy, the 2 H7N9 virus–infected case-patients in our study mounted strong proinflammatory cytokine responses (e.g., production of IL6, IL8, and monocyte chemotactic protein 1), even stronger than those observed in other nonimmunocompromised patients with H7N9 infection. On the other hand, levels of cytokines known to correlate with immune protection against influenza viruses, such as IL-2, IL-4 and IFN-g, which contribute to development of Th1, Th2, and Th17 cells, were observed to be much lower in the 2 case-patients reported here compared with other H7N9 virus–infected patients. Thus, disorders of innate immune and adaptive immune responses in these 2 case-patients might have contributed to the disease severity and fatal outcomes.

In summary, our findings strongly suggests nosocomial human-to-human co-transmission of H7N9 and pH1N1 viruses between 2 immunocompromised case-patients with hematologic diseases, with no evidence of transmission to others. The deaths of these 2 case-patients might have been attributable to co-infection with pH1N1 virus, delayed initiation of antiviral therapy, and the patients' immunocompromised status. These findings suggest the need for awareness and early testing for influenza in hematology units and for liberal use of early antiviral treatment if patients exhibit ILI symptoms. Implementing rigorous infection control practices might minimize cross-transmission. Avoiding the use of corticosteroids in patients with infection also needs to be emphasized.

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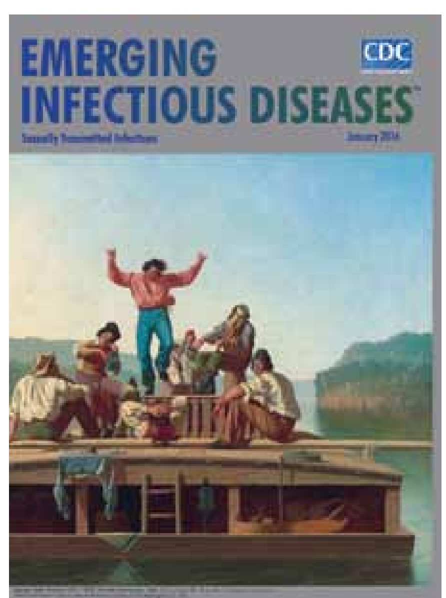

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RESEARCH

Quantifying Transmission of *Clostridium difficile* within and outside Healthcare Settings

David P. Durham, Margaret A. Olsen, Erik R. Dubberke, Alison P. Galvani, Jeffrey P. Townsend

To quantify the effect of hospital and community-based transmission and control measures on *Clostridium difficile* infection (CDI), we constructed a transmission model within and between hospital, community, and long-term care-facility settings. By parameterizing the model from national databases and calibrating it to *C. difficile* prevalence and CDI incidence, we found that hospitalized patients with CDI transmit *C. difficile* at a rate 15 (95% CI 7.2–32) times that of asymptomatic patients. Long-term care facility residents transmit at a rate of 27% (95% CI 13%–51%) that of hospitalized patients, and persons in the community at a rate of 0.1% (95% CI 0.062%–0.2%) that of hospitalized patients. Despite lower transmission rates for asymptomatic carriers and community sources, these transmission routes have a substantial effect on hospital-onset CDI because of the larger reservoir of hospitalized carriers and persons in the community. Asymptomatic carriers and community sources should be accounted for when designing and evaluating control interventions.

Infection with the nosocomial pathogen *Clostridium difficile* is a major risk in healthcare settings and long-term care facilities (LTCFs) and has an increasing prevalence in the broader community. Infection is diagnosed in ≥250,000 hospitalized persons annually in the United States (*1*). Colonization of the gut microbiota with *C. difficile* can be innocuous and asymptomatic. However, antimicrobial drugs disrupt the normal intestinal microbial architecture and can enable proliferation of *C. difficile* (*2*). An insufficient host antibody response to *C. difficile* toxins A and B can then lead to *C. difficile* infection (CDI). CDI is a severe diarrheal disease that is concentrated among elderly persons and those with extended hospital stays or residing in LTCFs. The relative risk for CDI, given recent antimicrobial drug exposure, differs greatly among antimicrobial drug classes and ranges from no relative risk when receiving tetracyclines to a 20-fold relative risk when receiving clindamycin (*2*). Despite an increasing interest in *C. difficile* biology and the epidemiology of CDI, fundamental questions about reservoirs and routes of transmission remain unanswered.

Molecular typing and contact tracing studies have estimated that 10%–38% of CDI cases that occur ≥48 hours after hospital admission (termed hospital-onset CDI) can be attributed to transmission from known symptomatic contacts within the hospital (*3–6*). These estimates suggest that a substantial proportion of CDI arises from other sources, such as transmission from patients with asymptomatic colonization or community acquisition (*3*,*5*,*7*,*8*). The relative role of these routes of transmission to the epidemiology of *C. difficile* is crucial for determining effectiveness of hospital-based measures to control infection. In addition, toxin-targeting treatments, such as vaccines, nontoxigenic *C. difficile*, and monoclonal antibodies, might protect against CDI but are unlikely to prevent asymptomatic colonization with *C. difficile* (*9*). To predict the effectiveness of these emerging therapies, it is critical to understand the role of asymptomatic carriers in CDI epidemiology.

Mathematical models of *C. difficile* colonization have generated insights regarding the epidemiologic role of antimicrobial drugs on CDI outbreaks (*10*). Such models have also quantified the effect of hospital-based control interventions (*11–14*) and demonstrated the crucial roles of asymptomatic colonization and patients with exposure before hospital admission in sustaining hospital transmission (*7*,*13*). Most studies have focused on the hospital setting. To fully understand the epidemiology of the pathogen and to inform decisions regarding control strategies, it is crucial to quantify the relative transmission of *C. difficile* in the hospital and in the broader community (*8*).

To evaluate the relative role of asymptomatic hospital transmission, symptomatic hospital transmission, LTCF transmission, and community transmission, we integrated diverse clinical and epidemiologic data into a dynamic model of *C. difficile* transmission within and among hospitals, LTCFs, and community settings in the United States. We parameterized our model by using Medicare and Healthcare Cost and Utilization Project databases and data from published epidemiologic and clinical research. To estimate infectivity of symptomatic and asymptomatic

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patients in the hospital; corresponding infectivity of persons in LTCFs and in the community; and average risks for acquiring *C. difficile* in the hospital, LTCF, and the community, we fit our model to estimated toxigenic *C. difficile* colonization and CDI incidence in each of these settings. Furthermore, we calculated the effect on CDI incidence of targeting key aspects of CDI epidemiology with control interventions in each of the 3 settings.

Methods

Definitions

We refer to acquisition of *C. difficile* from human sources as *C. difficile* transmission and acquisition of *C. difficile* from nonhuman sources as nonhuman acquisition. Asymptomatic persons carrying *C. difficile* are referred to as colonized. Persons carrying *C. difficile* and symptomatic for diarrheal disease associated with *C. difficile* are referred to as persons with CDI.

Model Structure

Previous models have focused almost exclusively on the hospital setting (7,8,10,12). We constructed a new model that encompasses C. difficile transmission and symptomatic CDI within a hospital, an LTCF, and an associated mid-sized community and quantifies patient movement between these settings. We parameterized our model with data from a combination of sources, including published literature, the US Census, national hospital and LTCF surveys, and the Healthcare Cost and Utilization Project and Medicare databases (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/4/15-0455-Techapp1.pdf).

We structured our model in compartments (Figure 1) composed of patients who are currently receiving antimicrobial drugs, those who have a history of antimicrobial drug use and an increased risk for CDI, or those who do not have a recent history of receiving antimicrobial drugs. Consistent with clinical observations (*15*), we assumed that the increased risk for CDI after antimicrobial drug use reverted to normal in an average of 45 days. Uncolonized patients could become asymptomatically colonized with *C. difficile* because of transmission from asymptomatic patients, transmission from patients with CDI, or through acquisition from background sources in the community. Asymptomatically colonized patients could remain asymptomatic, spontaneously clear their colonization, or develop symptomatic CDI. Patients with CDI could recover and be at temporarily increased risk for recolonization, could recover and remain colonized and at risk for recurrence, or could die from the disease. We included 3 CDI and recurrence classes, each with a successively higher likelihood of recurrence, to reflect clinical observations of the increasing likelihood of recurrence after multiple CDI episodes

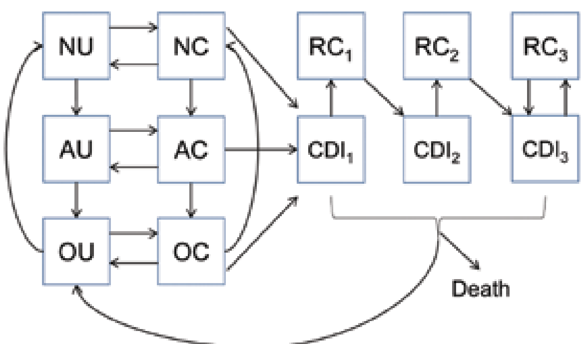

Figure 1. Compartmental model structure for *Clostridium difficile* infection (CDI) within each setting (hospital, long-term care facility, and community). Patients are classified as not receiving antimicrobial drugs (N), are receiving antimicrobial drugs (A), having a recent history of receiving antimicrobial drugs (O), uncolonized (U), asymptomatically colonized (C), symptomatically infected (CDI), or colonized and subject to recurrence (RC) of CDI. Arrows indicate changes in individual epidemiologic status. Subscripts indicate primary, secondary, or tertiary CDI.

(*16*–*18*). We assumed that all patients with CDI were first asymptomatically colonized before symptoms developed.

We embedded this epidemiologic model within a model of patient flow between the hospital, LTCF, and community (Figure 2), parameterized from national hospital and long-term-care-facility survey data. Patients with CDI remained hospitalized for an additional 3.1 days (95% CI 2.3–4.0 days) (*19*–*21*). Patients with CDI had a 96% (95% CI 93%–99%) probability of being given a diagnosis and subjected to isolation protocols that reduced transmission by 53% (95% CI 37%–72%) (*22*–*25*). We further assumed that persons in the community and in an LTCF in whom CDI developed were hospitalized with probabilities of 26% (95% CI 23%–28%) and 27% (95% CI 23%–32%), respectively (Table 1) (*26*,*27*).

Demographics

To represent demographically stratified CDI risk between the 3 settings, we modeled 5 demographic groups: persons <50 years of age, those 50–65 years of age without concurrent conditions, those 50–65 years of age with concurrent conditions, those >65 years of age without concurrent conditions, and those >65 years of age with concurrent conditions. Therefore, our full model consisted of base epidemiology (Figure 1) applied to each of the 5 demographic groups, and each group populated and moved between the hospital, LTCF, and the community (Figure 2) at rates calibrated from published C. difficile literature, US hospital discharge and census data, and Medicare and Healthcare Cost and Utilization Project databases (online Technical Appendix Table 4). We assumed that colonized patients with concurrent conditions are at greater risk for development of CDI (online Technical Appendix).

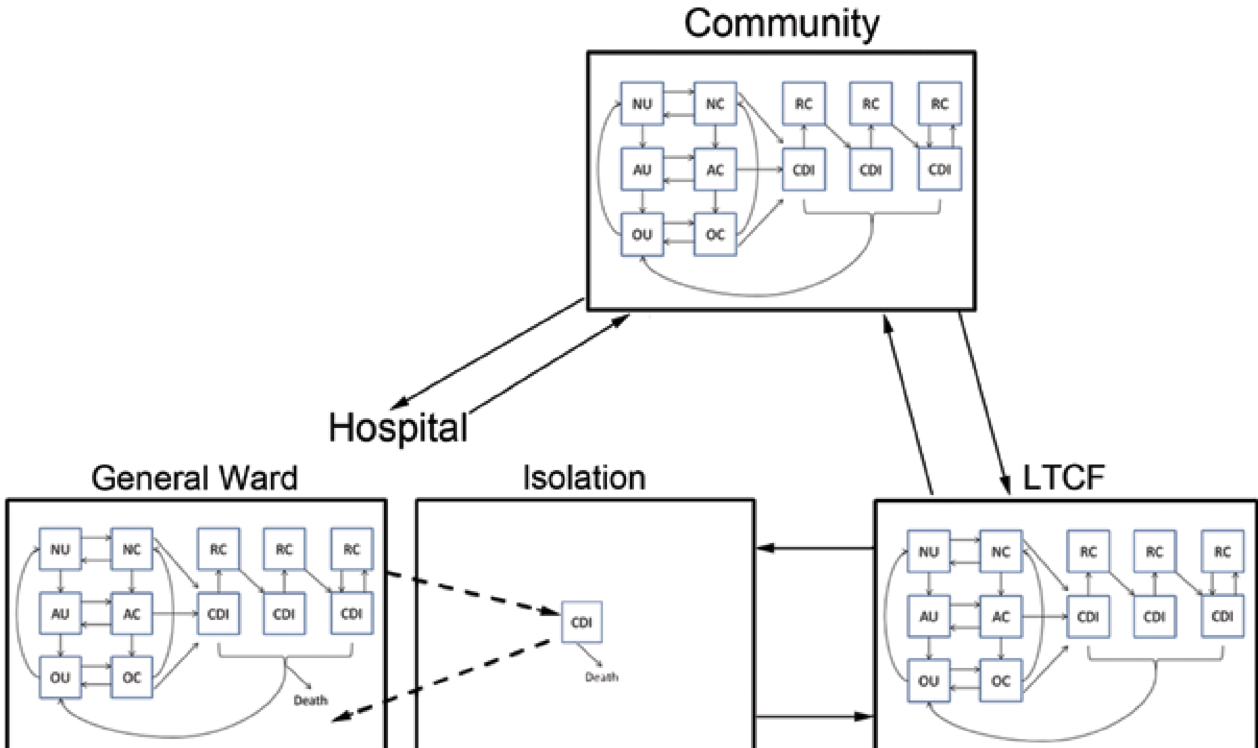

Figure 2. Transitions between settings (hospital, LTCF, and the non–healthcare community) for model structure of *Clostridium difficile* infection (CDI). Transitions were parameterized at demographically calibrated, age-specific rates. Hospitalized patients with CDI who were given a diagnosis are subject to enhanced isolation protocols that reduce transmission. All hospitalized CDI patients are discharged at a slower rate than non–CDI patients, which reflects longer hospitalization attributable to CDI. N, patients not receiving antimicrobial drugs; A, patients receiving antimicrobial drugs; O, patients with a recent history of receiving antimicrobial drugs; U, uncolonized patients; C, asymptomatically colonized patients; RC, symptomatically infected patients or colonized patients and subject to recurrence; LTCF, long-term care facility. Solid arrows indicate changes in individual epidemiologic status and patient movement between the hospital, community, and LTCF. Dashed arrows indicate isolation of CDI patients.

Transmission

We specified 5 *C. difficile* transmission rates: 1) the base CDI rate at which patients without a diagnosis and symptomatic CDI transmit in the hospital, 2) the base asymptomatic rate at which asymptomatically colonized patients transmit in the hospital, 3) the LTCF transmission rate representing the relative infectivity of persons in LTCFs compared with patients in the hospital, 4) the community transmission rate representing the relative infectivity of persons in the community compared with patients in the hospital, and 5) the rate of *C. difficile* acquisition from nonhuman reservoirs. We further defined the force of colonization as the rate at which uncolonized patients become asymptomatically colonized with *C. difficile* and specified 3 separate force-of-colonization rates: 1) the hospital, 2) LTCF, and 3) the community.

For the force of colonization in the hospital, we specified that nonisolated symptomatic patients with CDI transmit at the base CDI rate, that isolated patients with CDI transmit at the base CDI rate multiplied by the probability that isolation measures are insufficient, and that asymptomatically colonized patients transmit at the base asymptomatic rate. We assumed direct contact mixing and density-dependent transmission, which is consistent with the observation that larger hospitals have greater CDI incidence than smaller hospitals (*36*). Environmental contamination and transmission mediated by healthcare workers were implicitly included by our calibration of the base CDI rate and the base asymptomatic rate. Hospital hygiene was separated into 2 components: overall hospital hygiene, which influenced transmission from asymptomatically colonized patients and from undiagnosed patients with CDI; and the probability of, and effectiveness of, enhanced isolation protocols for patients given a diagnosis of CDI.

For the force of colonization in the LTCF, we made 3 assumptions. First, enhanced isolation protocols were not available. Second, patients with CDI transmit at the base CDI rate multiplied by the LTCF transmission rate modifier. Third, asymptomatically colonized patients transmit at the base asymptomatic rate multiplied by the LTCF transmission rate modifier.

Table 1. Epidemiologic and clinical model parameters for infection with *Clostridium difficile**

Parameter description	Prior rate (95% CI)†	Posterior rate (95% CI)†	Reference
Epidemiology			
All-cause CDI mortality rate, %			(*28*)
Age, y			
<50	4.7 (2.6–7.6)	4.5 (2.6–7.5)	
50–64	12 (8.7–16)	12 (8.5–16)	
>65	16.6 (14–19)	17 (14–19)	
Rate at which patients complete antimicrobial drug course	0.22 (0.17–2.29)	0.22 (0.17–2.29)	(*29*)
Rate at which recurrence develops in recovered patients	0.13 (0.24–1)	0.2 (0.32–1.05)	(*30*)
Rate at which patients not receiving antimicrobial drugs at increased risk for CDI revert to normal risk	0.038 (0.012–0.062)	0.033 (0.014–0.056)	(*15*)
Rate of recovery from CDI	0.099 (0.090–0.11)	0.099 (0.092–0.11)	(*22*)
Probability that a patient recovering from primary CDI will have >1 recurrence	22 (13–34)	24 (15–36)	(*16,17*)
Probability that a patient recovering from a first recurrence will have a second recurrence	33 (19–48)	34 (20–48)	(*16,17*)
Probability that a patient recovering from multiple recurrences will have an additional recurrence	56 (42–70)	56 (41–68)	(*17,18*)
Relative risk for CDI developing while a patient receives antimicrobial drugs	8.9 (4.9–13.)	8.3 (4.2–12)	(*2,15*)
Relative risk for CDI among persons 50–65 y of age vs. those <50 y of age	2.2 (1.4–3.4)	2.2 (1.5–3.0)	(*31*)
Relative risk for CDI among persons >65 y of age compared with those <50 y of age	2.9 (1.9–4.4)	3.2 (2.1–4.3)	(*31*)
Spontaneous clearance of asymptomatic *C. difficile* colonization	0.020 (0.015–0.025)	0.021 (0.016–0.026)	(*32*)
Hospital protocols			
All-cause fraction of community-onset CDI in patients who are hospitalized	0.26 (0.23–0.28)	0.26 (0.23–0.28)	(*26*)
All-cause fraction of LTCF-onset CDI in patients who are hospitalized	0.27 (0.23–0.32)	0.27 (0.23–0.32)	(*27*)
Increased attributable length of stay for hospitalized patients with CDI	3.1 (2.3–4.0)	3.1 (2.3–4.1)	(*19–21*)
Effectiveness of enhanced infection control measures in reducing transmission	53 (37–72)	52 (37–68)	(*22,23*)
Probability that a patient with CDI is properly identified and given enhanced infection control measures	0.96 (0.93–0.99)‡	0.96 (0.94–0.99)	(*24,25*)
Antimicrobial drug use rates			
Prescription rate among persons in community			(*33,34*)
Age, y			
<50	0.0013 (0.00095–0.0017)	0.0014 (0.00095–0.0018)	
50–64	0.0014 (0.00097–0.0018)	0.0014 (0.00097–0.0017)	
>65	0.0017 (0.0013–0.0021)	0.0017 (0.0013–0.0022)	
Prescription rate among patients in hospital	0.37 (0.22–0.66)	0.37 (0.21–0.68)	(*29*)
Prescription rate among patients in LTCF	0.0054 (0.0027–0.009)	0.0052 (0.0026–0.0087)	(*35*)

*CDI, *C. difficile infection*; LTCF, long-term care facility.
†Parameter rates are per day unless otherwise indicated.
‡A total of 73% of sites initiated protocols before laboratory confirmation and 27% initiated protocols after confirmation. Sensitivity was 86% for laboratory tests, which yielded an effective diagnosis rate of 0.73 + 0.27 × 0.86 = 0.96.

For the force of colonization in the community, we assumed that *C. difficile* could be acquired from nonhuman reservoirs (*37*), that patients with CDI transmit at the base CDI rate multiplied by the community transmission rate modifier, and that asymptomatically colonized patients transmit at the base asymptomatic rate multiplied by the community transmission rate modifier. Because there are insufficient published data with which to statistically differentiate between human transmission in the community and nonhuman acquisition, we estimated the force of colonization directly during our model calibration and then calculated the upper bounds for the community transmission rate modifier and for the rate of nonhuman acquisition.

Although age, history of antimicrobial drug use, and concurrent conditions are predictors of diarrheal CDI, they are not predictors of asymptomatic *C. difficile* colonization (*38,39*). Therefore, we assumed that the rate at which symptomatic CDI developed in colonized patients was dependent on age, antimicrobial drug use, concurrent conditions, and hospitalization status. Transmission parameters and force of colonization were independent of age, antimicrobial drug use or concurrent conditions (online Technical Appendix).

Calibration

We used the Markov Chain Monte Carlo Metropolis algorithm (40) to calibrate our stochastic model and combined prior parameter densities (Table 1) with epidemiologic data, including asymptomatic prevalence and CDI incidence in the hospital, LTCF, and community (online Technical Appendix Table 2). This analysis yielded an ensemble of 1,000 parameter sets that estimated the joint posterior distribution for parameters with prior literature estimates (Table 1) for the 5 transmission parameters and for the base rate at which CDI developed in asymptomatically colonized persons (Table 2). Details of coding, the stochastic model, and calibration are provided in the online Technical Appendix.

Epidemiologic Analysis

To estimate relative infectivity of a hospitalized patient with CDI compared with a hospitalized asymptomatically colonized patient, accounting for isolation protocols, we computed the ratio of 1) the base CDI transmission rate from a hospitalized patient with CDI multiplied by the probability that the patient is either not given a diagnosis or that isolation protocols are improperly implemented to 2) the base asymptomatic transmission rate from a hospitalized, asymptomatically colonized patient. To generate a posterior distribution for this ratio, we repeated this calculation for each of the 1,000 runs in our posterior sample. To estimate the average risk for a person to become exposed to and colonized with C. difficile, for each of the runs, we computed the average force of colonization within the hospital, community, and LTCF.

To estimate an upper bound for the community transmission rate and for nonhuman acquisition, we first computed the daily average community force of colonization, which represents the sum of *C. difficile* transmission from other persons in the community plus acquisition from nonhuman reservoirs. By setting the nonhuman acquisition rate to 0, we calculated an upper bound for the community transmission rate. Likewise, by setting the community transmission rate to 0, we calculated an upper bound for nonhuman acquisition. We repeated this step for each of the 1,000 runs and generated posterior distributions for the upper bounds of the community transmission rate and the nonhuman acquisition rate.

Control Strategy Analysis

To quantify the effect of transmission control interventions on CDI incidence, we varied each of the following factors: CDI diagnosis rate of a hospitalized patient with CDI, effectiveness of isolation protocols for a patient given a diagnosis, overall hospital hygiene, improvements in community transmission, and improvements in LTCF transmission across a range from 0 to double the model-fitted maximum likelihood estimate and while sampling all other model parameters from their posterior distributions. We used linear regression to determine the reduction for hospital-onset CDI, community-onset CDI, and LTCF-onset CDI incidence per 1% improvement in each transmission control intervention.

To compute the effect of different classes of antimicrobial drugs on CDI incidence, we varied the antimicrobial drug risk ratio in the hospital from 1, which is representative of low-risk antimicrobial drugs (e.g., tetracyclines), to 20, which is representative of high-risk antimicro- bial drugs (e.g., clindamycin) (*2*). While varying the antimicrobial drug risk ratio, we sampled all other parameters, including

Table 2. Calibrated posterior estimates of previously unknown epidemiologic parameters for infection with *Clostridium difficile**

Parameter description	Posterior rate (95% CI)
Hospital force of colonization†	0.023 (0.017–0.032)
Base CDI transmission rate within hospital†	1.2×10^{-2} ($0.65–2.1 \times 10^{-2}$)
Base CDI transmission rate within hospital accounting for isolation/control measures†	6.0×10^{-3} ($3.6–9.7 \times 10^{-3}$)
Base asymptomatic transmission rate within hospital†	4.0×10^{-4} ($2.4–5.5 \times 10^{-4}$)
Relative transmission from patients with CDI compared with asymptomatically colonized patients, accounting for isolation/control measures‡	15 (7.2–32)
LTCF force of colonization†	3.7×10^{-3} ($0.96–7.7 \times 10^{-3}$)
LTCF transmission rate, relative to hospital‡	0.13 (0.068–0.22)
LTCF transmission rate, relative to hospital, accounting for hospital CDI isolation/control measures‡	0.27 (0.13–0.51)
Community force of colonization†	1.2×10^{-3} ($0.50–2.3 \times 10^{-3}$)
Community transmission rate, relative to hospital‡§	5.2×10^{-4} ($3.3–8.9 \times 10^{-4}$)
Community transmission rate, relative to hospital, accounting for hospital CDI isolation/control measures‡ §	1.0×10^{-3} ($0.62–2.0 \times 10^{-3}$)
Rate of community acquisition from nonhuman reservoirs§	1.2×10^{-3} ($0.50–2.3 \times 10^{-3}$)
Base rate of CDI developing in hospital†¶	2.1×10^{-4} ($1.0–4.7 \times 10^{-4}$)
Base rate of CDI developing in LTCF†¶	8.6×10^{-5} ($1.1–22 \times 10^{-5}$)
Base rate of CDI developing in community†¶	6.3×10^{-6} ($2.9–12 \times 10^{-6}$)
Base rate of CDI developing given concurrent conditions†¶	2.6 (0.78–6.8)

*CDI, *C. difficile* infection; LTCF, long-term care facility.
†Parameter rates are per day.
‡Parameter rate expresses relative risk.
§Parameter rate represents an upper bound on the risk for transmission or acquisition within the community.
¶For a detailed decomposition of the rate of development of CDI, see the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/22/4/15-0455-Techapp1.pdf).

community and LTCF antimicrobial drug risk, from their posterior distributions, thereby obtaining 95% CIs for our estimates of the effect of antimicrobial drug class on CDI incidence. We repeated this analysis for antimicrobial drug risk in the community and the LTCF. We then calculated changes in hospital-onset CDI, community-onset CDI, and LTCF CDI incidence as hospital, community, and LTCF risk for antimicrobial drug use were varied.

Results

Epidemiology

For within the hospital, we computed that the ratio of transmission from an isolated symptomatic patient with CDI with transmission from an asymptomatic patient was 15 (95% CI 7.2–32) (Table 2). This high ratio indicates that a symptomatic patient with CDI contributes more to transmission than does an asymptomatically colonized patient, even after accounting for C. difficile protocols. Within the LTCF, the transmission rate from a person with CDI to an uncolonized person is 27% (95% CI 13%–51%) that of the hospital, and the transmission rate from an asymptomatically colonized person to an uncolonized person is 13% (95% CI 6.8%–22%) that of the hospital. Within the community, the transmission rate from a person with CDI to an uncolonized person is 0.1% (95% CI 0.062%–0.2%) that of the hospital, and the transmission rate from an asymptomatically colonized person to an uncolonized person is 0.052% (95% CI 0.033%–0.089%) that of the hospital (Table 2).

To estimate the average risk for a person to become exposed to and be colonized with *C. difficile*, we computed the force of colonization. We calculated that an uncolonized person in the hospital has a probability of 2.3% (95% CI 1.7%–3.2%) per day of acquiring *C. difficile* and becoming a carrier (with or without symptoms); an uncolonized person in the community has a probability of 0.12% (95% CI 0.050%–0.23%) per day, and a person in an LTCF has a probability of 0.37% (95% CI 0.096%–0.77%) per day (Table 2). These results provide a quantitative estimate of the average risk for *C. difficile* exposure to persons in each setting.

Control Strategy

To estimate the effect of transmission control interventions on CDI incidence, we computed the percentage reduction in hospital-onset CDI, community-onset CDI, and LTCF CDI per percentage improvement in hospital CDI diagnosis rate, effectiveness of isolation protocols, overall hospital hygiene, transmission in the community, and transmission in an LTCF (Figure 3). We found that CDI diagnosis rate, effectiveness of isolation, overall hospital hygiene, and transmission in the community, but not transmission in an LTCF, affected hospital-onset CDI. In addition, community-onset CDI and LTCF CDI were not affected by hospital-based transmission interventions.

As the relative risk for antimicrobial drug class prescribed within each of the settings was increased, the CDI incidence likewise increased within that setting (Figure 4). However, there was no relationship between the antimicrobial drug class prescribed within a location and CDI incidence in another location. Specifically, we estimated that for every unit increase in antimicrobial drug risk ratio, the CDI incidence increased by 160% (95% CI 98%–320%) in the hospital, 33% (95% CI 13%–83%) in the LTCF, and 6.4% (95% CI 3.9%–13%) in the community. These results indicate that the effect of antimicrobial drug risk on CDI incidence is intertwined with *C. difficile* transmission dynamics, which differ between the hospital, LTCF, and community.

Discussion

Through stochastic simulation and Bayesian model calibration, we estimated C. difficile transmission rates within and outside the healthcare setting. We also quantified the effect on CDI incidence of control interventions that reduce these transmission rates. We found that a person with CDI in an LTCF transmits at a rate 27% that for a comparable patient in the hospital, and a colonized person or a person

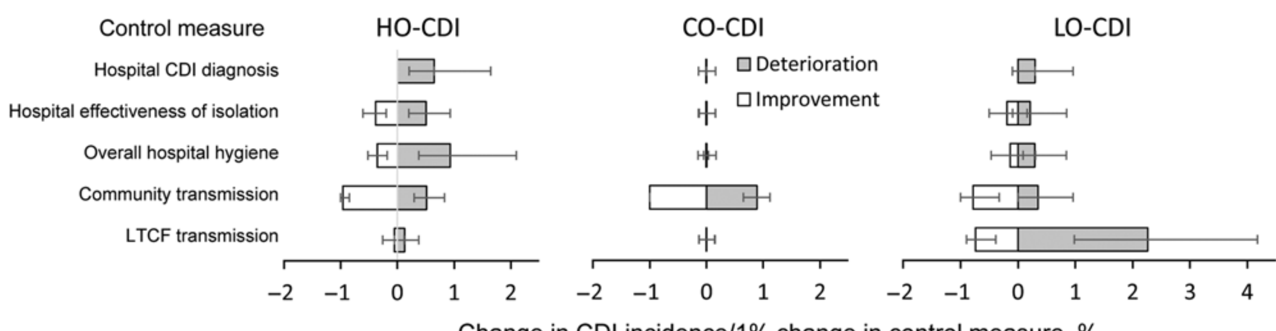

Figure 3. Effectiveness of *Clostridium difficile* infection (CDI) control parameters on incidence of infection quantified as percentage change in hospital-onset CDI (HO-CDI), community-onset CDI (CO-CDI), and long-term care facility (LTCF)–onset CDI (LO-CDI), quantified as percentage change in incidence per 1% change in each of 5 transmission parameters. Error bars indicate 95% CIs. LTCF, long-term care facility.

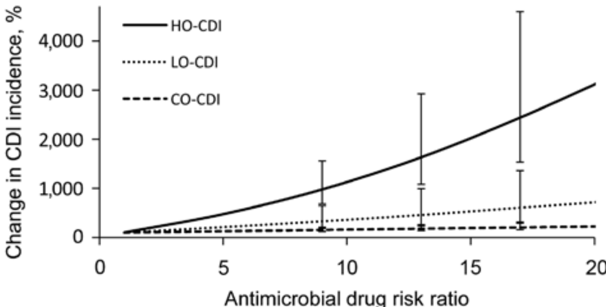

Figure 4. Increase in *Clostridium difficile* infection (CDI) incidence from use of antimicrobial drugs for in hospital-onset (HO-CDI), community-onset (CO-CDI), and long-term care facility–onset (LO-CDI) illnesses classified by drug risk ratio for CDI. *Clostridium difficile* infection (CDI) incidence from use of antimicrobial drugs for low through high CDI risk. Change in CDI incidence is measured as a multiple of the CDI incidence for an antimicrobial drug risk ratio = 1.0. Error bars indicate 95% CIs.

with CDI in the community transmits C. difficile to others at a rate <0.1% that of a comparable patient in the hospital. Despite the lower community transmission rate, we found that because of the much larger pool of colonized persons in the community, interventions that reduce community transmission hold substantial potential to reduce hospital-onset CDI by reducing the number of patients entering the hospital with asymptomatic colonization. Moreover, our results show that in the hospital, symptomatic CDI patients under isolation and infection control measures nonetheless transmit CDI to uncolonized patients at a rate that is 15 times greater than that of asymptomatic carriers. This higher rate of transmission indicates that toxin-targeting treatments (such as vaccines); nontoxigenic C. difficile; and monoclonal antibodies, which might protect against symptomatic CDI but not against asymptomatic colonization, could be effective tools for reducing not only primary CDI cases but also for further transmission (9).

Our epidemiologic results underscore the need for incorporating and understanding transmission dynamics within and outside healthcare settings when evaluating *C. difficile* control strategies. Although *C. difficile* transmission rates are lower among asymptomatically colonized persons, residents of LTCFs, and persons in the community than in hospitalized patients with symptomatic CDI, overall CDI incidence is driven by several factors: transmission, antimicrobial drug use, and underlying population health. We found that, per unit increase in relative antimicrobial drug risk, CDI incidence increases by a factor of 160% in the hospital and 33% in the LTCF but only by a factor of 6.4% in the community. This finding is a consequence of amplification by concentration.

When we compared patients in the hospital and LTCF with persons in the community, we found that patients are closer to each other, are more frequently receiving antimicrobial drugs, and tend to have poorer overall health or may be immunocompromised. These attributes combine to yield a greater risk for infection and transmission. This finding of amplification-by-concentration has major implications for antimicrobial drug risk management: those antimicrobial drugs strongly associated with CDI, such as clindamycin, cephalosporins, and fluoroquinolones (*2*), will have a more detrimental effect on overall CDI incidence in a high-transmission setting, such as a hospital, than they will in a moderate-transmission setting, such as an LTCF, or in a low-transmission setting, such as the community.

We found no major effect of hospital-based transmission interventions on LTCF-onset CDI or of LTCF-based transmission interventions on hospital-onset CDI. This finding suggests that although *C. difficile* can be introduced by a patient who acquired the bacteria in the hospital, CDI outbreaks in LTCFs are driven primarily from within and are best mitigated by targeted transmission interventions within the facility. Likewise, any interventions to reduce transmission within an LTCF will have limited effect on hospital-onset CDI because LTCF transmission interventions will not influence continued introduction of *C. difficile* to the hospital from the community.

The control strategies we evaluated (Figure 3) are representative of a broad range of interventions. For example, an improvement in hospital isolation effectiveness could be achieved through enhanced hospital staff adherence to precautions, or alternatively through an increased capacity to keep a patient with CDI in isolation for the duration of the disease. An improvement in the LTCF transmission rate could be achieved through an improvement to LTCF staff hygiene and cleanliness, through an increased availability of private facilities for residents, or through the isolation of LTCF residents with CDI.

Although there are few data with which to differentiate the sources of community-associated *C. difficile*, we were able to use a community *C. difficile* colonization study (*37*) to calibrate our model. From our calibrated model, we estimated the overall community force of colonization and calculated an upper bound for the community transmission rate. Future studies of similar design but with greater statistical power than the study used for our calibration (*37*), which survey healthy, nonhospitalized adults for asymptomatic *C. difficile* carriage while differentiating community risk factors, would provide the necessary data with which our model could directly quantify transmission from human sources and acquisition from nonhuman reservoirs.

Our analyses demonstrated that *C. difficile* transmission among healthcare settings and the community is interconnected, and there are comparable effects of community-based transmission and hospital-based transmission on hospital-onset CDI. We found that the effect of antimicrobial

drug use on CDI incidence is modulated by transmission dynamics, with specific antimicrobial drugs exacerbating incidence, and doing so to a greater degree in high-transmission settings than in low-transmission settings. These results underscore the need for empirical quantification of community-associated transmission and the need of understanding transmission dynamics in all settings when evaluating *C. difficile* interventions and control strategies.

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Microevolution of Monophasic *Salmonella* Typhimurium during Epidemic, United Kingdom, 2005–2010

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Microevolution associated with emergence and expansion of new epidemic clones of bacterial pathogens holds the key to epidemiologic success. To determine microevolution associated with monophasic *Salmonella* Typhimurium during an epidemic, we performed comparative whole-genome sequencing and phylogenomic analysis of isolates from the United Kingdom and Italy during 2005–2012. These isolates formed a single clade distinct from recent monophasic epidemic clones previously described from North America and Spain. The UK monophasic epidemic clones showed a novel genomic island encoding resistance to heavy metals and a composite transposon encoding antimicrobial drug resistance genes not present in other *Salmonella* Typhimurium isolates, which may have contributed to epidemiologic success. A remarkable amount of genotypic variation accumulated during clonal expansion that occurred during the epidemic, including multiple independent acquisitions of a novel prophage carrying the *sopE* gene and multiple deletion events affecting the phase II flagellin locus. This high level of microevolution may affect antigenicity, pathogenicity, and transmission.

Salmonella enterica is one of the most common enteric pathogens of humans and animals. An estimated 94 million cases of nontyphoidal salmonellosis occur worldwide each year, causing considerable illness and death; in the United States, the associated economic burden estimated by the US Centers for Disease Control and Prevention is >$2 billion US per year (*1*,*2*).

S. enterica consists of >2,500 serovars, of which *S. enterica* serovar Typhimurium (*Salmonella* Typhimurium) is the most ubiquitous in zoonotic reservoirs for human infection and the environment (*3*). Over the past half century, the epidemiology of *Salmonella* Typhimurium has been characterized by successive waves of dominant multidrug-resistant clones (*4*). During 1966–2010 in Europe, where variants are distinguished by definitive (phage) type (DT), *Salmonella* Typhimurium DT9, DT204, DT104, and DT193 emerged successively as multidrug-resistant strains (*5*). Epidemic strains dominate for 4–15 years before being replaced by a new dominant phage type. The emergence and spread of *Salmonella* Typhimurium DT104 was global (*6*) and largely responsible for the increased multidrug-resistant *Salmonella* isolates in Europe and North America in the 1990s (*7*). As DT104 incidence has waned in the United Kingdom, monophasic variants of *Salmonella* Typhimurium with the antigenic formula 1,4,[5],12:i:- have emerged (*8*), although it is not clear if this current monophasic *Salmonella* Typhimurium epidemic is related to other epidemics of monophasic variants previously reported in North America (*9*), Spain (*10*), and elsewhere in Europe (*11*). Analysis of the genomic deletions in the phase II flagellum locus responsible for the monophasic phenotype suggested that multiple independent clones may be emerging in the United States and Europe (*9*).

The first description of a monophasic *Salmonella* Typhimurium epidemic in Europe was that of a "Spanish clone," which emerged rapidly during 1997 and was characterized by a deletion in the allantoin–glyoxylate operon and the fljAB operon, phage type U302, and resistance pattern ACSuGSTSxT (resistant to ampicillin, chloramphenicol, sulfonamide, gentamicin, streptomycin, tetracycline, and co-trimoxazole) (*10*). Since this time, many European countries have reported increased incidence of this serotype, particularly associated with pig herds (12–15) but later with cattle (*16*,*17*). However, in contrast to the Spanish clone, these current monophasic *Salmonella* Typhimurium epidemic strains have commonly been associated with phage types DT193 or DT120 and a predominant

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ASSuT tetraresistance pattern (resistant to ampicillin, streptomycin, sulfonamide, and tetracycline), suggesting that the epidemics are distinct.

The molecular basis for the success of epidemic clones of bacterial pathogens has implications for the surveillance and management of infectious diseases. Epidemiologic success depends on selective advantage of epidemic clones, resulting from their unique genotype. The current multidrug-resistant *Salmonella* 4,[5],12:i:- epidemic in the Europe was first reported around 2005 and is mainly associated with isolates of phage types DT193 and DT120 (18).

We investigated the phylogenetic relationship of 206 strains of *Salmonella* Typhimurium (*Salmonella* 1,4,[5]:i:1,2) and monophasic *Salmonella* Typhimurium (*Salmonella* 1,4,[5],12:i:-), isolated from humans, livestock, or contaminated food from the United Kingdom or Italy from 1993 through 2010. We report the whole-genome sequence variation of *Salmonella* Typhimurium and *Salmonella* 1, 4,[5],12:i:- isolates from the United Kingdom and Italy and the application of these data to phylogenetic reconstruction of the epidemic. We address the questions of whether the monophasic *Salmonella* Typhimurium isolates in the United Kingdom are part of a single epidemic and how they are related to previously circulating biphasic and monophasic *Salmonella* Typhimurium strains.

Materials and Methods

We used bacterial isolates from strain collections held by the Animal and Plant Health Agency (Addlestone, UK); Public Health England (Colindale, London, UK); or the National Regional Laboratory for *Salmonella,* Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Italy). The serotype and phage type were determined as previously described (*19*). The presence of the *fljB* locus and the occupancy of the *thrW* locus was initially determined by PCR amplification as previously described (*11*). Strain selection was intended to represent the diversity of *Salmonella* Typhimurium in the United Kingdom and not to be representative of the epidemiology (online Technical Appendix 1, http://wwwnc.cdc.gov/EID/article/22/4/15-0531-Techapp1.xlsx).

To determine antimicrobial drug sensitivity, we tested isolates from animals in the United Kingdom and Italy for susceptibility to antimicrobial drugs according to standard procedure (20). Resistance or susceptibility were interpreted on the basis of British Society for Antimicrobial Chemotherapy break points; we report the intermediate category as resistant. We determined antimicrobial drug sensitivity of isolates from human patients in the United Kingdom by using a modified break-point technique on Iso-Sensitest agar (Oxoid, Basingstoke, UK) (online Technical Appendix 2, http://wwwnc.cdc.gov/EID/article/22/4/15-0531-Techapp2.pdf). The MIC for copper sulfate was the concentration at which bacterial growth optical density 600 nm was >0.1 after culture (without shaking) at 37°C for 24 hours in Luria Bertani (Oxoid) broth buffered with 25 mmol/L HEPES (4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid) at pH7. We then determined the whole-genome sequence by using the HiSeq Illumina (http://www.illumina.com) platform, sequence analysis, de novo assembly, annotation, and PCR amplification (online Technical Appendix 2).

Results

Salmonella 4,[5],12:i:- Strains

We determined that contemporary *Salmonella* 4,[5],12:i:- strains in the United Kingdom are part of a single clonally expanding clade. We constructed a maximum-likelihood phylogeny of all 97 monophasic and 142 *Salmonella* Typhimurium strains (online Technical Appendix 1) by using 12,793 variable sites in the genome, with reference to the whole-genome sequence of reference strain SL1344, excluding single-nucleotide polymorphisms (SNPs) in prophage, insertion sequence elements, and repetitive sequences (Figure 1). Most (77 of 97) monophasic strains were from a single distinct clade that seemed to be part of the current monophasic *Salmonella* Typhimurium epidemic because they were the most abundant and most recently isolated strains. However, older monophasic isolates were also found in at least 3 other clades within the *Salmonella* Typhimurium tree (Figure 1, indicated with *). A clade containing 8 isolates including 2 DT191a (Figure 1, indicated with†) was closely related to a *Salmonella* 1,4,[5],12:i:- isolate from the North American epidemic strain CVM23701 (*9*). Only 6 SNPs distinguished this isolate from strain H07 474 0455. In addition, a clade containing 6 *Salmonella* Typhimurium var. Copenhagen (4,12:i:1,2) strains (e.g., H070160417) and a clade containing 4 isolates (e.g., H103720606) contained monophasic strains.

Phylogenetics of Monophasic *Salmonella* Typhimurium

A maximum-likelihood phylogenetic tree, reconstructed by using variable sites within the whole-genome sequence with reference to the draft genome sequence of a representative strain from within the epidemic (strain SO4698-09), indicated a clonally expanding clade with a maximum root-to-tip distance of ≈70 SNPs. This finding indicated that all strains in the tree shared a common ancestor in the recent past (Figure 2). All isolates from this monophasic clade were of sequence type 34. The phage type of monophasic epidemic isolates varied according to phylogeny. Most isolates were DT193 (38 of 62 typed) or DT120 (9) and various other phage types including DT7 (3), DT191a (1), DT21 (1), DT21var (1), U311 (3), U302 (2), and RDNC (3). However, although virtually all isolates in subclades

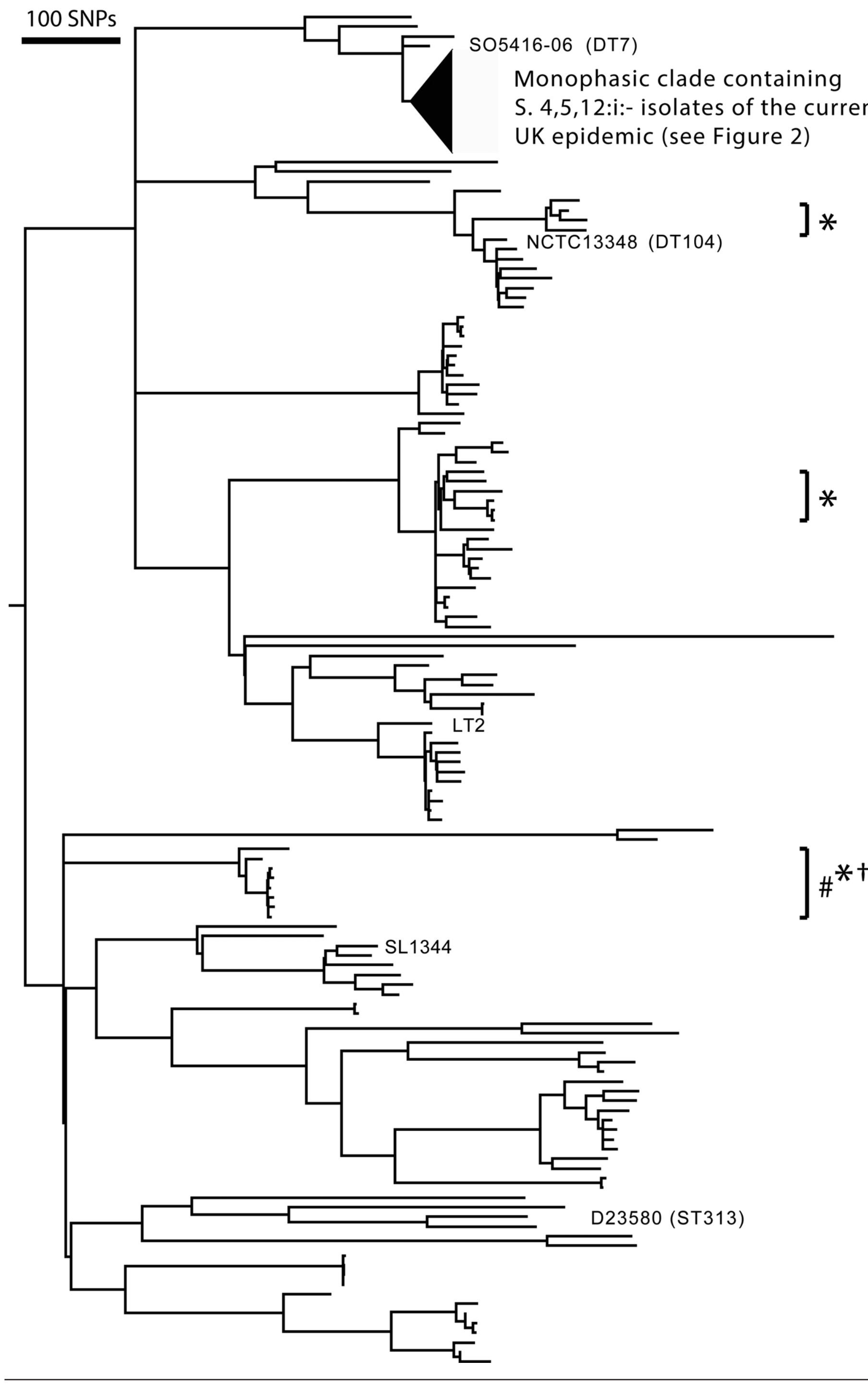

Figure 1. Phylogeny of *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) and *Salmonella* 1,4,[5],12:i:- isolates from the United Kingdom and Italy, 2005–2010. Maximum-likelihood tree of 212 *Salmonella* Typhimurium and monophasic isolates was constructed by using 12,793 single-nucleotide polymorphisms (SNPs) outside of prophage elements, insertion sequence elements and sequence repeats identified by reference to the whole-genome sequence of *Salmonella* Typhimurium strain SL1344. The tree is rooted with *Salmonella* Enteritidis whole-genome sequence as an outgroup (note shown). The lineage containing the *Salmonella* 1,4,[5],12:i:- current UK epidemic group is conflated for simplicity (filled triangle). The designation of the isolates (left column) and phage type are shown (right column). *Monophasic isolates outside of the main epidemic clade. †Monophasic clade closely related to the monophasic clone CVM23701 from North America (*9*). DT, definitive (phage) type; ND, not determined. Scale bar indicates the approximate number of SNPs determined by genetic distance and the number of SNPs used to construct the tree. An expanded version of this figure is available online (http://wwwnc.cdc.gov/EID/article/22/4/15-0531-F1.htm).

A and B were DT193, the phage type was highly variable in subclade C. Biphasic DT193 strains (e.g., 4061-1997; Figure 1) isolated before 2005 were not direct ancestors of the current monophasic *Salmonella* Typhimurium epidemic because they were present on a distinct lineage. Indeed, DT193 isolates were present on 4 distinct lineages within the phylogenetic tree, highlighting the polyphyletic nature of this phage type (Figure 1). Isolates from UK animals in subclade C were relatively scarce; 1 of 21 isolates in this subclade was from a UK animal. Instead, isolates from this subclade came predominantly from humans in the United Kingdom and humans and animals in Italy. In contrast, isolates from subclade A were mostly (18 of 32) of livestock origin; only 5 were of human origin. Clade B contained an approximately equal number of human and livestock isolates. Furthermore, although isolates from UK pigs were

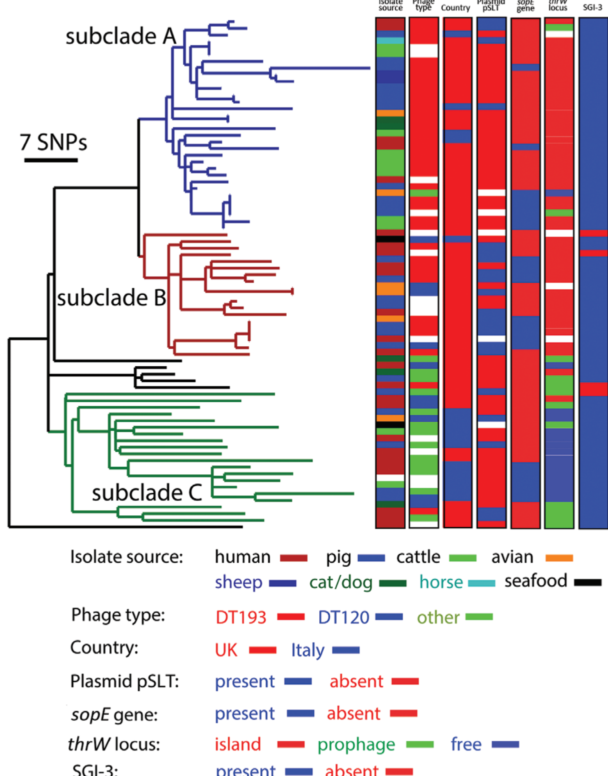

Figure 2. Phylogeny of *Salmonella* 1,4,[5],12:i:- epidemic clade isolates from the United Kingdom and Italy, 1993–2010. Maximum-likelihood tree of 77 *Salmonella* 1,4,[5],12:i:- isolates rooted with *Salmonella* Typhimurium strain SL1344 was constructed by using 1,058 single-nucleotide polymorphisms (SNPs) outside of prophage elements, insertion sequence elements, and sequence repeats identified with reference to whole-genome sequence of *Salmonella* Typhimurium strain SO4698-09. Subclades A (blue lineages), B (red lineages), and C (green lineages) are indicated. Strain designations are color coded for isolates from humans (red) and animals (blue). Epidemiologic data for the source of isolate, phage type, country of origin, presence of the virulence plasmid (pSLT), presence of the *sopE* gene, occupancy of the *thrW* locus, and presence of *Salmonella* genetic island 3 are indicated (right). Scale bar indicates the approximate number of SNPs determined by genetic distance and the number of SNPs used to construct the tree. An expanded version of this figure is available online (http://wwwnc.cdc.gov/EID/article/22/4/15-0531-F2.htm).

present in all 3 subclades, isolates from UK cattle were present only in subclade A, consistent with epidemiologic reports that the epidemic originated in pig herds and later spread to cattle herds (*17*). Despite analysis inclusion of only 6 isolates from birds, these were distributed throughout the tree, suggesting multiple transmission events into these animal populations. The distribution of isolates from humans and livestock (pigs, cattle, and sheep) within subclades of the phylogenetic tree of UK monophasic isolates was also strikingly uneven. Most (64 of 77) isolates were ASSuT tetraresistant, and the corresponding resistance genes were detected in de novo assembled sequences (online Technical Appendix 2 Figure 1), suggesting that the most recent common ancestor (MRCA) of the clade had this complement of resistance genes. However, during clonal expansion, 7 strains had lost their resistance genes entirely and another 7 had an altered complement of resistance genes.

Novel Genetic Island Encoding Resistance to Heavy Metals

A large novel genomic island (designated SGI-3) specific to the monophasic *Salmonella* Typhimurium epidemic clade is inserted at the *yjdC* locus (online Technical Appendix 2 Figure 2) in strain SO4698-09. The island contained ≈90 genes, some of which had sequences similar to those associated with plasmid transfer and conjugation, and an integrase gene, suggesting that the island may have originated by integration of a plasmid. Determination of the accessory genome indicated that the island was present in 74 of 77 isolates within the monophasic clade (Figure 2) but was absent from all strains from outside the clade. Ancestral state reconstruction performed by using ACCTRAN (*21*) (online Technical Appendix 2 Figure 3, panel A) suggested that this island was probably introduced shortly before clonal expansion of the monophasic clade. Three clusters of genes similar to genes involved in resistance to heavy metals are present on the island. Consistent with the island contributing to enhanced resistance to copper sulfate, a common animal feed additive, the MIC ($p = 0.015$) for copper sulfate was significantly greater for isolates within the monophasic *Salmonella* Typhimurium clade (24.2 ± 1.9 mmol/L) than for *Salmonella* Typhimurium isolates from outside this clade (21.2 ± 1.1 mmol/L) that did not encode the island (online Technical Appendix 2 Figure 4).

Genotypic Variation in the *fljBA* and *thrW* Loci and Loss of the Virulence Plasmid

The monophasic phenotype results from the absence of phase-2 flagellin monomer FljB. The presence of the *fljBA* genes and the neighboring genome sequence of *Salmonella* Typhimurium and monophasic variants, determined by mapping raw sequence read data to the *fljB* locus region of the SL1344 whole-genome sequence (online Technical Appendix 2 Figure 5, panel A), indicated that the UK epidemic strains are monophasic because of multiple independent deletion events that occurred during clonal expansion. Four *Salmonella* Typhimurium isolates (2 DT7 isolates [SO5416–06 and H09164 0090], 1 DT135 isolate [SO6221–07], and 1 DT177 isolate [H08390 0191]) that were closely related and shared a common ancestor with the monophasic epidemic strains (Figure 1) encoded the entire

fljBA locus, indicating that the MRCA with these strains and the epidemic strains was biphasic. In contrast, 67 of 77 monophasic *Salmonella* Typhimurium strains from the epidemic clade lacked at least part of the *fljBA* locus, resulting from deletions ranging in size and with a distribution that was consistent with the phylogenetic relationship of the strains (online Technical Appendix 2 Figure 5, panel A). The 8 epidemic strains that did not have a deletion in the *fljB* locus were deeply rooted in the tree, consistent with multiple deletion events (1–36 kb) occurring since clonal expansion of the clade. Most deletions shared a common junction in the intergenic region of *fljB* and *iroB*. Because it was not possible to assemble short read sequence data across the *fljB* locus deletion region, to investigate the nature of the deletion, we generated long read sequence data for a representative isolate SO4698–09 by using the PacBio sequencing platform (Pacific Biosciences, Menlo Park, CA, USA). A single contig assembly of these data revealed a 15,726-bp deletion of the genome relative to SL1344 and a 27,473-bp insertion of a novel sequence (online Technical Appendix 2 Figure 5, panel B). The inserted sequence was similar to sequences of several genes from transposon Tn21, mercury resistance genes (*merTABCDE* and *merR*), and antimicrobial drug resistance genes, consistent with the resistance profile of this strain (*strA*, *strB*, *sul2*, *tet[B]*, and $bla_{TEM\text{-}1}$). The composite transposon insertion was not present in closely related isolates (e.g., SO5416-06) (Figure 1) that were outside of the monophasic clade, suggesting that it was acquired by the MRCA of the monophasic clade and not before clonal expansion. The deletions in the *fljB* locus of monophasic strains from outside the main clade from the United Kingdom were distinct from that in the UK monophasic clade but identical to those described for strains from epidemics in North America (e.g., CVM23701) (*9*) and Spain (e.g., 1115/25) (*10*) (online Technical Appendix 2 Figure 5, panel A).

In addition to hypervariability at the *fljB* locus, isolates from the epidemic group exhibited sporadic loss of the virulence plasmid pSLT. The pattern of plasmid loss within the clade could be most parsimoniously explained by loss during clonal expansion. Of note, the loss of pSLT was not uniform across the monophasic tree. Although only 13% and 20% of isolates tested contained pSLT in subclades A and C, respectively, in contrast, >70% of isolates in subclade B contained the plasmid (Figure 2).

sopE Virulence Gene

The *sopE* virulence gene was acquired on a novel prophage, mTmV (monophasic *Salmonella* Typhimurium V), by multiple independent events during clonal expansion of the epidemic clade. The *thrW* locus of contemporary monophasic *Salmonella* Typhimurium isolates has been reported to harbor either a prophage, a novel genetic island, or neither (*11*). In strain SO4698-09, the *thrW* locus contains the novel genetic island described previously but also an additional prophage element encoding the *sopE* gene that together total 55 kb. Determination of the accessory genome by using the Roary pan genome pipeline (*22*) indicated that 23 of 77 monophasic isolates from the epidemic clade contained the *sopE* gene (Figure 2). SopE is a guanine exchange factor involved in subversion of the host enterocyte cytoskeleton, a key component of the infection process (*11*,*23*,*24*). The *sopE* gene was present in 6 distinct clusters of the monophasic clade, and ancestral state reconstruction indicated that multiple independent acquisitions followed by clonal expansion of the *sopE* -positive variant was the most likely explanation for their distribution (online Technical Appendix 2 Figure 3, panel B). The *sopE* gene of strain SO4698-09 is present on a 55-kb region, designated mTmV phage, which was absent from strain SL1344 and shared the greatest similarity with the *Shigella flexneri* V prophage (online Technical Appendix 2 Figure 6) (*25*). The mTmV phage from SO4698-09 was not related to the FELS-2 prophage of *Salmonella* Typhimurium strain SL1344, which also encodes the *sopE* gene, except in a 2,443-bp region that encoded the *sopE* gene and flanking sequence. Examination of partial assemblies of other monophasic strains encoding *sopE* revealed that the gene was associated with the same prophage and inserted between the genome region corresponding to the *thrW* locus. These data indicated that a novel *sopE* phage entered the genome on at least 6 occasions during the clonal expansion of the epidemic clade. Because the *sopE* gene was present in phylogenetic clusters toward the terminal branches of the monophasic clade tree and subsequently exhibited clonal expansion, we addressed the question of whether the proportion of strains that encoded the *sopE* gene in our strain collection each year changed during 2005–2010. The frequency distribution for each year was determined from collated data from 59 strains for which date of isolation and sequence data were available and an additional 41 randomly selected monophasic strains from the United Kingdom for which the presence of the *sopE* gene was determined by PCR (Figure 3; online Technical Appendix 2 Table). Increased frequency, ranging from none in 2005 and 2006 to 40% in 2010, suggested that acquisition of this gene may have conferred a competitive advantage.

Discussion

We identified a remarkable level of microevolution during clonal expansion of the epidemic. Such expansion may affect the antigenicity, pathogenicity, and transmission of monophasic *Salmonella* Typhimurium.

The phylogenetic relationships of *Salmonella* 1,4,[5],12:i:- isolated from the United States and Europe

since the late 1990s is unclear from reports to date. Our analyses suggest that at least 3 distinct epidemics have been associated with *Salmonella* 1,4,[5],12:i:- and that most of the monophasic isolates from livestock and humans in the United Kingdom since 2006 are not directly related to isolates from either the epidemic in Spain around 1997 (*10*) or the epidemic in the United States around 2004 and 2007 (*9*). Instead, the UK epidemic is related to that reported in Germany and elsewhere since around 2005 (*11*). The US clone is characterized by a large deletion in the *fljB* locus and acquisition of a prophage, neither of which were present in the UK monophasic clone. Furthermore, the whole-genome sequence for a single isolate from the US epidemic (CVM23701) placed this isolate in a small clade of monophasic isolates from the United Kingdom isolated around 1995, distinct from the current UK clade. The clone from Spain is characterized by variable size deletions in the fljB locus, all distinct from deletions observed in the UK isolates, and a deletion in the allantoin metabolism locus, also absent from the main UK clade. The MRCA of the UK *Salmonella* 1,4,[5],12:i:- epidemic in our strain collection was shared with a biphasic *Salmonella* Typhimurium isolate with DT7 (strain H091640090), a relatively rare phage type that has not been associated with epidemics in the epidemiologic record. The common ancestor with strain H091640090 probably existed in the recent past (≈20 years) because only ≈10 SNPs have accumulated in the genome since the lineages diverged, according to the short-term substitution rate (1–2 SNPs/genome/y) previously reported for Salmonella epidemics (*26,27*).

Because virtually all monophasic strains from the current epidemic clade encoded SGI-3 but isolates from outside the clade did not, initiation of clonal expansion was probably accompanied by the acquisition of this genomic island. SGI-3 encodes resistance to heavy metals, including copper and zinc, which is potentially relevant because these are supplements commonly added to pig feed as micronutrients and general antimicrobials (*28*). Indeed, in the European Union, heavy metals have been used increasingly in response to the ban on nonspecific use of antimicrobial drugs in animal feed for growth promotion (*29*). Concentration of heavy metals in pig intestines may represent substantial selective pressure contributing to the success of this clone. Indeed, a recent study reported that an enhanced MIC (20–24 mmol/L) compared with the baseline MIC (16 mmol/L) for copper sulfate was significantly more likely to be found in isolates from pig feces (*30*).

A remarkable feature of the monophasic *Salmonella* Typhimurium epidemic in the United Kingdom is the considerable number of polymorphisms that affect coding capacity that occurred during the short period (≈10–15 years) of clonal expansion of the epidemic clade. These include a complex pattern of deletions in the *fljB* locus and surrounding genome sequence, insertions in the thrW locus, and acquisition of a novel phage carrying the sopE gene. These polymorphisms seem to be stable and not deleterious because they all appear in parts of the tree that have subsequently undergone further clonal expansion. Deletions in the *fljB* locus that occurred subsequent to the initial clonal expansion of the epidemic clade accounted for the monophasic phenotype exhibited by most of these isolates. The high frequency of deletions in this locus may be the result of a composite Tn21-like transposable element that is inserted in the hin–iroB intergenic region, a well-known characteristic of such insertions (*31*).

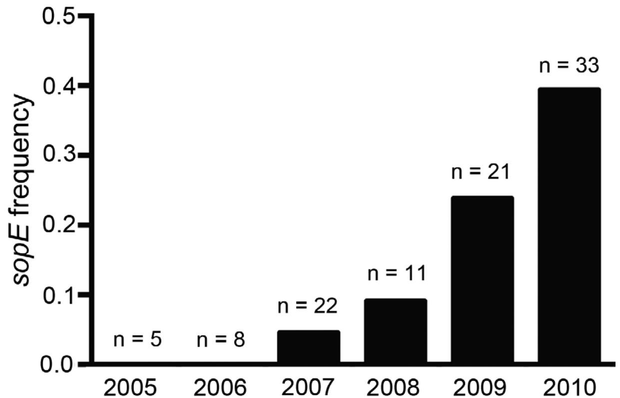

Figure 3. Frequency (proportion) of carriage of the *sopE* gene in *Salmonella* 1,4,[5],12:i:- epidemic isolates from the United Kingdom and Italy for each year during 2005–2010. The presence of the *sopE* gene was detected in draft genome assemblies by sequence comparison or by PCR amplification of genomic DNA by using primers specific for the *sopE* gene of randomly selected monophasic isolates from each year. The number of isolates investigated for each year is indicated above the bar.

The acquisition of the sopE gene on a novel prophage element that occurred through multiple recent independent events may strongly affect the pathogenesis and epidemiology of the current epidemic. Lysogeny by phages carrying the sopE gene has been associated with epidemic strains of *Salmonella* Typhimurium and of other *Salmonella* serotypes (*32*). The expression of SopE may increase the fitness of the pathogen, a possibility consistent with the observation that recent acquisition of the sopE gene by monophasic epidemic isolates has been followed by an increase in the frequency of sopE-positive isolates. The ability to induce inflammatory diarrhea is a main strategy for the transmission of *Salmonella* Typhimurium. SopE is a guanine exchange factor that activates both cdc42 and rac1; sopE2 activates only cdc42 (*33*). All *Salmonella* Typhimurium strains sequenced to date encode the sopE2 gene that exhibits 59% identity with SopE. The additional activity of SopE has a marked effect on the outcome of the interaction of *Salmonella* Typhimurium with the intestinal mucosa, resulting in

increased amounts of salmonellae in the intestinal lumen and shedding in the feces. SopE expression results in increased production of host nitrate, a valuable electron acceptor used by *Salmonella* Typhimurium for respiration (34).

In conclusion, our findings indicate that the current monophasic *Salmonella* Typhimurium clone associated with many animal species and human clinical infections in the United Kingdom arose recently. Subsequent microevolution in a short time has resulted in considerable genotypic variation affecting antigens, virulence factors, and resistance loci. Some genomic features, such as resistance to heavy metals, may have resulted in initial selection for the current clone, while more recent horizontal gene transfer or deletions and plasmid loss may have generated variation selected during the epidemic.

Addendum

It has come to the authors' attention that the designation "Salmonella Genetic Island 3 (SGI-3)" has been previously assigned to a 31-kb genomic island in a strain of Salmonella Mississippi (http://dx.doi.org/10.1371/journal.pone.0041247). To avoid confusion in the literature, we propose that the SGI-3 referred in our manuscript be designated SGI-4 in future reference.

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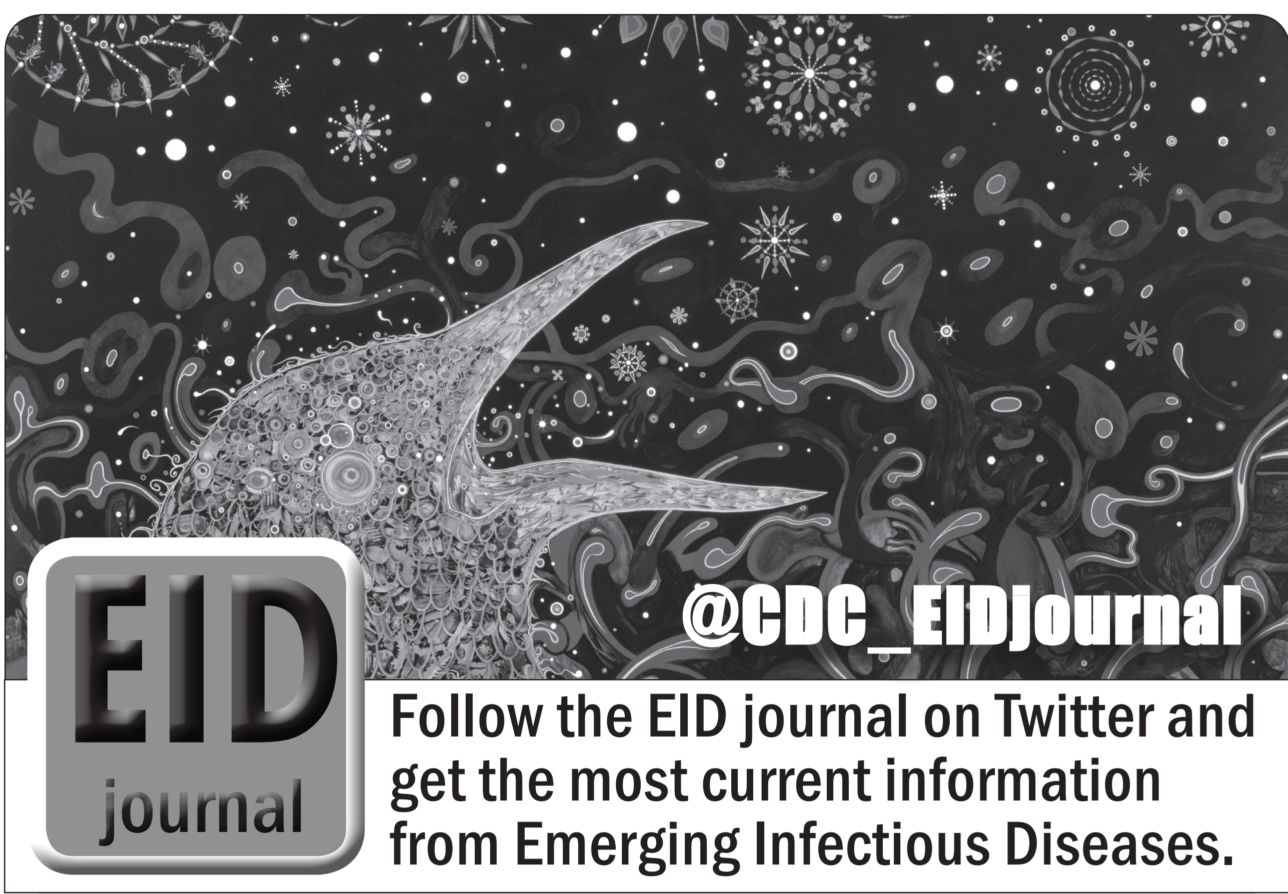

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Molecular Typing and Epidemiology of Human Listeriosis Cases, Denmark, 2002–2012[1]

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Denmark has a high incidence of invasive listeriosis (0.9 cases/100,000 population in 2012). We analyzed patient data, clinical outcome, and trends in pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) of *Listeria monocytogenes* strains isolated in Denmark during 2002–2012. We performed 2-enzyme PFGE and serotyping on 559 isolates and MLST on 92 isolates and identified some correlation between molecular type and clinical outcome and patient characteristics. We found 178 different PFGE types, but isolates from 122 cases belonged to just 2 closely related PFGE types, clonal complex 8 and sequence type 8. These 2 types were the main cause of a peak in incidence of invasive listeriosis during 2005–2009, possibly representing an outbreak or the presence of a highly prevalent clone. However, current typing methods could not fully confirm these possibilities, highlighting the need for more refined discriminatory typing methods to identify outbreaks within frequently occurring *L. monocytogenes* PFGE types.

Listeriosis is a foodborne infection that can cause life-threatening illnesses, including bloodstream infections (BSI) and central nervous system (CNS) infections. Listeriosis is caused by the Gram-positive and ubiquitous bacterium *Listeria monocytogenes* and mainly affects the elderly, immunocompromised persons, and pregnant women. Even though pregnant women often have mild or no clinical symptoms, *L. monocytogenes* can cause severe systemic infection in fetuses and neonates; infection in utero can lead to preterm birth or abortion and stillbirth (*1*).

Most listeriosis cases are sporadic, but outbreaks do occur. Ready-to-eat products, such as delicatessen meats, soft cheeses and smoked seafood, have repeatedly been identified by foodborne disease control programs as sources of outbreaks and products that put humans at risk for listeriosis (*2–4*). For surveillance of outbreaks and trace-back of contaminated sources, highly discriminative molecular subtyping methods are instrumental in supporting the epidemiologic investigation. Pulsed-field gel electrophoresis (PFGE) has been considered the standard typing method for *L. monocytogenes* (*5*,*6*). The advantage of PFGE is mainly its high discriminative power. However, compared with sequence-based typing methods, such as multilocus sequence typing (MLST), PFGE has ambiguity in interpreting data and lacks standard nomenclature; thus, PFGE has difficulties in readily comparing international data. In addition, MLST provides information on phylogenetic relationship. *L. monocytogenes* is genetically heterogeneous and can be divided into 4 genetic lineages (I–IV) that have different pathogenic properties. Isolates from food and from human cases most frequently belong to lineages I and II (*7*).

In Denmark, the annual incidence of listeriosis increased from 0.5 cases per 100,000 population in 2002–2003 to a peak of 1.8 cases in 2009 and 0.9 cases in 2012, and is now among the highest incidences reported globally (*8*,*9*). Similar increasing trends have been reported from other European countries during the same period (*4*). The high but variable incidence calls for further examination of the possible explanations. We retrospectively analyzed trends related to patient data and PFGE- and MLST-types of *L. monocytogenes* strains occurring in Denmark during 2002–2012. In addition, we assessed the possible association between clinical aspects of the disease and strain genotype.

Materials and Methods

Case Information

This study comprises all culture-confirmed cases of invasive listeriosis in humans in Denmark during 2002–2012. In Denmark, listeriosis is notifiable by diagnostic laboratories to the reference laboratory at Statens Serum Institut (SSI) in Copenhagen. Reportable information is patient age and sex, sample isolation site, date of specimen collection, and hospital and hospital department at which the specimen was collected. The case definition for listeriosis used

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in Denmark is in accordance with the case definition by the European Commission (*10*). Cases are categorized according to the site of isolation of *L. monocytogenes* (usually blood or cerebrospinal fluid), clinical diagnosis, or both. Hence, case-patients are grouped according to those with BSIs, CNS infections, pregnancy-associated infections, or other infections. Pregnancy-associated infections comprise listeriosis in neonates in the first month of life and maternal–fetal infections. A pregnancy-associated infection is counted as a single case and is reported as an infection in the mother. From the Danish Civil Registry System, we collected information on vital status to estimate the case-fatality rate (CFR) for non–pregnancy-associated cases. We defined a fatal case as death occurring within 30 days of the date the diagnostic specimen was collected.

Characterization of Bacterial Isolates

The diagnostic laboratories not only notify SSI of listeriosis cases but also refer isolated bacteria to SSI for phenotypic confirmation and typing. Serotypes were established by agglutination with BD Difco Listeria O Antisera Type 1 and 4 (Becton, Dickinson and Company, Sparks, MD, USA) on all isolates and with PCR serogrouping on a subset of isolates (*11*). During 2006–2012, *L. monocytogenes* isolates from human cases were routinely typed by PFGE at the SSI as a means to survey for outbreaks. PFGE was performed according to the PulseNet protocol, using the restriction enzymes *Asc*I and *Apa*I (*6*). For the isolates from 2002 through 2005, PFGE was performed retrospectively as part of this study. We used BioNumerics software version 6.6 (Applied Maths, Sint-Martens-Latum, Belgium) to analyze gels and assign bands. The combined *Asc*I and *Apa*I profiles defined the pulsotype. Isolates were considered having the same pulsotype if they had identical band patterns (no single-band differences) with both enzymes. We calculated similarity between band patterns by using the Dice coefficient, with optimization and tolerance set at 1% for both enzymes. We constructed a dendrogram based on the combined *Asc*I and *Apa*I profiles using UPGMA. Based on this dendrogram, MLST, and PCR serogroup, the genetic lineage of each isolate was determined (*7*,*12*).

We selected 92 isolates for MLST analysis. Isolates were selected in proportion to the number of cases from each year, the different clinical manifestations, the distribution of geography and age groups, and so that they represented both common and rare pulsotypes (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/22/4/15-0998-Techapp1.pdf). For 74 isolates, MLST alleles were extracted from whole-genome sequencing data from Illumina platforms (Illumina Inc., San Diego, CA, USA) by mapping the raw reads to each of the 7 MLST loci sequences from *L. monocytogenes* strain EDG-e. Mapping was done using an in-house pipeline based on BWA (Burrows–Wheeler Aligner, http://bio-bwa.sourceforge.net/) and SAMtools (Sequence Alignment/Map, http://www.htslib.org/). For 18 isolates, MLST alleles were determined by conventional PCR and Sanger sequencing according to the MLST scheme by Ragon et al. (*12*). MLST sequence type (ST) and clonal complex (CC) were assigned by using the Institut Pasteur *L. monocytogenes* MLST sequence type database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Lmono.html). Isolates of the same 2-enzyme pulsotype generally belong to the same CC and, in most instances, the same ST (*13*); therefore, isolates of the same pulsotypes as those typed by MLST were assigned to a presumptive ST and CC. We defined a cluster as the occurrence of at least 3 cases with indistinguishable pulsotypes within a period of 14 weeks (*14*).

Statistical Analyses

Difference in age between groups of patients was assessed by using the Wilcoxon rank-sum test. Categorical variables were compared using χ^2 or Fisher exact test, when appropriate. Relative risks (RRs) with accompanying 95% CIs were calculated; $p \leq 0.05$ using 2-sided tests indicated statistical significance. We used SAS version 9.4 software (SAS Institute, Inc., Cary, NC, USA) for statistical calculations.

Results

Description of Cases and Origin of Bacterial Isolates

In Denmark, 570 cases of invasive listeriosis were notified during 2002–2012; of these, 52% were in women. All patients were hospitalized. For 559 (98%) cases, an isolate of *L. monocytogenes* was referred to SSI. Of the infections, 73% were BSIs, 19% were CNS infections, 4% were pregnancy-associated infections, and 4% were other infections (Table 1). On average, the proportion of BSIs varied from 69% in 2002–2004 to 78% in 2005–2009 and fell to 66% in 2010–2012; the proportion of CNS infections increased from 16% in 2002–2004 to 25% in 2010–2012. Median age of patients with non–pregnancy-associated infections was 71 years; no difference in age was found between patients with BSIs and CNS infections. Of all registered cases, 150 resulted in death within 30 days of the sample collection date; 95 of patients with fatal disease were ≥70 years of age. The overall CFR for non–pregnancy-associated cases was 27% (range 17%–40%, by year). CFR varied with age: 22% for patients <70 years of age versus 33% for patients ≥70 years ($p = 0.004$). Overall, we observed similar CFRs for CNS infections and BSIs and for male and female patients.

Genetic Lineage and Serotype

Cluster analysis of combined *Asc*I and *Apa*I pattern divided the isolates into 3 genetic lineages (online Technical

Table 1. Characteristics of reported cases of human listeriosis by year, Denmark, 2002–2012*

Year	No. cases reported	Incidence per 10^5 population	Median patient age, y (range)†	Infection type, no. (%)				CFR, %†
				CNS	BSI	Pregnancy-associated	Other‡	
2002	28	0.52	69 (1–90)	6 (21)	18 (64)	1 (4)	3 (11)	26
2003	28	0.52	76 (23–95)	5 (18)	18 (64)	3 (11)	2 (7)	24
2004	41	0.76	74 (44–98)	5 (12)	31 (76)	4 (10)	1 (2)	27
2005	44	0.81	68 (23–95)	4 (9)	39 (89)	0 (0)	1 (2)	23
2006	58	1.07	70 (8–91)	13 (22)	43 (74)	2 (2)	0 (0)	20
2007	59	1.08	67 (19–96)	11 (19)	45 (76)	0 (0)	3 (5)	32
2008	55	1.00	70 (1–93)	4 (7)	45 (82)	1 (2)	5 (9)	33
2009	98	1.78	74 (44–98)	18 (18)	74 (76)	4 (4)	2 (2)	29
2010	61	1.10	75 (2–91)	15 (25)	40 (66)	6 (10)	0 (0)	40
2011	48	0.86	70 (2–96)	9 (19)	36 (75)	0 (0)	3 (6)	25
2012	50	0.90	74 (24–93)	15 (30)	30 (60)	2 (4)	3 (6)	17
Total	570	0.95	71 (1–98)	105 (18)	419 (74)	23 (4)	23 (4)	27

*BSI, blood stream infection; CFR, case-fatality rate; CNS, central nervous system.
†Calculated for non–pregnancy-associated cases only.
‡Includes peritonitis, pleuritis, arthritis, abscesses, and osteitis.

Appendix Figure 1), as did PCR serogrouping and MLST. We found that 42% of isolates belonged to lineage I and 58% to lineage II; 1 isolate belonged to lineage III/IV (PCR serogroup L). Within the lineage I isolates, 82% (193/235) were serotype 4 and 18% (42/235) were serotype 1. Of the 42 serotype 1 lineage I isolates, 12 were typed by PCR serogrouping and were PCR serogroup IIb. All lineage II isolates belonged to serotype 1, predominantly serogroup 1/2a, as determined by PCR serogroup (online Technical Appendix Figure 1).

Molecular Typing

PFGE divided the strains into 122 *Asc*I and 140 *Apa*I profiles, for a total of 178 combinations (pulsotypes), which we identified by year (Figure 1). During the 11-year period, 116 pulsotypes (representing 21% of typed cases) occurred only once, and 56 pulsotypes (representing 43% of cases) were seen 2–13 times. The 5 most common pulsotypes represented 82, 40, 40, 17, and 14 cases.

The 92 isolates selected for MLST belonged to 53 pulsotypes and 28 different STs. These 53 pulsotypes represented 69% (387/559) of all reported isolates. The 10 most frequent STs among these 387 isolates were CC8/ST8 (121 cases), CC2/ST2 (52 cases), CC6/ST6 (48 cases), CC1/ST1 (36 cases), CC9/ST9 (22 cases), CC155/ST155 (27 cases), CC7/ST7 (15 cases), and CC5/ST5 and CC14/ST399 (10 cases each) (online Technical Appendix Figure 2).

The 2 most common pulsotypes, 40–42 and 38–48, were represented by 122 (82 and 40, respectively) isolates. These pulsotypes differed by only 2 bands in the *Asc*I

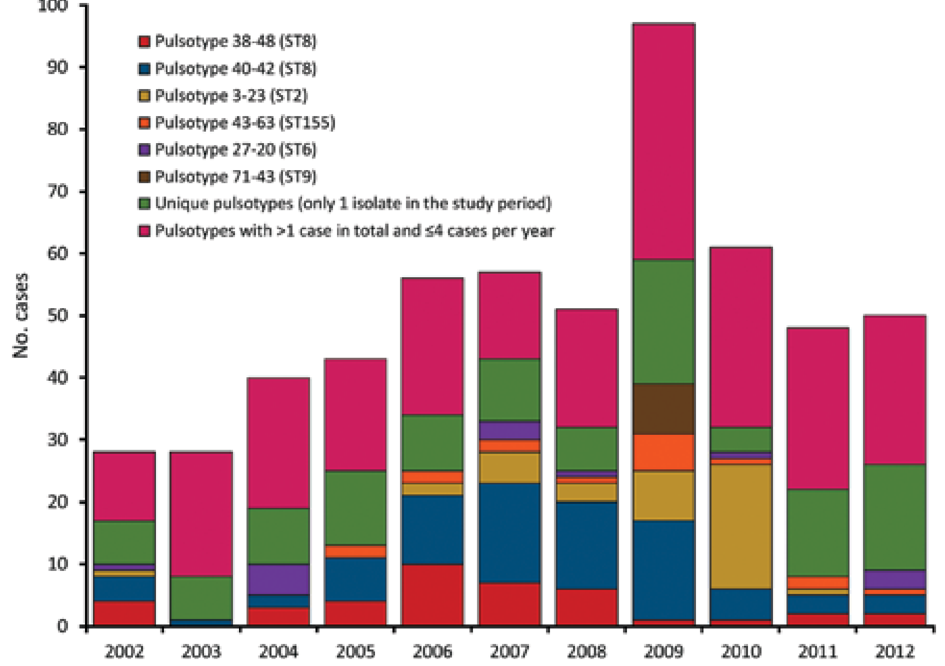

Figure 1. Distribution of pulsotypes of *Listeria monocytogenes* isolates from humans with listeriosis, Denmark, 2002–2012.

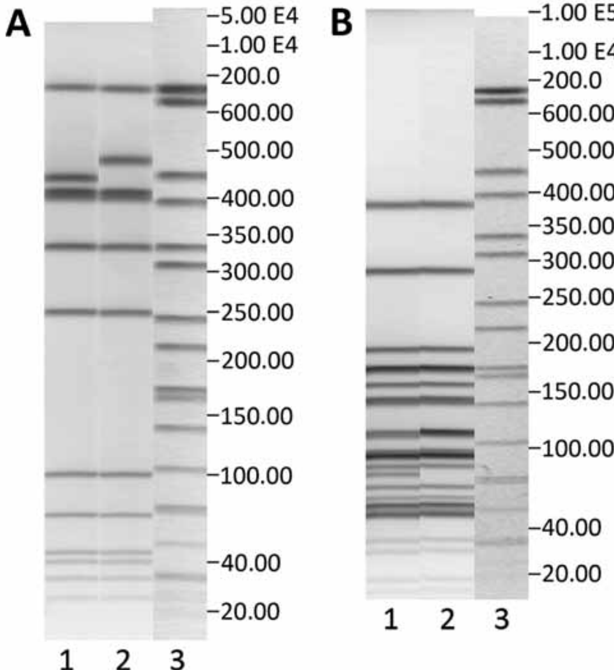

Figure 2. Pulsed-field gel electrophoresis (PFGE) profiles with *Asc*I (A) and *Apa*I (B) restriction enzymes of the 2 most common pulsotypes, 40–42 and 38–48, in Denmark, 2002–2012. A) Lane 1, pulsotype 38–48, GX6A16.0038 DK; lane 2, pulsotype 40–42, GX6A16.0040; lane 3, markers. B) Lane 1, pulsotype 38–48, GX6A12.0048 DK; lane 2, pulsotype 40–42, GX6A12.0042 DK; lane 3, markers. Both pulsotypes belong to clonal complex 8.

pattern and 1 band in the *Apa*I pattern (Figure 2). During 2005–2009, a total of 36% (range 18%–40%) of the isolates belonged to these 2 pulsotypes (Figure 1). Isolates of pulsotypes 40–42 and 38–48 were ST8, except for 1 isolate belonging to ST120. Both ST8 and ST120 belong to CC8. Pulsotype 3–23, which was found in 40 cases and mostly seen in 2009–2010, was CC2/ST2.

Relationship between Typing and Epidemiologic Data

We summarized the subtyping and clinical data related to the pulsotypes associated with ≥8 cases in this study (Table 2). Isolates of pulsotypes belonging to genetic lineage I generally were responsible for a higher proportion of CNS and pregnancy-associated cases than isolates of pulsotypes belonging to genetic lineage II. Isolates belonging to the 2 most common pulsotypes, 40–42 and 38–48, which belong to CC8, were responsible for high proportions of BSIs, corresponding to the high number of BSIs seen in the years when these types were predominant. Of note, none of the CC8 isolates caused pregnancy-associated cases. In contrast, isolates of pulsotype 3–23 (CC2/ST2) alone caused 6 pregnancy-associated cases in 2009–2010. The remaining 17 pregnancy-associated cases were caused by 14 pulsotypes.

Over time, the distribution of lineages by clinical manifestation (BSI and CNS infection) and age (for BSI) revealed that the number of BSIs in patients ≥60 years of age, especially with lineage II isolates, showed an increasing tendency, peaking in 2009 and then declining (Figure 3). Overall, a slightly increasing tendency was seen for CNS infections caused by lineage I isolates.

We found a statistically significant difference in the clinical manifestation of disease caused by lineage I and II. BSIs were more common in patients infected with lineage II strains than in those infected with lineage I strains (79.8% vs. 66.8%, respectively; $p<0.001$), CNS infections were more common among patients with lineage I strains than those with lineage II strains (22.5% vs. 14.9%, respectively; $p<0.001$), and more pregnancy-associated cases were caused by lineage 1 than lineage II strains (7.7% vs. 1.6%, respectively; $p<0.001$). We found no difference in the age of patients infected with isolates of genetic lineage I or II; median age was 71 years for both groups of patients. We observed a moderate effect of genetic lineage on the CFR; 29.6% of patients infected with lineage II strains died, versus 23.0% infected with lineage I strains.

The RR of death from infection with a lineage II strain compared with a lineage I strain was 1.28 (95% CI 0.95–1.73). However, among persons with CNS infections, the effect of lineage was greater (RR 3.31; 95% CI 1.55–7.09), but age was an effect modifier in the sense that for patients <70 years of age, the RR of death from CNS infection with a lineage II strain, versus a lineage I strain, was 6.07 (95% CI 1.44–25.45), and for patients ≥70 years of age, the RR was 2.11 (95% CI 0.88–5.05). Effects of lineage and age on CFR were not seen among patients with BSIs.

Cluster Detection

In the retrospective analysis of PFGE data, we detected 29 clusters comprising 174 cases (Table 3). The 3 most frequent pulsotypes each formed more than 1 cluster. Overall, 13 clusters involved the 2 pulsotypes belonging to CC8, and 8 of the 13 clusters occurred during 2005–2009 where these 2 types were highly prevalent. We found 4 clusters defined by pulsotypes that were seen only at the time of the cluster (n = 3) or in the year the cluster was present (n = 1). One of these 4 clusters, the cluster with pulsotype 71–43, was a confirmed outbreak (*15*).

Discussion

This study provides insights into the dynamics of the pulsotypes of *L. monocytogenes* isolates through a period with an increasing incidence of listeriosis in Denmark. We found that during 2005–2009, an increase in the number of cases was mainly driven by the emergence of isolates of *L. monocytogenes* of genetic lineage II (serotype 1/2a). Throughout the study period, we found a high proportion of

Table 2. Clinical and subtype data associated with the most common PFGE pulsotypes of *Listeria monocytogenes* isolates from persons with listeriosis, Denmark, 2002–2012*

Pulsotype	ST/CC	Genetic lineage (PCR serogroup)	No. cases	Median patient age, y (range)†	Infection type, no. (%)				CFR, %†
					BSI	CNS	Pregnancy-associated	Other‡	
40–42	8/8	II (IIa)	82	71 (8–91)	68 (83)	11 (13)	0 (0)	3 (4)	30
38–48	8/8§	II (IIa)	40	69 (24–91)	31 (78)	7 (18)	0 (0)	2 (5)	25
3–23	2/2	I (IVb)	40	69 (1–90)	23 (58)	11 (28)	6 (15)	0 (0)	29
43–63	155/155	II (IIa)	17	75 (42–95)	15 (88)	1 (6)	0 (0)	1 (6)	41
27–20	6/6	I (IVb)	14	74 (57–98)	11 (79)	3 (21)	0 (0)	0 (0)	29
25–1	1/1	I (IVb)	13	67 (2–83)	6 (46)	6 (46)	0 (0)	1 (8)	23
41–43	9/9	II (IIc)	11	72 (53–93)	9 (82)	1 (9)	0 (0)	1 (9)	45
22–2	1/1	I (IVb)	11	72 (37–90)	5 (46)	4 (36)	2 (18)	0 (0)	11
44–61	155/155	II (IIa)	10	76 (49–91)	7 (70)	2 (20)	1 (10)	0 (0)	33
71–43¶	9/9	II (IIa)	8	77 (44–94)	7 (88)	1 (13)	0 (0)	0 (0)	25
67–92	1/1	I (IVb)	8	74 (63–98)	7 (88)	1 (13)	0 (0)	0 (0)	25
27–18	6/6	I (IVb)	8	52 (50–76)	3 (38)	4 (50)	1 (13)	0 (0)	29
12–38	7/7	II (IIa)	8	73 (51–90)	3 (38)	5 (63)	0 (0)	0 (0)	13
35–26	59/59	I (IIb)	8	70 (55–80)	4 (50)	2 (25)	2 (25)	0 (0)	17

*Data are for pulsotypes found in ≥8 cases. Pulsotype is for combined *Asc*I and *Apa*I pattern. BSI, blood stream infection; CC, clonal complex; CFR, case-fatality rate; CNS, central nervous system; PFGE, pulsed-field gel electrophoresis; ST, sequence type.
†Calculated for non–pregnancy-associated cases only.
‡Includes peritonitis, pleuritis, arthritis, abscesses, and osteitis.
§One of the 5 representative isolates selected for multilocus sequence typing for this pulsotype was ST120/CC8.
¶Confirmed outbreak described previously (*15*).

sporadic cases caused by unique or infrequent pulsotypes. Nevertheless, a remarkably high proportion, 36%, of the infections in 2005–2009 were caused by isolates of CC8 with 2 closely related pulsotypes.

CC8 is globally distributed (*16*). In Switzerland, CC8 was the most prevalent clone during 2011–2013 (*17*), and in Canada, a CC8/ST120 clone caused both sporadic cases and outbreaks during 1988–2010 (*18*). The PFGE types of the CC8 isolates from Canada are quite similar to the CC8 pulsotypes reported in our study (*19*). In line with the epidemiologic findings in Canada, the CC8 clone in Denmark caused no pregnancy-associated infections and mostly caused infections in the elderly. This propensity for CC8 pulsotypes to infect older persons rather than pregnant women may reflect different food preferences between the 2 groups at risk for listeriosis as well as the frequency of exposure to food substances contaminated with CC8 *L. monocytogenes*; alternatively, this propensity may be related to the virulence potential of this specific clone. Recent research has suggested that the CC8 strains from Canada possess a strong capacity for biofilm formation, which may support persistence within food production environments and subsequent contamination of foods (*20*). Similar abilities could be harbored by the

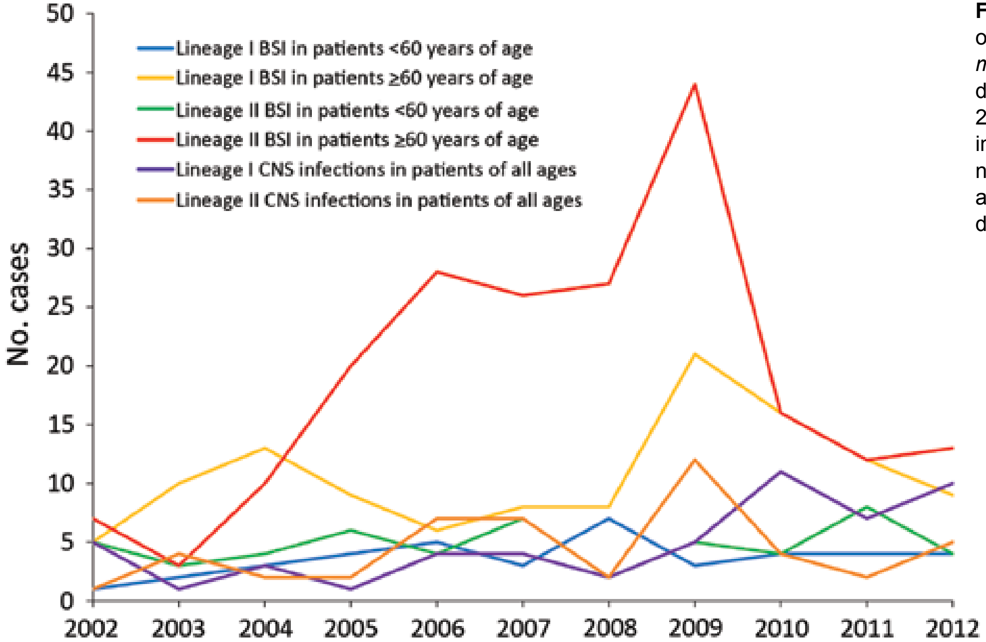

Figure 3. Distribution of lineages of *Listeria monocytogenes* isolates by disease manifestation, Denmark, 2002–2012. Only blood stream infections (BSIs) and central nervous system (CNS) infections are shown. For BSIs, the distribution by age is also shown.

Table 3. Clusters of identical pulsotypes of *Listeria monocytogenes* isolates, Denmark, 2002–2012*

Year(s)	No. cases	Time range for the cluster, d	Genetic lineage (PCR serogroup)	ST/CC	Pulsotype
Clusters with pulsotypes other than the 3 highly frequent types					
2003†	3	40	I (ND)	224/224	181–218
2004	3	90	I (IVb)	6/6	27–20
2005‡	3	97	I (ND)	5/5	31–30
2005–2006	3	42	I (IVb)	1/1	25–1
2006	3	66	II (IIa)	155/155	44–61
2006†	4	103	II (ND)	391/89	21–34
2009	4	41	II (IIa)	155/155	43–63
2009†§	8	5	II (IIa)	9/9	71–43
2009	4	130	I (IIb)	59/59	35–26
2009	3	97	I (IVb)	1/1	67–92
2010	3	60	II (IIc)	9/9	41–43
2010	3	28	I (IVb)	1/1	22–2
2011	4	108	II (IIa)	18/18	101–126
Clusters with the 3 highly frequent pulsotypes					
2004	3	84	II (IIa)	8/8	38–48
2005	3	81	II (IIa)	8/8	38–48
2006–2007	11	191	II (IIa)	8/8	38–48
2007	6	164	II (IIa)	8/8	38–48
2008	3	81	II (IIa)	8/8	38–48
2005	5	86	II (IIa)	8/8	40–42
2006–2007	12	230	II (IIa)	8/8	40–42
2007–2008	23	421	II (IIa)	8/8	40–42
2008–2009	6	83	II (IIa)	8/8	40–42
2009–2010	15	310	II (IIa)	8/8	40–42
2010	3	44	II (IIa)	8/8	40–42
2011	3	55	II (IIa)	8/8	40–42
2012	3	75	II (IIa)	8/8	40–42
2007	3	46	I (IVb)	2/2	3–23
2009–2010¶	8	148	I (IVb)	2/2	3–23
2010#	19	253	I (IVb)	2/2	3–23

*A cluster was defined as $\geq$3 cases with identical pulsotypes within 14 weeks. ST, sequence type; CC, clonal complex; ND not determined.
†This type only seen this 1 time during the study period.
‡This type only seen in 2005.
§Confirmed outbreak with meals-on-wheels food delivery as source of infection, described previously (*15*).
¶Cluster investigated but no common source identified.
#May be part of the former cluster (marked with ¶) with this pulsotype 3–23.The cluster included 5 pregnancy-associated cases. Cluster investigation could not determine a common source of infection, but smoked salmon was suspected based upon interview data.

CC8 isolates from Denmark, but further examination is needed to confirm this.

Nakari et al. (*21*) found that an increase in listeriosis in Finland in 2010 was partly caused by a specific *Asc*I type, *Lm*96, which caused 19% of human cases and was the most prevalent type found in food isolates in Finland in 2010. Of note, *Lm*96 was identical to the *Asc*I profile 38 in Denmark, the second most common *Asc*I profile belonging to CC8. The type was found in a specific fishery production plant in Finland and in cold-smoked rainbow trout products from the plant. A persistent contamination of the plant was suspected because the same PFGE type had been found in earlier years. An enquiry via the Food and Waterborne Diseases Network revealed that *Lm*96 was a common profile in several European countries and had been isolated from various food categories in addition to fishery products (*21*).

The fact that closely related or even identical pulsotypes have been found in the same period raises the question whether the contamination of food could originate from a common source (e.g., a fish farm). Studies have shown that *L. monocytogenes* isolates found in fish farms and raw fish material often belonged to the same pulsotypes as isolates from the final product, indicating that raw fish material can be important sources of *L. monocytogenes* contamination of final fishery products (*22*,*23*). For several years, cold-smoked fishery products have been among the important suspected sources of infections in Denmark. Ready-to-eat fishery products are consumed in large amounts and are the food category most often found to be contaminated with *L. monocytogenes* in the European Union (*3*,*4*,*24*). Lambertz et al. (*25*) compared isolates of *L. monocytogenes* recovered in 2010 from ready-to-eat foods and processing plants in Sweden with clinical isolates obtained from listeriosis patients during 2005–2010. They found that the most common human pulsotype was also the most common pulsotype among the isolates from ready-to-eat foods, and 17 of 19 food isolates of this pulsotype originated from fishery products from a processing plant not in Sweden. Similar

findings were made in Norway by Lunestad et al. (*26*). Whether the CC8 isolates described in our study reflect the presence of a common pulsotype with many unrelated sources or a long-term cluster with a common source of infection is difficult to determine.

Since 2006 in Denmark, 2-enzyme PFGE has been used to type human isolates of *L. monocytogenes*. Listeriosis clusters caused by *L. monocytogenes* pulsotypes 40–42 and 38–48 were defined separately and restricted to predefined periods of weeks. During 2006–2007, basic epidemiologic information was collected and, if possible, patients were contacted, but no standardized questionnaire existed. However, no correlation between cases and a common source of infection was established, and it was concluded the cases were caused by common *L. monocytogenes* pulsotypes (Steen Ethelberg, unpub. data). Our findings show that the prevalence of both pulsotypes increased during 2005–2009 and substantially decreased beginning in 2010. This pattern could indicate that a common source caused infections through several years. Some pulsotypes are more common than others, and isolates of these pulsotypes might not be epidemiologically linked. Consequently, common pulsotypes create challenges in defining cluster detection levels (*27*). Our findings show that with a cluster detection level of 3 cases with identical pulsotypes within a period of 14 weeks, possible true clusters of rare pulsotypes are discovered, but the number of detected clusters associated with common pulsotypes is apparently overestimated. Having different cluster detection levels based on the frequency of the pulsotypes may enhance the chance of detecting true clusters (*27*,*28*). On the other hand, because clones of *L. monocytogenes* can persist in food processing plants for several years, exhibiting little genetic variation and causing infections spanning several years (*29*), narrow temporal cluster definitions may not always be appropriate for cases of listeriosis with identical pulsotypes. Our results point to the need for typing methods that can provide higher resolution of common pulsotypes but also phylogenetically link related pulsotypes and, thus, improve the epidemiologic investigations of suspected outbreaks. Whole-genome sequencing seems to be able to fulfill these needs, as has already been shown in several outbreaks with listeriosis (*19*,*30*,*31*). In addition, optimizing epidemiologic information through routine interviews of all cases of listeriosis has also proven powerful in finding common sources of infections as well as defining outbreaks involving strains of different genotypes (*2*,*32*,*33*). Last, real-time comparison of subtyping results between isolates recovered from foods and humans would substantially increase the knowledge on possible sources of infection and enhance the chances of successful outbreak investigations.

We found a substantial increase in BSIs among patients ≥60 years of age with lineage II isolates during 2005–2009, which, for the most part, explained the increased incidence seen in those years. Our findings concur with those from studies in England and Wales, in which Gillespie et al. (*34*) found that an increase in cases of listeriosis in 2001–2007 was mainly related to persons ≥60 years of age with bacteremia. Similarly, other European countries have reported an increase in infections caused by serotype 1/2a (*35*,*36*). Our study did not include data on concurrent conditions, socioeconomic factors, or medications used by persons with listeriosis, which could have contributed with further explanatory variables, as in the study by Gillespie et al. (*34*), in which patients with cancer and patients receiving stomach acid inhibitors were mainly affected by the increase.

We found some associations between specific molecular types and clinical manifestations. The pulsotype 3–23, belonging to CC2/ST2 and serotype 4b, accounted for 6 of 23 pregnancy-associated infections; 5 of these occurred in autumn 2010 and were probably linked. Pulsotypes belonging to CC1, CC2, and CC6 showed high proportions of CNS infections, which is in accord with our finding that lineage I was associated with more CNS infections and pregnancy-associated cases. In contrast with a previous study from Denmark (*37*), we found that the CFR for patients infected with lineage II (serotype 1/2a and 1/2c) isolates was slightly higher than that for patients infected with lineage I (serotype 4b and 1/2b) isolates. Compared with lineage I, lineage II was significantly associated with a higher mortality rate for patients <70 years of age with CNS infections but not for patients ≥70 years of age with CNS infections. However, this study would have been strengthened if we had had information on concurrent conditions, as they could be confounding this association.

Our findings show that retrospective typing of isolates gives new insight into the epidemiology of listeriosis. By PFGE typing, we found a high diversity of *L. monocytogenes* in clinical cases but also a small number of frequent types representing a substantial fraction of all cases. Possibly, these types represent epidemiologically linked cases (outbreaks) or, alternatively, ubiquitous types present in many unrelated food sources and infections. New discriminatory typing methods are necessary to clarify the clonality of these common types. In the near future, whole-genome sequencing is likely to be the method of choice for such analyses. Several studies have reported on the genetic diversity of *L. monocytogenes* based on MLST, making it possible to compare typing data globally (*13*,*16*,*36*,*38*). By the addition of MLST, we could compare types our study with those in other countries, making it clear that some of the common clones in our study had also been found in other countries, thus paving the way for a better understanding of internationally occurring clones. To enhance the surveillance of listeriosis, continuous typing with highly discriminatory methods combined with timely collection of

patients' histories of food intake could significantly improve the chances of detecting, solving, and stopping outbreaks. Moreover, human and food isolates should be typed by the same methods and compared on a regular basis.

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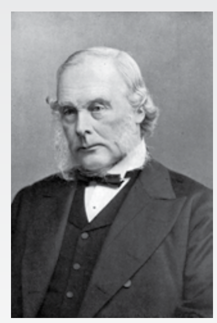
etymologia

Listeria [lis-te're-ə]

A genus of small, gram-positive, rods, *Listeria* was first isolated by Murray in 1924 as *Bacterium monocytogenes*. In 1927, Pirie proposed the genus *Listerella* in honor of British surgeon Sir Joseph Lister (1827–1912), an early advocate of antiseptic surgery. It was not until 1939 that Pirie realized that this genus had already been taken by a slime mold (also named in honor of Lister, by Jahn in 1906). In 1940, he proposed the alternative name *Listeria*. The mouthwash Listerine was also named after Lister, in 1979 by Lawrence and Bosch, when it was marketed as a surgical antiseptic.



Joseph Lister, 1902. Photographer unknown. Weltrundschau zu Reclams Universum 1902, Public Domain, https://commons.wikimedia.org/w/index.php?curid=9647607

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Limited Dissemination of Extended-Spectrum β-Lactamase– and Plasmid-Encoded AmpC–Producing *Escherichia coli* from Food and Farm Animals, Sweden

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Extended-spectrum β-lactamase (ESBL)– and plasmid-encoded ampC (pAmpC)–producing *Enterobacteriaceae* might spread from farm animals to humans through food. However, most studies have been limited in number of isolates tested and areas studied. We examined genetic relatedness of 716 isolates from 4,854 samples collected from humans, farm animals, and foods in Sweden to determine whether foods and farm animals might act as reservoirs and dissemination routes for ESBL/pAmpC-producing *Escherichia coli*. Results showed that clonal spread to humans appears unlikely. However, we found limited dissemination of genes encoding ESBL/pAmpC and plasmids carrying these genes from foods and farm animals to healthy humans and patients. Poultry and chicken meat might be a reservoir and dissemination route to humans. Although we found no evidence of clonal spread of ESBL/pAmpC-producing *E. coli* from farm animals or foods to humans, ESBL/pAmpC-producing *E. coli* with identical genes and plasmids were present in farm animals, foods, and humans.

In 2012, the Panel on Biologic Hazards of the European Food Safety Authority (EFSA) concluded that a risk exists for transmission of extended-spectrum b-lactamase (ESBL)– and plasmid-encoded AmpC (pAmpC)–producing *Enterobacteriaceae* from farm animals, particularly poultry, to humans through the food chain (*1*). This conclusion is problematic because ESBL and pAmpC hydrolyze extended-spectrum cephalosporins, which are one of the most widely used antimicrobial drug classes (*2*). Extended-spectrum cephalosporins are also listed by the World Health Organization as being critically useful antimicrobial drugs in human medicine (*2*). Therefore, the high frequency of ESBL/pAmpC-producing *Escherichia coli* reported for farm animals, particularly broilers, in Europe is of great concern (*3*). A recent systematic review by Lazarus et al. (*4*) reported the same conclusion as the EFSA but also that the magnitude of transmission and its geographic extent are still unclear. In addition, these authors reported a lack of studies on a national level and no comparisons of isolates from animals or food with isolates from human asymptomatic carriage (*4*).

In Sweden, antimicrobial drugs are used less often in animals and humans than in other countries in Europe and the frequency of ESBL/pAmpC-producing *E. coli* is lower (*5,6*). However, one exception in Sweden is the large frequency of pAmpC-producing *E. coli* in poultry and domestic chicken meat (*7–10*). In addition, Sweden has a low population density of humans and animals. Therefore, the situation in Sweden with regards to dissemination of ESBL/pAmpC-producing *E. coli* could be different from that for previous studies from other countries in Europe, which reported potential transmission of ESBL/pAmpC-producing *E. coli* from farm animals by foods to humans (*11–13*).

The objective of this study was to investigate potential dissemination of ESBL/pAmpC-producing *E. coli* isolates among foods, farm animals, patients with bloodstream infections, and presumed healthy human carriers in the community in Sweden. This objective was achieved by comparing genetic relatedness of ESBL/pAmpC-producing isolates from foods intended for retail markets, farm animals, and humans in Sweden. The study was conducted on a national level, tested a large collection of isolates from diverse sectors, and was undertaken in cooperation with governmental agencies for human health, animal health, and food safety. We aimed to improve overall knowledge regarding the influence of farm animals and foods on the frequency of ESBLs and pAmpCs in humans.

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Materials and Methods

Datasets and Isolates

A total of 716 ESBL/pAmpC-producing E. coli isolates from 4,854 samples obtained during 2010–2013 were available for analysis (Table 1). Isolates were from community carriers (n = 103); patients with bloodstream infections (n = 387); broilers (n = 32), laying hens (n = 10), pigs (n = 3), calves (n = 9), and chicken meat (n = 74) from Sweden; imported chicken meat (n = 84); imported beef/pork (n = 16); and imported leafy greens (n = 2). ESBL/pAmpC-producing E. coli isolates have not been found in samples of beef or pork from Sweden (5). All isolates were obtained by using equivalent methods. Samples from farm animals and foods were screened by using cefotaxime, and samples from community carriers were screened by using cefpodoxime. Isolates from bloodstream infections have reduced susceptibility to ceftazidime or cefotaxime and were submitted to the Public Health Agency of Sweden by 18 clinical microbiology laboratories (S. Ny et al., unpub. data).

Isolates from broilers and chicken meat in Sweden obtained during 2010, community carriers, and bloodstream infections were subjected to ESBL/pAmpC gene sequencing, multilocus sequence typing (MLST), transfer of plasmids, and subsequent plasmid replicon typing (*7–9*) (S. Ny et al., unpub. data). When data was lacking in previous studies (*10,14,15*; M. Egervärn et al., unpub. data), analyses were performed in our study as described.

Characterization of Isolates

MLST was performed by using an MLST Database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli), and sequence types (STs) were identified by using BioNumerics versions 7.0 or 7.1 (Applied Maths, Ghent, Belgium). Transfer of plasmids carrying genes encoding ESBL/pAmpC was performed by electroporation to ElectroMax DH10B cells (Life Technologies, Carlsbad, CA, USA), and transformation of plasmids was confirmed by detection of genes as described (*8*). Plasmid replicon typing was performed on transformants by using the PBRT Kit (Diatheva, Fano, Italy). For transformants positive for incompatibility group *inc*I1, the plasmid was subjected to plasmid MLST (pMLST) by using a Plasmid MLST database (http://pubmlst.org/plasmid/).

Statistical Analysis

Descriptive statistics were used to describe different datasets. Further analysis was undertaken by creating different profiles on the basis of ST type, replicon type, and ESBL/pAmpC gene.

Results

Overlap of Genes Encoding ESBL and pAmpC

Overlap between sectors (humans, farm animals, and foods) was defined as identical genetic traits in at least 1 isolate from human samples and 1 isolate from farm animal or food samples. A total of 24 genes encoding ESBL or pAmpC were identified across all sectors. bla_{CMY-2} and $bla_{CTX-M-1}$ were the only genes present in all sectors and the

Table 1. Characteristics of *Escherichia coli* isolates from various sources tested for ESBL and pAmpC, Sweden*

Category and source	Year	No. samples	% Positive for ESBL	% Positive for pAmpC	Reference
Human					
Community carriers	2011–2013	2,134	4.3	0.4	S. Ny et al., unpub. data
Bloodstream infections	2011–2012	387†	92.5	7.5	S. Ny et al., unpub. data
Farm animals					
Broilers	2010	100	6.0	28.0	(*7,9*)
Laying hens	2012	69	4.4	8.7	(*14*)
Pigs	2011	184	1.6	0	(*14*)
Calves	2011–2012	729	0.7	0.5	(*15*)
Meat					
Domestically produced chicken	2010	100	4.0	40.0	(*8*)
Domestically produced chicken	2013	59	0	50.8	(*14*)
Imported chicken (Europe)	2010–2011, 2013‡	109	19.3	21.1	(*10*), S. Börjesson et al., unpub. data
Imported chicken (South America)	2010–2011	43	90.7	4.7	(*10*)
Imported beef (Europe)	2010–2011	136	5.9	0	(*10*)
Imported beef (South America)	2010–2011	42	0	0	(*10*)
Domestically produced pork	2011	100	0	0	(*14*)
Imported pork (Europe)	2010–2011	119	5.9	0.8	(*10*)
Leafy greens					
Sweden	2012–2013	147	0	0	M. Egervärn et al., unpub. data
Imported	2012–2013	375	0.5	0	M. Egervärn et al., unpub. data
Mixed origin	2012–2013	108	0	0	M.Egervärn et al., unpub. data

*ESBL, extended-spectrum β-lactamase; pAmpC, plasmid-encoded AmpC.
†These are not samples, but hospital isolates from bloodstream infections submitted to the Public Health Agency of Sweden and confirmed as producers of ESBL and pAmpC.
‡Only samples from chicken meat from Denmark.

only genes identified in poultry from Sweden (laying hens and broilers) and chicken meat (domestically produced) (Figure 1; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/4/15-1142-Techapp1.pdf). These genes were also commonly detected in chicken meat from Europe. *bla*$_{\text{CTX-M-1}}$ was the most common gene in isolates from imported beef/pork, and it was also identified in 2 isolates from imported leafy greens. ESBL/pAmpC-producing isolates in chicken meat from South America had a different gene distribution dominated by *bla*$_{\text{CTX-M-2}}$ and *bla*$_{\text{CTX-M-8}}$ (Figure 1) *bla*$_{\text{CMY-2,}}$ *bla*$_{\text{CTX-M-1,}}$ *bla*$_{\text{CTX-M-2}}$, and *bla*$_{\text{CTX-M-8}}$ were present in isolates from community carriers and bloodstream infections, but were not the most common genes (Figure 1; online Technical Appendix).

The 2 most common genes identified in isolates from community carriers and bloodstream infections (*bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-14}}$) were not isolated from chicken meat or poultry from Sweden (Figure 1; online Technical Appendix). *bla*$_{\text{CTX-M-15}}$ was present in isolates from pigs and calves in Sweden (n = 4) and imported beef (n = 2), and *bla*$_{\text{CTX-M-14}}$ was detected in 1 isolate from imported pork. Other genes identified in isolates from humans that also were detected in farm animals or meat were *bla*$_{\text{CTX-M-3}}$ (pig from Sweden), *bla*$_{\text{SHV-12}}$ (imported chicken meat), and *bla*$_{\text{TEM-52}}$ (pigs from Sweden, and imported chicken meat and pork) (Figure 1; online Technical Appendix).

Overlap of Plasmid Replicon Types

We found a difference between sectors with regards to replicon types of plasmids carrying ESBL/pAmpCs genes (Figure 2). In isolates from chicken meat and poultry from Sweden, incI1 and incK plasmids were primarily detected. IncI1 was the most common replicon type in isolates from imported beef/pork. IncI1 and incK plasmids were also detected in isolates from humans, but to a lesser extent (Figure 2). Isolates from humans had mainly plasmids belonging to different incF replicon types, which were present only occasionally in isolates from poultry in Sweden, chicken meat, and imported beef/pork. Some plasmids could not be transferred by electroporation. This finding was especially common for human isolates carrying *bla*$_{\text{CTX-M-15}}$ and isolates carrying *bla*$_{\text{CTX-M-2}}$ from imported chicken meat (S. Ny et al., unpub. data) (*10*).

Overlap of Plasmids and Genes Encoding ESBL and pAmpC

The following combinations found in foods or farm animals were also identified in humans isolates: incK-*bla*$_{\text{CMY-2}}$, incI1-*bla*$_{\text{CMY-2}}$, incI1- *bla*$_{\text{CTX-M-1}}$, incI1-*bla*$_{\text{CTX-M-15}}$, incI1-*bla*$_{\text{CTX-M-8}}$, incFII-*bla*$_{\text{CTX-M-1,}}$ incFII-*bla*$_{\text{CTX-M-14,}}$ incI1-*bla*$_{\text{TEM-52}}$, incN-*bla*$_{\text{CTX-M-1}}$, and incA/C-*bla*$_{\text{CMY-2}}$ (online Technical Appendix). In poultry and chicken meat from Sweden, the combinations incK-*bla*$_{\text{CMY-2}}$, incI1-*bla*$_{\text{CMY-2}}$, and incI1- *bla*$_{\text{CTX-M-1}}$ were primarily identified. These combinations were also identified in imported chicken meat and pigs/calves from Sweden (online Technical Appendix). In isolates from humans, these 3 combinations constituted in 9% and 4% of isolates from community carriers and bloodstream infections respectively. The combination incI1-*bla*$_{\text{CTX-M-8}}$ was identified in chicken meat from South America and was also identified in 2 isolates from bloodstream infections (online Technical Appendix) (S. Ny et al., unpub. data).

The 2 most common combinations identified in isolates from humans that overlapped with farm animals or foods were incI1-*bla*$_{\text{CTX-M-15}}$ and incFII-*bla*$_{\text{CTX-M-15.}}$ These combinations were identified in 1 isolate from pig/calves

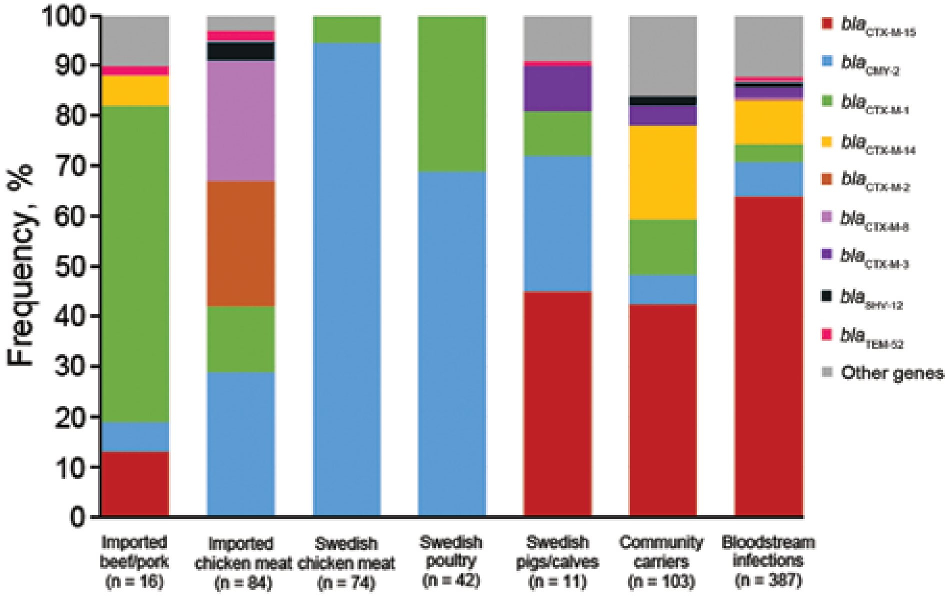

Figure 1. Frequency of overlapping extended-spectrum β-lactamase– and plasmid-encoded AmpC genes in *Escherichia coli* isolates from various sources, Sweden. Data for leafy greens were excluded because there were only 2 isolates (both *bla*$_{\text{CTX-M-1}}$) from this source.

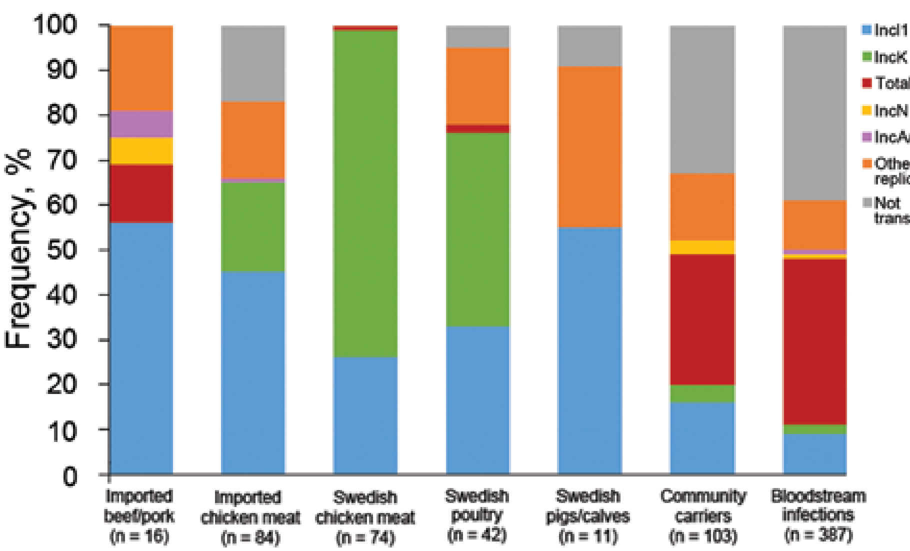

Figure 2. Frequency of overlapping plasmid replicon types containing extended-spectrum β-lactamase– and plasmid-encoded AmpC genes in *Escherichia coli* isolates from various sources, Sweden. Data for leafy greens were excluded because there were only 2 isolates (incl1 and incl2). Other replicon types also include nontypeable plasmids. Gray bar indicates plasmids that could not be transferred by electroporation, primarily isolates with $bla_{CTX-M-15}$ and $bla_{CTX-M-2}$.

in Sweden and 2 isolates from imported beef/pork (online Technical Appendix).

Because incI1 plasmids with identical genes overlapped in different sectors (online Technical Appendix), pMLST was performed to further discriminate between incI1 plasmids. Eight different overlapping incI1 plasmid STs (pSTs) were identified in isolates from community carriers (6 isolates), bloodstream infections (10 isolates), poultry from Sweden (15 isolates), chicken meat from Sweden (17 isolates), imported chicken meat (23 isolates), calves/pigs from Sweden (2 isolates), imported beef/pork (7 isolates), and leafy greens (1 isolate) (Table 2).

Overlap of *E. coli* STs and Plasmid Genes Encoding ESBL and pAmpC

When we compared clonal distributions on the basis of *E. coli* MLST, plasmid replicon type, and ESBL/pAmpC genes, 3 overlapping combinations (ST155-incI1-$bla_{CXT-M-1}$, ST10-incI1-$bla_{CXT-M-15}$ and ST57-incK-bla_{CMY-2}) were identified (online Technical Appendix). These 3 combinations constituted 17 (2%) of 715 isolates tested. For the combination ST155-incI1-$bla_{CXT-M-1}$, incI1 from community carriers and imported chicken meat belonged to pST3, and the 3 incI1 plasmids from poultry in Sweden belonged to a pST3-like incI1 (nontypeable *sog*S gene) (Table 2). The incI1 in the isolate from bloodstream infections with the overlapping combination ST10-incI1-$bla_{CXT-M-15}$ belonged to pST172, and the others belonged to pST37 (community carriers) and pST31 (calves from Sweden and imported beef). This finding decreased the total number of overlapping clones from 17 to 11: 2 from community carriers, 3 from imported chicken meat, and 6 from poultry in Sweden. Thus, the only clonal overlap identified consisted of isolates from farm animals, foods and community carriers.

Discussion

ESBL/pAmpC-producing *E. coli* in food and farm animals in Sweden appears to have had a limited effect on presence among human community carriers and the increasing problem with ESBL/pAmpC in healthcare facilities in this country, as shown in this study by low clonal overlap of isolates from community carriers, imported chicken meat, and poultry from Sweden. In addition, no overlap of isolates from bloodstream infections and isolates from foods or animals was identified. Thus, our findings confirmed results of previous analysis of risk factors associated with community carriage in Sweden, which indicated that preferred diets of humans do not increase the risk of becoming a carrier of ESBL/pAmpC-producing *E. coli* (S. Ny et al., unpub. data). However, one risk factor identified for being a community carrier was travel to Asia and Africa, which is supported by previous studies showing an increased carriage rate in healthy residents of Sweden after visits to these areas (*16,17*).

The finding that farm animals are a limited dissemination route to humans has also been reported in a study conducted in the United Kingdom, the Netherlands, and Germany, which showed limited overlap of ESBL-positive *E. coli* isolates of human and animal origin (*18*). However, another study in Germany reported a major set of similar subtypes of ESBL-positive *E. coli* (*11*). Unfortunately it is difficult to make further comparisons between the results of the current study and these 2 studies because different molecular methods were used (*11,18*). In addition, the other 2 studies did not include pAmpC-producing *E. coli* in their evaluations.

Nonetheless, results of the current study indicate that limited dissemination of ESBL/pAmpC genes or plasmids could have occurred between sectors. This conclusion is based on the fact that 4 plasmid/gene combinations (incK-bla_{CMY-2}, incI1-bla_{CMY-2}, incI1- $bla_{CTX-M-1}$, and incI1-$bla_{CTX-M-8}$)

Table 2. Overlapping plasmid multilocus sequence types for 114 incI1 plasmids carrying ESBL/pAmpC genes in *Escherichia coli* isolates from various sources, Sweden*

Plasmid multilocus sequence type	ESBL/pAmpC gene	Category (no.)	*E. coli* multilocus sequence type
3	$bla_{CTX-M-1}$	Community carriers (4), bloodstream infections (1), poultry (9), chicken meat from Sweden (3), imported chicken meat (2), imported beef/pork (4), leafy greens (1)	57, 80, 88, 117, 131, 135, 141,155, 219, 602, 752, 744, 847, 1335 1594,1607,1640, 4162, 4367
3-like†	$bla_{CTX-M-1}$	Bloodstream infections (1), poultry (3)	62,155
7	$bla_{CTX-M-1}$	Blood stream infections (3), imported chicken meat (5)	117, 453, 746, 752, 2500, 4373
31	$bla_{CTX-M-15}$	Community carriers (1), bloodstream infections (1), pigs or calves (1), imported beef/pork (2)	10, 162, 349
114	$bla_{CTX-M-8}$	Bloodstream infections (2), imported chicken meat (9)	10, 155, 533 1304, 1773, 4362
36	bla_{TEM-52}	Bloodstream infections (1), imported chicken meat (2), pigs or calves (1), imported pork/beef (1)	10, 86,155, 297, 2169
2	bla_{CMY-2}	Bloodstream infections (1), chicken meat from Sweden (2)	10, 58, 299
12	bla_{CMY-2}	Community carriers (1), poultry (3), chicken meat from Sweden (12), imported chicken meat (5)	10, 38, 48, 58, 69, 117, 131, 206, 297, 1079

*ESBL, extended-spectrum β-lactamase; pAmpC, plasmid-encoded AmpC.
†The *sog*S gene could not be detected by PCR.

identified in isolates from humans can be considered to be animal associated. IncK-bla_{CMY-2} and incI1-bla_{CMY-2} was the most prevalent combination in isolates from poultry in Sweden and domestically produced chicken meat and were also found in pigs and calves in Sweden.

Furthermore, IncI1-$bla_{CTX-M-1}$ and incI1-$bla_{CTX-M-8}$ were common in imported foods, primarily chicken meat, and incI1-$bla_{CTX-M-1}$ was detected in farm animals and chicken meat in Sweden. It has also been suggested that incI1 and incK plasmids might have emerged from animal reservoirs (*19*). Results of our study support this suggestion because a clear difference in plasmids carrying the genes encoding ESBL/pAmpC from farm animal/foods and human isolates was observed (Figure 2). The hypothesis that ESBL/pAmpC genes and plasmids from foods/animals could have disseminated to humans in Sweden is further supported by the fact that incI1 plasmids with the same pMLST types were identified in different sectors (Table 2; online Technical Appendix).

In our study, incI1-pST3-$bla_{CTX-M-1}$ and incI1-pST12-bla_{CMY-2} plasmids were identified in isolates from humans, farm animals, and foods. IncI1-pST3-$bla_{CTX-M-1}$ is of particular interest because it has been commonly identified in poultry in Europe and might have spread to other animal species and humans (*12,20,21*). Another example of possible ESBL/pAmpC plasmid spread from foods to humans was detection of 2 bloodstream infection isolates carrying incI1-pST114-$bla_{CTX-M-8}$ (Table 2). A recent study in the Netherlands that used whole-genome sequencing also concluded that it is not clonal dissemination, but rather plasmids and genes that are being disseminated between human and animals, mainly poultry (*22*).

The results of this study also confirm the conclusion of the EFSA that chicken meat can be a dissemination route for ESBL/pAmpC to humans, with poultry serving as the reservoir (*1*). However, this reservoir so far appears to have had a restricted effect on bloodstream infections and community carriers in Sweden. Only 2 of 103 isolates from community carriers were identified as possibly being associated with poultry on the basis of ST plasmid–gene combinations of isolates. Our results indicate that, in Sweden, 0.09% of the population might be expected to carry poultry-associated isolates. In addition, only 3% of isolates from bloodstream infection and 5% from community carriers carried identical plasmid–gene combinations to those identified in isolates from chicken meat and poultry in Sweden.

These results contradict the findings of studies in the Netherlands, which report larger clonal overlap (≥40%) of ESBL-producing isolates (pAmpC was not included) from humans, broilers, and chicken meat (*12,13*). Two other studies from the Netherlands reported that 19% of human clinical isolates have identical plasmid-ESBL gene combinations and 4% plasmid-bla_{CMY-2} combinations as those identified for poultry (*12,23*). The considerable difference between Sweden and the Netherlands is difficult to explain, but it could be influenced by differences in human and animal population densities, farming intensity, climate, biosafety, and hygiene practices. The low rate of antimicrobial drug use in poultry in Sweden compared with that in the Netherlands might have also influenced the differences (*6*). With regards to isolates from pigs/calves and imported pork/beef, these isolates had gene and plasmid–gene combinations comparable with those of human isolates. In addition, prevalences were much lower in isolates from pigs/calves and imported pork/beef than in chicken meat/poultry. Thus, it is likely that most of these isolates could be examples of human-to-animal spread.

The results of our study suggest that, to control the increase in ESBL/pAmpC in the human healthcare system in Sweden, minimizing transmission between humans should be prioritized. However, the high prevalence of ESBL/pAmpC in chicken meat and poultry in Sweden is

problematic, and precautionary efforts should be made to reduce this prevalence in Sweden and internationally. Changes in the molecular epidemiology of ESBL/pAmpC might occur quickly, and foods and farm animals could play a more critical role in the near future. Therefore continuous monitoring and comparative analyses of ESBL/pAmpC from farm animals, foods, and humans, as well as limiting spread within and between different sectors are needed. To reduce the frequency and transfer of ESBL/pAmpC, continued collaboration between professionals and agencies working in human healthcare, animal healthcare, and the food industry is needed. In addition, collaboration with environmental professionals is also essential.

In Sweden, foods and farm animals appear to be limited contributors to ESBL/pAmpC-producing *E. coli* in community carriers and its increasing prevalence in human bloodstream infections. However, foods might function as a dissemination route, and farm animals might function as a potential reservoir for genes encoding ESBL/pAmpC and plasmids carrying these genes. On a gene/plasmid level, there is an overlap between food/farm animals, primarily poultry, and humans, but compared with results from other studies in Europe, this overlap is limited.

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prions *Plasmodium knowlesi* cholera tularemia *Eptesicus fuscus* syncytium *Klebsiella* Kaposi *Leptospira* sapovirus yaws *Rickettsia* *Vibrio vulnificus* Quinine variola *Campylobacter* *Acinetobacter* Chagas disease rotavirus Lyssavirus *Aspergillus* botulism *Escherichia coli* syphilis knemidocoptic mange *Babesia* hemozoin *Naegleria fowlerii* *Ehrlichia* Leishmaniasis *Bordetella* *Anopheles* rabies Verona integron Zika virus Herpesvirus vaccination Artemisinin Dengue *Shigella* Borna disease virus Ebola *Franciscella tularensis* typhus Rickettsia orf *Coxiella burnetii* kobuvirus *Candida* Q fever *Orientia tsutsugamushi* Bocavirus chimera *Brucella* Norovirus tuberculosis quarantine Mange tetanus Malaria measles Chikungunya pertactin *Borrelia* Leprosy influenza Calcivirus quarantine Peste des petits ruminants O'nyong-nyong virus *Pseudoterranova azarasi* meliodosis Diphtheria Bonferroni correction pertussis Merkel cells *Ignatzschineria* Glanders *Yersinia*

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Post-Ebola Syndrome, Sierra Leone

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Thousands of persons have survived Ebola virus disease. Almost all survivors describe symptoms that persist or develop after hospital discharge. A cross-sectional survey of the symptoms of all survivors from the Ebola treatment unit (ETU) at 34th Regimental Military Hospital, Freetown, Sierra Leone (MH34), was conducted after discharge at their initial follow-up appointment within 3 weeks after their second negative PCR result. From its opening on December 1, 2014, through March 31, 2015, the MH34 ETU treated 84 persons (8–70 years of age) with PCR-confirmed Ebola virus disease, of whom 44 survived. Survivors reported musculoskeletal pain (70%), headache (48%), and ocular problems (14%). Those who reported headache had had lower admission cycle threshold Ebola PCR than did those who did not ($p<0.03$). This complete survivor cohort from 1 ETU enables analysis of the proportion of symptoms of post-Ebola syndrome. The Ebola epidemic is waning, but the effects of the disease will remain.

Thousands of persons have now survived Ebola virus disease (EVD). During efforts to control the current Ebola-Zaire outbreak, attention has focused on containing spread of infection and improving survival. In Sierra Leone, 4,051–5,115 persons are confirmed to have survived from among 8,704 confirmed cases and 3,589 confirmed deaths (*1*).

Survivors report a range of sequelae loosely described as post-Ebola syndrome. Follow-up clinics were not always planned as part of the emergency response. However, survivors from the Ebola treatment unit (ETU) at the 34th Regimental Military Hospital (MH34), Wilberforce Barracks, Freetown, Sierra Leone, were all followed up in an outpatient clinic within 2 weeks after discharge. Although resources to care for survivors, including basic equipment (e.g., adequate stethoscopes), were scarce, each survivor was seen by a physician who made contemporaneous structured notes, which afforded an opportunity to document post-Ebola syndrome during these first weeks.

What proportion of Ebola survivors have sequelae is not clear. Little is known about post-Ebola syndrome or whether it is an entity distinct from an appropriate response to the traumatic events of EVD. Abdominal pain, vision loss, hearing loss, impotence, bleeding, psychological problems, and general weakness were listed qualitatively as symptoms of post-Ebola syndrome after the Ebola-Sudan outbreak in Uganda in 2000 (*2*). Arthralgia and ocular diseases were noted in 19 survivors (selected according to availability) who were followed up after the 1995 Ebola-Zaire outbreak in Kikwit (*3*,*4*); in the same outbreak, arthralgia, myalgia, abdominal pain, extreme fatigue, and anorexia were more common in Ebola survivors than in their household contacts (*5*). From the current outbreak, survivors reported arthralgia and anorexia (which in this context includes loss of appetite without weight loss) in a telephone-administered questionnaire in Guinea several months after discharge (*6*). Because none of these studies comprised an unselected cohort of survivors, interpretation of proportions was difficult. Other reports referred to anecdotes of pain, weakness, difficulty hearing, and mental disturbances (*7*,*8*). These observations suggest complaints that might be expected. Descriptions of the proportions of survivors needing care for the most common problems are needed to plan health care for the thousands of survivors. We report the symptoms described by all EVD survivors from 1 ETU in the initial weeks after discharge.

Methods

The MH34 ETU can accommodate 30 persons with confirmed EVD plus 20 persons with suspected EVD; it also contains a doffing area. MH34 opened on December 1, 2014, with 115 staff, including 3 physicians and catered to patients who fell ill in western Freetown and surrounding areas. The ETU admitted 355 patients (84 PCR-positive patients) and discharged 44 survivors during December 2014–March 2015. The area for persons with confirmed EVD is a permanent building with several 1–4-bed rooms that have electric lighting and ceiling fans. Three hot meals per day are provided, generally rice with protein, such as fish or chicken; each meal is provided with 2 bags of water, and more water is freely available. Staff members of this small ETU are all permanent Sierra Leonean healthcare workers.

Patients were admitted to the confirmed Ebola ward when Ebola virus (EBOV) infection was confirmed by real-time PCR. For some patients, a cycle threshold (C_t) result also was available. Although C_t results were not standardized between PCR platforms or between laboratories, a low

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C_t reflects a high viral load. Patients were staged on arrival to the ETU, as follows:

- Stage 1: influenza-like illness (i.e., fever, myalgia. lethargy, fatigue, headache, sore throat, conjunctival injection).
- Stage 2: multisystem features, including "wet" gastrointestinal symptoms (vomiting, diarrhea), neurologic symptoms (headaches, confusion), vascular symptoms and signs (capillary leak, respiratory distress, hypotension), rash.
- Stage 3: internal and external bleeding, multiorgan failure.

Patients were treated for Ebola with supportive care (*9*). Antimicrobial drugs were administered empirically, and artesunate, paracetamol, and 500 mL intravenous Ringer's lactate were administered on arrival. Ongoing treatment included further boluses of intravenous fluid; antiemetic medication and proton pump inhibitors were administered in accordance with clinical need. Some patients participated in a compassionate use open nonrandomized study of a single unit of convalescent whole blood (CWB), results of which are pending.

Discharge criteria were as follows: 2 consecutive negative PCR results for Ebola virus on separate days; medical fitness, in the opinion of his/her physician; and adequate social provisions, including release of the house and household members from quarantine. During the convalescent period, many patients ate >1 serving of each meal, 3 time per day. Although they were not routinely weighed, most patients visibly gained weight.

On leaving the ETU, all survivors were issued a survivor's certificate and invited to a follow-up appointment within 2 weeks after discharge. Some survivors were seen before this appointment because of clinical need.

Contact with survivors was maintained by mobile phone. Confirmation of identification has not proved problematic because the survivors and healthcare workers had come to know each other well. Appointments are made by mobile phone and unscheduled visits by patients to the hospital. All survivors attended their follow-up visits. Patients were examined by 1 of 3 experienced physicians.

A follow-up appointment was established as a standard of care in this ETU from the outset at the height of the epidemic. Handwritten clinical notes documented presenting complaints, symptoms, and signs. These notes were subsequently used to develop appropriate preprinted clinical documentation. Age, sex, presenting complaints, and history of transfusion with CWB were noted for each patient. Preexisting conditions were rare in this cohort of patients and not included in this data extraction. At that time, facilities and equipment for survivors were limited; for example, all stethoscopes had been incinerated; blood pressure cuffs, ophthalmoscopes, and specialist opinions were not available.

Data Analysis

We determined 95% CIs and conducted hypothesis testing of binomial outcomes (binomial frequency test) continuous outcomes (Mann-Whitney U) and analyzed them using Stata version 9 (StataCorp LP, College Station, TX, USA). Graphics were produced by using Stata version 9 and R version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Demography

During December 1, 2014–March 30, 2015, the MH34 ETU treated 84 persons with PCR-confirmed EVD. Of these, 44 (52%) survived; 23 were female, and patient ages were 8–70 years (median 35 years; interquartile range [IQR] 20–37 years); age was not documented for 1 patient (Figure 1; Table 1).

Acute EVD Episode

Information about the acute EVD episode was available for 12 (27%) of the 44 survivors. Sex and ages of these 12 survivors did not differ significantly from those of the full set. For these 12 persons, median length of ETU stay was 15.5 days (range 9–17 days, IQR 13.5–16.6 days). For the 11 survivors for whom C_t results were available, median C_t at ETU admission (admission C_t) was 28 (range 23–37, IQR 23–31). Two patients were admitted in clinical stage 1 and 9 in clinical stage 2.

Twenty-three (52%) survivors received CWB. Ages of survivors receiving transfusions did not differ significantly from those of survivors who did not ($p = 0.8$). The frequencies of symptoms did not differ significantly between survivors

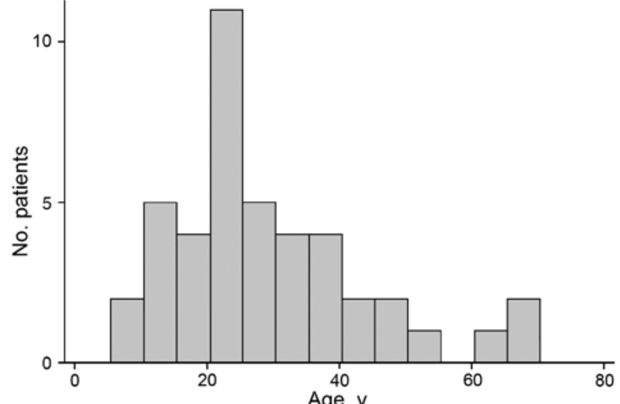

Figure 1. Age distribution of patients at Ebola survivors clinic at the 34th Regimental Military Hospital, Wilberforce Barracks, Freetown, Sierra Leone. Cycle threshold levels at hospital admission by age are shown in Table 1.

Table 1. C_t results at hospital admission for 44 patients with post-Ebola syndrome, by sex, Sierra Leone*

Category	Patient sex M	Patient sex F	All patients
No. patients	21	22	44
C_t at admission			
Median	30	25	25
Range	10–52	8–70	8–70
IQR	22–37	20–34	20–37

*Age distribution of all patients is shown in Figure 1. C_t, cycle threshold; IQR, interquartile range.

who received CWB and those who did not (p = 0.5). Our study was not designed to assess efficacy or toxicity of CWB.

Post-Ebola Syndrome Complaints

At the time of data extraction, each survivor had attended at least 2 appointments. All survivors had ≥1 post-Ebola complaint (median 2, maximum 5). A total of 117 separate complaints were reported: 31 (70% [95% CI 55%–83%]) patients had musculoskeletal pain, 21 (48% [95% CI 32%–63%]) had headaches, and 6 (14% [95% CI 5%–27%]) had ocular problems.

In their initial follow-up appointment, patients who reported headache had had admission C_t results that were significantly lower (correlating to a higher viral load) than those who did not subsequently report headache (with headache: n = 6, median C_t 24 [IQR 23–28]; without headache: n = 5, median C_t 31 [IQR 30–31]; p<0.03 by Mann-Whitney U test) (Table 2; Figure 2). There was no significant difference in admission C_t or clinical stage, or length of stay in the ETU for acute Ebola or clinical stage, between patients who had ocular problems or musculoskeletal pain and those who did not (Table 2; Figure 2).

One patient died after deteriorating respiratory symptoms and left-sided pleural effusion. He was a 25-year-old man in whom EVD was diagnosed on January 26; he received supportive care and 1 unit of CWB. His first negative PCR result was on February 8 and his confirmatory negative test on February 11; he was discharged home. At his 14-day follow-up visit, he had weight loss, cough, and dyspnea on exertion. At his second outpatient appointment, he was admitted to the general medical ward of MH34 on March 3 with left-sided pleural effusion. A pleural tap yielded only a small quantity of blood-stained fluid that was insufficient for analysis. He died on March 8, 2015, one month after his recovery from acute EVD. In adherence to safe-burial policy, a postmortem examination was not performed. His diagnosis remains unclear, but postviral effusion is possible, with tuberculosis pleural effusion a differential diagnosis.

Table 2. C_t results at hospital admission for patients with post-Ebola syndrome who reported 1 of the 3 most common symptoms, Sierra Leone*

Category	Ocular problems	Musculoskeletal pain	Headache
Yes, no. patients	3	7	6
C_t at admission			
Median	31	25	24
IQR	25–37	23–30	23–28
No, no. patients	8	4	5
C_t at admission			
Median	27	29	31
IQR	23–29	25–34	30–31
p value†	0.2	0.5	**0.03**

*Box-and-whisker plots illustrating symptom appearances are shown in Figure 2. Some patients reported ≥1 symptom. Boldface indicates significance. C_t, cycle threshold; IQR, interquartile range.
†By Mann-Whitney U test.

Musculoskeletal Pain

Because in our experience and in the local context the distinction between myalgia and arthralgia can be physician-dependent, we merged these complaints. However, for the purpose of comparisons with other studies, we determined that 12 (27% [95% CI 15%–42%]) of the 44 survivors had arthralgia, 15 (34% [95% CI 20%–50%]) had myalgia, and 4 (9% [95% CI 3%–22%]) had both (Table 3). We found no significant differences between the proportion of male and female survivors, or between children (<18 years of age) and adults, who had musculoskeletal pain.

Patients described musculoskeletal pain variously as problems with walking or moving or pain specific to 1 area (such as knees, thighs, or back) or generalized musculoskeletal pain. (21%–52%). Most often, patients

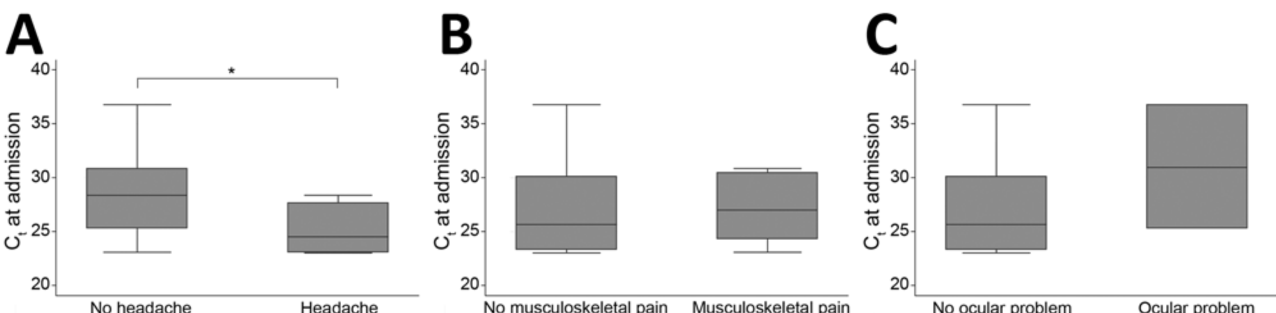

Figure 2. Comparison of the most common post-Ebola syndrome symptoms with admission C_t results, 34th Regimental Military Hospital, Wilberforce Barracks, Freetown, Sierra Leone. A) Headache, B) musculoskeletal pain, C) ocular problems. Specific C_t levels are shown in Table 2. *Indicates significant difference (p<0.03). C_t, cycle threshold.

Table 3. Musculoskeletal symptoms described by 31 patients with post-Ebola syndrome, Sierra Leone*

	Patient sex		
Area of pain	M	F	Total
Joints			
Joint, unspecified	5	9	14
Knee, unspecified	2	0	2
Right knee joint	0	1	1
Shoulder joint	1	1	2
Body			
Generalized body	4	4	8
Upper back	1	3	4
Musculoskeletal, unspecified	2	0	2
Left thigh	1	1	2
Lower limb	0	1	1
Right thigh	1	0	1
Gluteal muscle	1	0	1

*Values are no. patients. Some survivors reported >1 area of pain. The proportion of male and female survivors with musculoskeletal pain did not differ significantly (χ^2, p = 0.7).

characterized their musculoskeletal pain as a general pain rather than pain in a specific joint or area, as reflected in the recorded symptoms. Unspecified joint pain accounted for 14 of the 19 times joint pain was recorded (73% [95% CI 49%–90%]) and generalized body pain for 8 of the 19 times body pain was recorded (42% [95% CI 20%–67%]). Some patients recorded >1 symptom.

Examination indicated no joint inflammation or effusion, such as might be expected in a reactive condition, and patients retained full range of movement. Functional disability ranged from mild to moderate. For example, 1 man in his 20s continues to play football but now takes acetaminophen to facilitate this activity. A woman in her 40s requires assistance to step into a bath and cannot continue normal household work; she walked unaided into the clinic but needed assistance to step up into the clinic room and to sit and stand. Most of her musculoskeletal symptoms are relieved by simple analgesics.

Headache

Of the 21 (48% [95% CI 32%–63%]) survivors who reported having headache, 2 (10% [95% CI 1%–30%]) were girls 8 and 11 years of age. The proportion of male and female survivors reporting headaches did not differ significantly (p = 1 by χ^2 test). Headache was generally described as affecting the full head, with no diurnal pattern and being constant. Ocular symptoms might coincide, but no visual phenomena, such as might be found in migraines, were reported.

Ocular Symptoms

Among the 6 (14% [95% CI 5%–27%]) survivors who reported ocular problems, symptoms were eye pain, clear discharge, red eyes, and blurred vision (Table 4). These symptoms appeared within 2 weeks after discharge and were not present at or before ETU discharge.

Table 4. Ocular symptoms described by 6 patients with post-Ebola syndrome, Sierra Leone

Patient age, y/sex	Symptom
8/F	Eye pain
14/F	Clear eye discharge
20/F	Clear eye discharge
28/F	Red eyes and blurred vision on the left
29/F	Red eyes
46/M	Blurred vision

Eye discharge was treated with topical chloramphenicol. Ophthalmology services for survivors are currently under development.

Combinations of Musculoskeletal Pain, Headache, and Ocular Problems

Musculoskeletal pain and headache overlapped substantially. Eighteen (58% [95% CI 40%–75%]) of the 31 survivors with musculoskeletal pain reported headache, and 18 (86% [95% CI 64%–97%]) of the 21 survivors with headache reported musculoskeletal pain. Two (6% [95% CI 1%–21%]) survivors with musculoskeletal pain reported ocular problems, and 2 (33% [95% CI 4%–78%]) with ocular problems reported musculoskeletal pain. Two (6 % [95% CI 1%–30%]) survivors with headache reported ocular problems. One survivor had all 3 complaints (i.e., 3% [95% CI 1%–17%] of survivors with musculoskeletal pain; 5% [95% CI 0%–24%] of those with headache, and 17% [95% CI 0%–64%] with of those with ocular problems) (Figure 3).

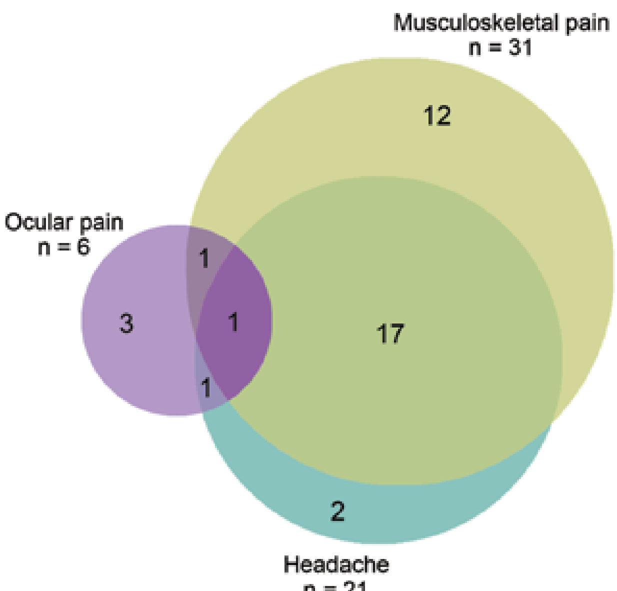

Figure 3. Scale Venn diagram illustrating the overlap between the 3 main symptom groups among persons with post-Ebola syndrome seen at the Ebola survivors clinic at the 34th Regimental Military Hospital, Wilberforce Barracks, Freetown, Sierra Leone. Seven patients did not have any of the 3 main symptom groups.

Other Symptoms

Twenty-six (59% [95% CI 43%–74%]) of the 44 survivors reported other symptoms. Five (11% [95% CI 4%–25%]) reported cough; 4 (9% [95% CI 3%–22%]) each reported abdominal pain, chest pain, or itching; 3 (7% [95% CI 1%–19%]) each reported insomnia, fever, or loss of appetite; 2 (5% [95% CI 1%–15%]) each reported labored speech, epigastric pain, or rash; and 1 (2% [95% CI 0%–12%]) reported weight loss, hiccups, increased appetite, chest pain, sneezing, diarrhea, vomiting, left sided weakness with facial nerve palsy, breathlessness, rash, dry flaky skin, earache, fever blister/cold sore, left scrotal swelling, nasal congestion, and tremors (Table 5).

Table 5. Post-Ebola complaints other than headache, musculoskeletal pain, or ocular problems among 44 survivors, Sierra Leone

Complaint	No. (%; 95% CI, %)
Cough	5 (11; 4–25)
Abdominal pain	4 (9; 3–22)
Chest pain	4 (9; 3–22)
Itching	4 (9; 3–22)
Insomnia	3 (7; 1–19)
Fever	3 (7; 1–19)
Loss of appetite	3 (7; 1–19)
Labored speech	2 (5; 1–15)
Epigastric pain	2 (5; 1–15)
Rash	2 (5; 1–5)
Other*	1 (2; 0–12)

*Weight loss, hiccups, increased appetite, chest pain, sneezing, diarrhea, vomiting, left sided weakness with facial nerve palsy, breathlessness, rash, dry flaky skin, earache, fever blister/cold sore, left scrotal swelling, nasal congestion, tremors.

Discussion

We documented symptoms of EVD survivors in the initial 3 weeks after negative Ebola virus PCR results and 2 weeks after ETU discharge. The dominant clinical features of this survivor cohort were musculoskeletal pain, headache, and ocular problems. Symptoms did not differ by survivor sex or age. Symptoms did not appear to be affected by use of CWB to manage acute EVD; however, this finding should be interpreted with caution because this report is not a prospective study and not designed to consider the effect of CWB on post-Ebola syndrome. Whether this collection of signs and symptoms after acute EVD constitutes a separate syndrome might be semantic. Because experience of survivors in the weeks after EVD, although varied, has common features, we propose that the term post-Ebola syndrome is useful to describe these phenomena.

Our findings are consistent with some aspects of previous reports (*2*,*5*) but vary from others. For example, the prevalence of extreme fatigue and anorexia reported in Kikwit and Guinea (*5*,*6*) was not dominant in the cohort reported here. This finding might be due to the period of inpatient convalescence of survivors at MH34 with substantial nutritional support.

We hypothesize that the pathogenesis of pain, particularly muscle pain, is a sequelae of widespread myositis or rhabdomyolysis during acute EVD. This hypothesis would be consistent with laboratory data reporting raised transaminases and disseminated intravascular coagulation from a previous outbreak of Ebola (*10*) in Sudan. Future research would benefit from a comparison of a survivor cohort with a matched group who had not had Ebola and, if this pain is more common in Ebola survivors (as was found in Kikwit [*5*]), further elucidation of its etiology would be useful in determining treatment strategies.

Post-Ebola syndrome includes, but is not restricted to, musculoskeletal pain, headache, and ocular problems. Because some complications occur weeks or months after acute onset of EVD, some symptoms might be underestimated in this cohort (*2*,*5*). Since these data were extracted, clinical facilities and documentation has improved, so future information is likely to be more detailed in terms of specific diagnosis, and scope, particularly in regard to psychosocial health and ophthalmology. Previous outbreaks have reported psychosocial problems (*2*), although they are not included in all reports (*5*). Psychosocial problems also were evident in the survivors in our study but not captured in the documentation. Improved collaboration with MH34's mental health team should improve both the care and documentation. Anecdotal evidence from the survivors' clinic suggests that more subtle neurologic problems, such as specific nerve palsies, might feature more heavily in a follow-up study.

Survivors who reported headache had had lower C_t results than did those who did not. Although patients with higher initial viral loads might be more likely to have central nervous system involvement, and then have a higher probability of headache as a post-Ebola sequelae; C_t values are not standardized among platforms or laboratories. This intertest variability, together with the small sample sizes in this data extraction, suggests any association should be interpreted with caution. We propose that this association warrants further investigation. Headaches could also represent ongoing tension headaches or might result from underlying undiagnosed changes in vision.

We would expect the criteria and definition of post-Ebola syndrome to continue to develop and that the survivors will continue to face fresh challenges. During the height of the Ebola epidemic, when these consultations took place, resources and equipment for assessing survivors were limited. Our survey documents symptoms only in the first 3 weeks after ETU discharge. Subsequent follow-up might be more detailed and benefit from increased resources, and symptoms continue to develop with time. Indeed, Ebola virus can cross the blood–brain barrier during the acute illness (*11*) and persists in some compartments for several months (*12*). Areas for development include comparison of symptoms to community controls, psychosocial

problems, causes of ocular problems and musculoskeletal pain, and longitudinal description of the clinical picture.

Because musculoskeletal pain is a common complaint in the general population in Sierra Leone, a community-controlled comparison is needed. In survivors of the Kikwit Ebola-Zaire outbreak in 1995, Rowe et al. reported that their key features—arthralgia, myalgia, abdominal pain, fatigue, and anorexia—were more common in survivors than in household contacts, whereas fever, headache, diarrhea, dyspnea, hiccups, and hemorrhage were the same in both groups (*5*). A topic for future research is the longitudinal course of recovery. Wendo et al. (*2*) reported that 1 year after the Ebola-Zaire outbreak in Uganda, 25% of patients were still reporting to clinic. Therefore, we can expect some survivors to have long-term clinical needs. The epidemic is waning but the effects of the disease it caused will remain.

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Dr. Scott is a clinical lecturer in pharmacology and infectious diseases at the Institute of Translational Medicine, University of Liverpool. Her primary research interests include characterizing PES and other emerging infectious diseases, clinical trials of EVD candidate therapeutics, and optimizing therapeutic regimens, including passive immunotherapy for EVD.

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Leprosy and Armadillos

Dr. Richard Truman, Chief of the National Hansen's Disease Program Laboratory Research Branch, discusses leprosy and armadillos.

EID PODCASTS

Armadillos used in leprosy research. Photo CDC, Dr. Charles Shepard, 1981

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Transmission of Middle East Respiratory Syndrome Coronavirus Infections in Healthcare Settings, Abu Dhabi

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Middle East respiratory syndrome coronavirus (MERS-CoV) infections sharply increased in the Arabian Peninsula during spring 2014. In Abu Dhabi, United Arab Emirates, these infections occurred primarily among healthcare workers and patients. To identify and describe epidemiologic and clinical characteristics of persons with healthcare-associated infection, we reviewed laboratory-confirmed MERS-CoV cases reported to the Health Authority of Abu Dhabi during January 1, 2013–May 9, 2014. Of 65 case-patients identified with MERS-CoV infection, 27 (42%) had healthcare-associated cases. Epidemiologic and genetic sequencing findings suggest that 3 healthcare clusters of MERS-CoV infection occurred, including 1 that resulted in 20 infected persons in 1 hospital. MERS-CoV in healthcare settings spread predominantly before MERS-CoV infection was diagnosed, underscoring the importance of increasing awareness and infection control measures at first points of entry to healthcare facilities.

Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel coronavirus first identified in the Middle East region in 2012. Epidemiologic aspects of this virus remain poorly defined, but human-to-human transmission of MERS-CoV in healthcare facilities is recognized as a means of spreading infection (*1–7*). In Saudi Arabia, the country with the greatest number of MERS-CoV infections, exposures in healthcare facilities have resulted in repeated outbreaks and have been linked to spread of disease after the virus has been introduced from other sources (e.g., zoonotic) (*5,6,8–11*). The 2015 outbreak in South Korea is a dramatic example of extensive healthcare-associated transmission after a single introduction of MERS-CoV by an infected traveler; that introduction resulted in >180 documented infections in hospitals lacking adequate infection prevention measures (*12,13*). Because healthcare settings have the potential to contribute substantially to the spread of MERS-CoV infections, improving our understanding of infection risk and transmission patterns remains an urgent priority.

By early September 2015, a total of 74 patients with laboratory-confirmed MERS-CoV infection were reported in the United Arab Emirates (UAE); most were reported from Abu Dhabi during March–April 2014, when the Arabian Peninsula had a sharp increase in infections, primarily involving healthcare workers (HCWs) and patients with recent healthcare exposure (*8,9,11*). The extensive case investigation and contact tracing by HCWs and the Health Authority of Abu Dhabi in response to this increase provide resources to inform our understanding of MERS-CoV infections acquired and spread in healthcare settings. We describe the epidemiologic and clinical characteristics of healthcare-associated MERS-CoV infections in Abu Dhabi and characterize the size and suspected transmission patterns in healthcare settings.

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Methods

Setting

Abu Dhabi is the largest of the 7 emirates of UAE. It has ≈2.3 million residents, including 1.9 million expatriates, and 35 hospitals (*14*).

Case and Contact Investigation Methods

In January 2013, a standardized public health protocol for MERS-CoV response was established in Abu Dhabi. Case-patients were defined as persons reported to the Health Authority of Abu Dhabi with laboratory confirmation of MERS-CoV infection by PCR performed on a respiratory sample (*15,16*). Our analysis included all case-patients reported during January 1, 2013–May 9, 2014. Activities involved in this investigation were reviewed by the US Centers for Disease Control and Prevention (CDC) and by the Health Authority of Abu Dhabi and were determined to be an urgent public health response that did not constitute human subjects research.

Health Authority staff conducted detailed investigations of case-patients and their close contacts, interviewing case-patients or family proxies to collect demographic, clinical, and risk-factor information during the 14 days before illness onset. Additional information about clinical exposures was collected from HCW case-patients (e.g., use of personal protective equipment [PPE]). After laboratory confirmation, all case-patients were hospitalized with airborne precautions until they had 2 consecutive PCR-negative MERS-CoV tests on specimens collected at least 48 hours apart. Close contacts, which included anyone who lived with, visited, provided care for, or had other similarly close contact with case-patients while they were symptomatic, were identified from interviews and other sources (e.g., hospital documentation). The contacts were interviewed and had nasopharyngeal, sputum, or tracheal aspirate samples collected for PCR testing, regardless of symptoms.

Sources of Exposure

Medical and public health records were used to categorize sources of exposure. Infections of case-patients who worked at, were admitted to, or visited a healthcare facility during the 14 days before symptom onset were considered to be healthcare-associated if exposure to a known MERS-CoV case-patient occurred exclusively in this setting. Healthcare exposure to a case-patient was characterized as either confirmed (i.e., persons who had been within 2 m of a symptomatic case) (*15,17*) or probable (i.e., persons who had been in the same hospital unit for >1 hour, had a common HCW, or had moved into a bed or dialysis station vacated by a symptomatic case) (*18*). Probable exposure was assumed for continuously hospitalized case-patients in whom symptoms of infection developed >14 days after admission.

Identification and Description of Clusters

Case investigations were used to construct cluster diagrams depicting suspected healthcare-associated transmission pathways. Healthcare-associated clusters were defined as ≥1 epidemiologically related, healthcare-associated case-patient in the same healthcare setting (i.e., with confirmed or probable exposure). Healthcare-associated clusters consisted of ≥1 source case (i.e., case-patient with the earliest date of symptom onset in the healthcare-associated cluster) and ≥1 secondary case (i.e., case-patient with confirmed exposure to the source case). Healthcare-associated clusters could also include tertiary and quaternary cases (i.e., case-patients exposed only to secondary and tertiary cases, respectively). Clinical records were used to assess probable exposures for healthcare-associated case-patients with no confirmed exposure; cases with confirmed exposure were not assessed for probable exposure because confirmed exposure was assumed to confer the greatest risk.

Laboratory Analysis

PCR

Nasopharyngeal swab, sputum, or tracheal aspirate samples were tested at a central laboratory (Molecular Diagnostic Laboratory at Sheikh Khalifa Medical City Hospital) in Abu Dhabi by using real-time reverse transcription PCR (rRT-PCR) for the upstream E gene and open reading frame 1 (*19,20*). A convenience sample of positive isolates was validated by using the nucleocapsid-based rRT-PCR assay at CDC (*21*).

Sequencing

Genetic sequencing was performed on a subset of isolates from 8 case-patients (7 from healthcare-associated clusters and 1 non–healthcare related). Full genome sequencing from original respiratory samples was determined by using the Sanger method (direct genome walking PCR) and next-generation sequencing approaches (Illumina MiSeq sequencer, http://www.illumina.com/systems/miseq.html) (*22,23*). Sequences were aligned by using MUSCLE (*24*) within the MEGA5 program (*25*).

Statistical Analysis

Descriptive analysis of healthcare-associated cases and clusters was conducted by using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA). Fisher exact test and independent *t*-test were used to compare clinical and demographic characteristics of source-cases and healthcare-associated cases; a 2-sided α level of 0.05 was used to determine significance.

Results

Case and Contact Investigation

Of 65 MERS-CoV case-patients identified during our investigation period (July 1, 2013–May 9, 2014) in Abu-Dhabi, 27 (42%) were healthcare associated; 19 (70%) of the 27 were HCWs; 6 (22%) were hospitalized patients,

Table 1. Descriptive epidemiology of 30 cases of MERS-CoV infection transmitted in healthcare settings, Abu Dhabi, January 1, 2013–May 9, 2014*

		Healthcare-associated case-patients†				
Demographic and clinical characteristic	Source case-patients, n = 3‡	All HCA case-patients, n = 27	HCWs, n = 19	Patients, n = 6	Visitors, n = 2	Signif§
Median age, y (range)	59 (30–83)	43 (27–82)	39 (27–63)	65 (40–73)	44 (34–54)	
Male sex	3 (100)	17 (63)	11 (58)	5 (83)	1 (50)	
Expatriate¶	1 (33)	26 (96)	18 (95)	6 (100)	2 (100)	0.02
Exposures within 14 d before symptom onset#						
Travel	0	2 (7)	1 (5)	0	1 (50)	
Camel	2 (67)	0	0	0	0	0.01
Symptoms						
Any symptoms reported	3 (100)	16 (59)	10 (53)	5 (83)	1 (50)	
Documented fever or symptom of respiratory illness**	3 (100)	13 (48)	8 (42)	5 (83)	0	
Documented fever (≥38.5°C)	3 (100)	9 (33)	6 (32)	3 (50)	0	
Shortness of breath	3 (100)	5 (19)	0	5 (83)	0	0.01
Fatigue/malaise	2 (67)	8 (30)	4 (21)	3 (50)	1 (50)	
Cough	2 (67)	7 (26)	4 (21)	3 (50)	0	
Cough with sputum production	2 (67)	2 (7)	0	2 (33)	0	0.04
Rhinorrhea	2 (67)	2 (7)	2 (11)	0	0	0.04
Muscle aches	2 (67	7 (26	5 (26)	1 (17)	1 (50)	
Chest pain	1 (33)	2 (7)	1 (5)	1 (17)	0	
Joint pain	2 (67)	2 (7)	2 (11)	0	0	0.04
Headache	2 (67)	4 (15)	3 (16)	1 (17)	0	
Sore throat	1 (33)	5 (19)	5 (26)	0	0	
Wheezing	1 (33)	3 (11)	1 (5)	2 (33)	0	
Vomiting/nausea	1 (33	1 (4)	0	1 (17)	0	
Medical history						
Any underlying conditions	2 (67)	15 (56)	7 (37)	6 (100)	2 (100)	
Diabetes mellitus	1 (33)	6 (22)	1 (5)	4 (67)	1 (50)	
Dementia	1 (33)	0	0	0	0	
Malignancy	1 (33)	0	0	0	0	
Receiving immunosuppressant	1 (33)	0	0	0	0	
Chronic pulmonary disease	0	2 (7)	0	2 (33)	0	
Renal disease	0	5 (19)	0	4 (67)	1 (50)	
Congestive heart failure	0	1 (4)	0	1 (17)	0	
Obese††	0	2 (7)	1 (5)	1 (17)	0	
Hypertension	0	12 (44)	5 (26)	5 (83)	2 (100)	
Hyperlipidemia	0	7 (26)	4 (21)	2 (33)	1 (50)	
Asthma	0	2 (7)	2 (11)	0	0	
Ischemic heart disease	0	3 (11)	1 (5)	2 (33)	0	
Severity of symptoms						
Care in ICU	3 (100)	5 (19)	0	5 (83)	0	0.01
Supplemental O_2 required	3 (100)	6 (22)	0	6 (100)	0	0.02
Intubated	3 (100)	3 (11)	0	3 (50)	0	<0.01
Died	2 (67)	2 (7)	0	2 (33)	0	0.04
Reason tested for MERS-CoV						
Screening as part of contact investigation	0	24 (89)	19 (100)	3 (50)	2 (100)	<0.01
Symptoms consistent with MERS-CoV	3 (100)	3 (11)	0	3 (50)	0	<0.01

*Values are no. (%) patients except as indicated. HCA, healthcare-associated; HCWs, healthcare workers; MERS-CoV, Middle East respiratory syndrome coronavirus; Signif, statistically significant.
†HCA case-patients include HCWs, patients, and hospital visitors but does not include source case-patients.
‡Source case-patients are those with the earliest date of onset of symptoms in an HCA cluster of case-patients.
§Comparison between type of case (source case vs. HCA case) determined by Fisher exact test. Only significant values are shown.
¶Nationalities: Philippines, India, Somalia, Bangladesh, Egypt, Jordan, Oman, Pakistan, Sudan, and Syria.
#For case-patients with no reported symptoms, date of positive sample collection was used in place of symptom onset.
**Symptoms of respiratory illness are cough, shortness of breath, or wheezing.
††Obesity status was determined by clinical staff.

and 2 (7%) were hospital visitors (Table 1). An additional 16 case-patients had worked at or visited a healthcare facility in the month before illness but did not meet the healthcare-associated case definition and were excluded from this analysis; 8 of the 16 excluded case-patients were HCWs with confirmed exposure to a symptomatic case-patient outside the healthcare setting (i.e., household); 8 had visited a healthcare facility but had no probable or confirmed exposure in this setting.

Source Cases

All 3 source case-patients in the healthcare-associated clusters were men with a median age of 59 years; 2 had a history of camel exposure in the 14 days before symptom

onset (Table 1). All were symptomatic, were admitted to intensive care, required supplemental oxygen, and were intubated; 2 died (67% case-fatality rate).

Healthcare-Associated Cases

Of the 27 healthcare-associated case-patients, 17 (63%) were male; median age was 43 years. None had a history of camel exposure during the 14 days before symptom onset. Disease severity varied by type of case; source case-patients had the greatest disease severity (Table 1). Fewer than half (42%) of HCW case-patients reported fever or symptoms of respiratory disease, and none required intensive care. The proportion of patients who died was significantly lower among healthcare-associated case-patients (2/27 [7%]) than among source case-patients (2/3 [67%]); death among healthcare-associated case-patients occurred only among hospital patients (2/6 [33%]).

Identification and Description of Clusters

From the epidemiologic and genetic investigation, we identified 3 healthcare-associated clusters at 3 hospitals during our investigation period. The clusters ranged in size from 3 to 21 case-patients (Figure 1).

Cluster I, July 2013

The source case-patient for cluster I (patient I-A; Figure 1) was an 82-year-old UAE resident who owned a farm with camels, had no travel history or contact with another case-patient, and was hospitalized in Abu Dhabi with respiratory symptoms in July 2013. Two days later, the patient was transferred by ambulance to another hospital ≈350 km away, where he tested positive by PCR for MERS-CoV, developed acute respiratory distress syndrome, and died.

Among 277 healthcare contacts identified in the 2 hospitals and among transport staff, 4 healthcare-associated case-patients were detected through PCR screening of respiratory specimens, including the nurse who accompanied the source case-patient in the ambulance (patient I-B; Figure 1) and 3 HCWs who were involved in the patient's evaluation or early care at the second hospital (1 physician, 2 nurses; patients I-C, I-D, and I-E; Figure 1). All infected HCWs had close contact with the case-patient without respiratory protection before the MERS-CoV diagnosis.

Cluster II, March–April 2014

The source case-patient for cluster II (patient II-A; Figure 1) was a 68-year-old UAE resident who owned a farm and reported direct contact with camels. He had no travel history, no contact with a known case, and no healthcare facility contact during the 14 days before symptom onset. In March 2014, this patient was hospitalized in Abu Dhabi with respiratory symptoms; MERS-CoV was diagnosed 4 days later.

Among 90 healthcare contacts identified, 2 healthcare-associated case-patients were detected. A secondary case-patient (patient II-B; Figure 1) who shared a room with the symptomatic source case-patient before the MERS-CoV diagnosis subsequently developed respiratory symptoms, was readmitted to the hospital, was diagnosed with MERS-CoV, and died. Screening of contacts identified a tertiary case-patient (patient II-C; Figure 1) who had a probable exposure to patient II-B in hemodialysis (before diagnosis) and no exposure to the source case-patient.

Cluster III, March–April 2014

The source case-patient for cluster III (patient III-A; Figure 1) was a 45-year-old expatriate who had no travel history, no animal contact, and no healthcare facility contact during the 14 days before symptom onset. He worked in the storage room at a paramedic dispatch station, a nonclinical facility located in a police station where no patient contact occurs. An extensive epidemiologic investigation of household and work contacts revealed no known exposure to a case before symptom onset and no link to cluster II, which occurred at a hospital >350 km away. No known case-patients were transported by paramedics in this unit.

On March 29, the source case-patient developed respiratory symptoms. From April 2–6, he was assessed at an emergency department (ED) in Abu Dhabi on 3 occasions for fever, cough, shortness of breath, and pneumonia. He was examined in an ED room (with a curtain divider) under standard precautions and was given a surgical mask and oxygen, which staff reported he removed repeatedly because of difficulty breathing. On April 6, he was admitted to a general medical unit (unit A), where he received care for 5 hours before being transferred to the intensive care unit (ICU) and placed on airborne infection isolation precautions. A MERS-CoV diagnosis was confirmed on April 9, and he died the next day.

PCR screening of respiratory specimens from 224 possible healthcare contacts from the ED, ICU, and medical wards identified 15 healthcare-associated case-patients (Figure 1). Ten were secondary case-patients who had exposure to the source case-patient during ED visits (patient III-B on April 2; patients III-C–K on April 6); 1 was a HCW who cared for the source case-patient in the ICU after the MERS-CoV diagnosis (patient III-L); and 4 were tertiary case-patients among HCWs who had no exposure to the source case-patient but had confirmed exposure to infected co-workers in radiology (patient III-M), the hospital transport unit (patient III-N), and the ED (patients III-O and III-P) (Figure 2). Attack rates among healthcare contacts with confirmed exposure to the source case-patient were estimated to be 16% (10/64 contacts) in the ED and radiology department before diagnosis and 5% (1/21 contacts) in the ICU after diagnosis.

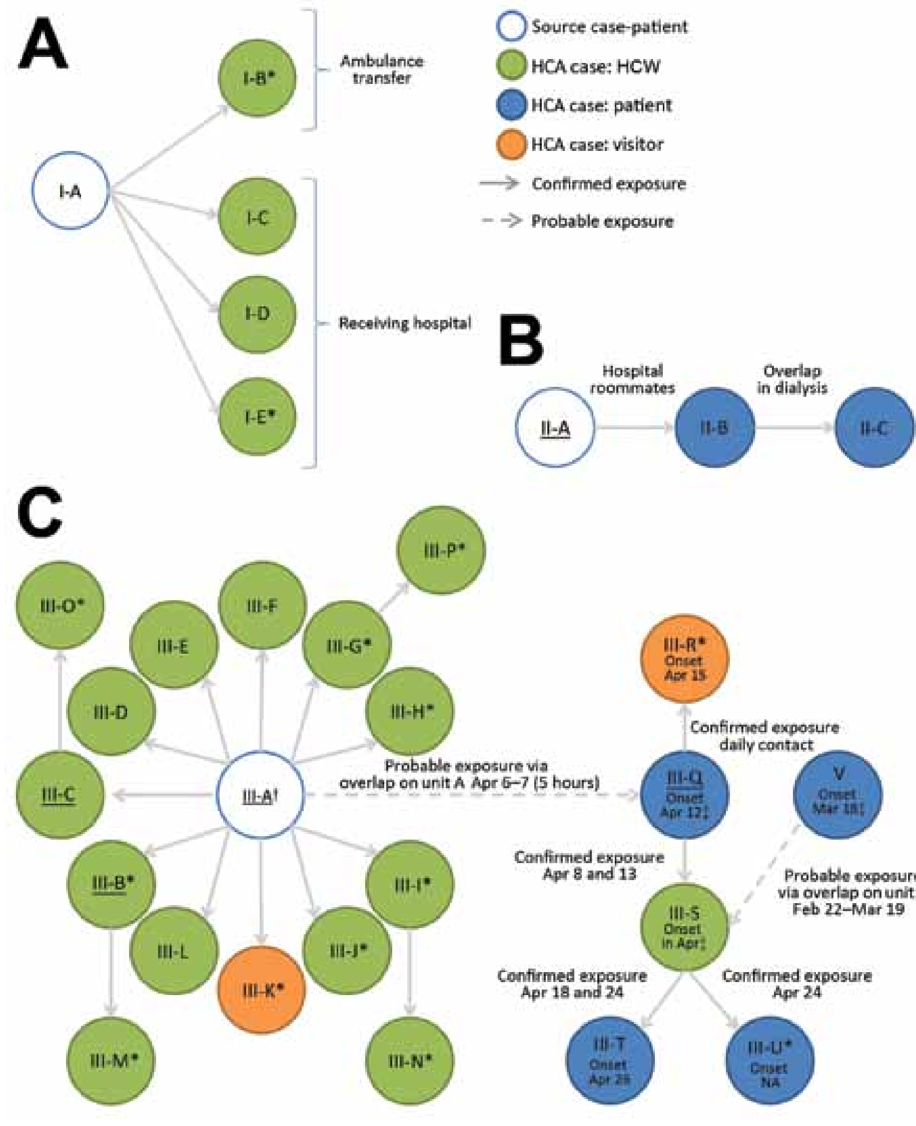

Figure 1. Transmission of Middle East respiratory syndrome coronavirus (MERS-CoV) infections in 3 healthcare setting clusters, Abu Dhabi, January 2013–May 2014. A) Cluster I; B) cluster II; C) cluster III. Individual patients are identified by cluster and a letter indicating the order in which cases occurred (e.g., I-A indicates the source case-patient for cluster I). Figure panels illustrate suspected chains of transmission of MERS-CoV infection within the 3 clusters. Each circle represents a case-patient. Arrows connect case-patients with likely source cases of MERS-CoV infection, with arrows pointing in the direction of transmission (i.e., from source case-patient to secondary case-patient). Descriptions adjacent to arrows indicate the timing or location of confirmed (shown with solid arrows) and probable (shown with broken arrows) exposures between the case-patients. Asterisks (*) indicate case-patients who reported no fever or symptoms of respiratory disease; underlining indicates cases for which isolates underwent genetic sequencing. †Dates of exposure and symptom onset for case-patients III-B–III-L are summarized in Figure 2. ‡After identification of MERS-CoV in case-patient V, healthcare workers in unit A were screened beginning March 24, 2014. MERS-CoV was not detected from a sputum specimen collected from case-patient III-S at this time. The MERS-CoV–positive specimen was collected on April 24, after identification of case-patient III-Q on the same ward. HCA, healthcare-associated; HCW, healthcare worker.

A second subcluster of illnesses was detected in unit A (i.e., general medical unit of hospital of admission) when a 74-year-old hospital patient (patient III-Q) who had been admitted to the unit in February developed new onset of shortness of breath on April 12 and had a MERS-CoV–positive sample collected the same day. During the 14 days before symptom onset, the patient was continuously hospitalized, had no travel history, no animal contact, and no confirmed exposure to a case-patient; however, she had resided in a room adjacent to patient III-A (source case-patient of cluster III) in unit A for 5 hours on April 6–7. During this period, no close contact with patient III-A occurred, and no documented common healthcare contacts or common equipment was identified; consequently, this case constitutes a probable rather than confirmed exposure.

Subsequent screening of 83 healthcare contacts of patient III-Q identified 2 healthcare-associated case-patients, including patient III-Q's daughter (patient III-R, tertiary case), who had been staying in the patient's hospital room, and a nurse who provided care to patient III-Q in unit A (patient III-S, tertiary case). Further screening of 12 patients who received care from the infected nurse while she was symptomatic identified 2 more case-patients (patients III-T and III-U, quaternary cases), who were bedbound chronic care patients hospitalized for >4 weeks before their MERS-CoV–positive sample collection date. No cases were identified among the 50 additional healthcare contacts screened from Unit A; these contacts included the remaining 42 HCWs who worked on Unit A and the 8 patients who had shared a room with patient III-T. In total, 20 healthcare-associated cases (12 secondary, 6 tertiary,

and 2 quaternary) are attributable to a single introduction in the hospital.

A final healthcare-associated case-patient who was also cared for in unit A was identified (patient V; Figure 1). This 40-year-old expatriate man was admitted to the hospital in February 2014 with shortness of breath and multiple concurrent conditions, including congestive heart failure. The patient was cared for in unit A and a dialysis unit during February–March; new fever and shortness of breath developed on March 18, and he tested positive for MERS-CoV and was transferred to the ICU, where he died. The case-patient was hospitalized during the 14 days before symptom onset and had no travel history, animal contact, or contact with a known case. No source case or secondary cases were identified when 141 healthcare-associated contacts were screened (including patient III-S, a HCW who submitted a PCR-negative respiratory specimen during this contact investigation).

Laboratory

PCR

All case isolates were laboratory confirmed as positive by rRT-PCR for the upstream E gene and open reading frame 1. Average time between sample collection and laboratory results was 1 day (range 0–3 days). All 23 PCR-confirmed case isolates included in the validation sample were verified by using the N2 assay at CDC.

Sequencing

Genetic sequencing was performed on a subset of 8 isolates: 7 from case-patients in healthcare-associated clusters

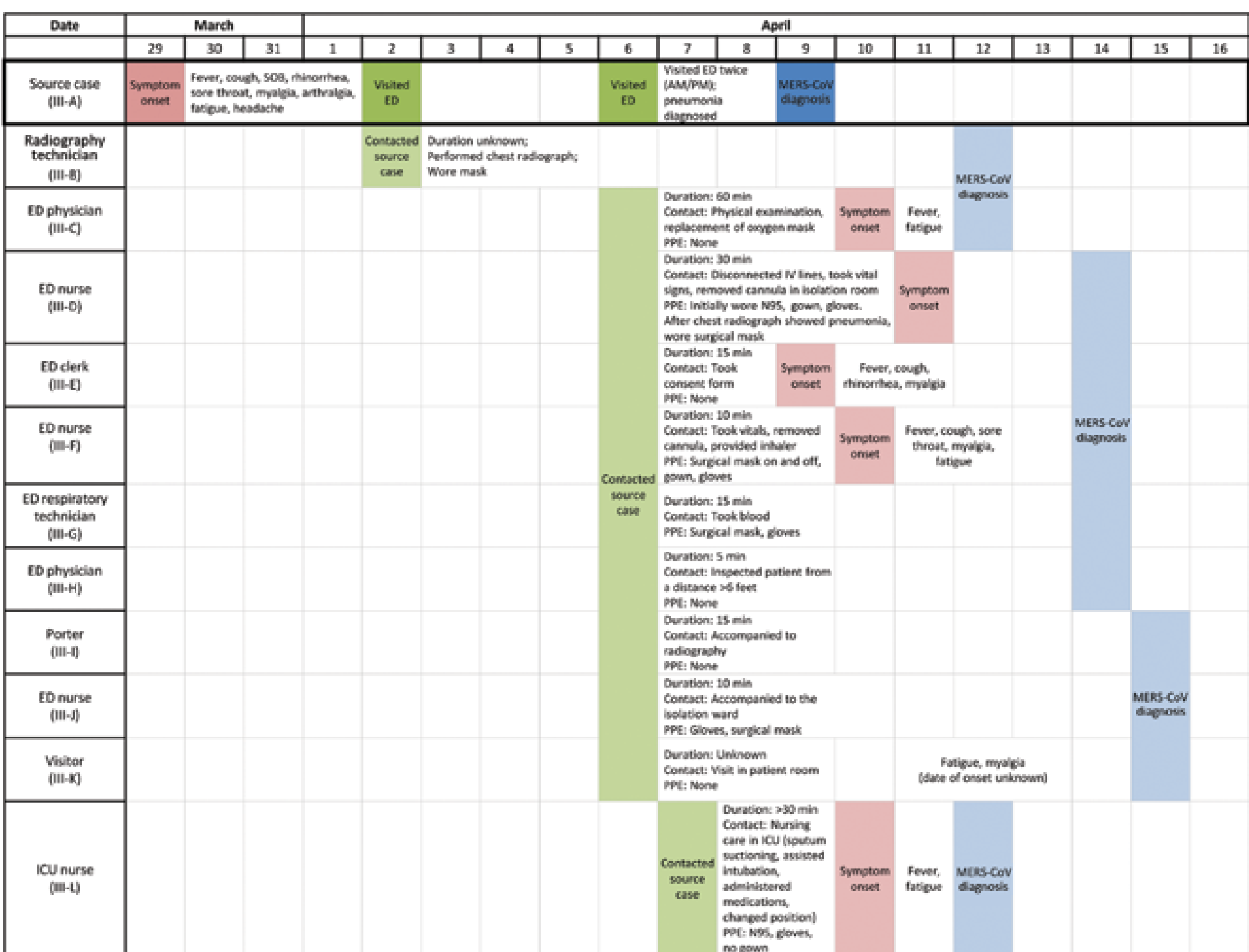

Figure 2. Timeline of exposures, symptom onset, and diagnosis of Middle East respiratory syndrome coronavirus (MERS-CoV) among secondary case-patients in a healthcare-associated cluster (cluster III), Abu Dhabi, 2014. Colored boxes indicate key dates for each case-patient: green boxes indicate date of interaction between source case (patient III-A) and healthcare providers; pink boxes indicate date of symptom onset; blue boxes indicate date of MERS-CoV diagnosis. For 5 case-patients who reported no symptoms, symptom onset is not listed; data exclude a secondary case with probable exposure (patient III-Q). SOB, shortness of breath; ICU, intensive care unit; PPE, personal protective equipment; duration, duration of exposure; ED, emergency department.

(patients II-A, III-A–III-C, III-O, III-Q, and III-R) and 1 from a non–healthcare-related case-patient from Abu Dhabi (Table 2). Full genome sequences were deposited in GenBank (accession nos. KP209306–KP209313). The genome sequences are similar (>99%) to other known MERS-CoV and clusters most closely with camel-derived MERS-CoV strains (GenBank accession nos. KJ650295–KJ650297) obtained in Al-Hasa, Saudi Arabia, in 2013, suggesting potential camel origin. Comparing complete genome sequences to the source case for cluster III (patient III-A) showed that all 6 MERS-CoV sequences from cluster III are considered to be genetically related, with <2 nt differences in the genome. The sequence for the source case from cluster II (patient II-A) is not considered related (11-nt difference). The non–healthcare-associated case from 2013 is even more divergent (30-nt difference).

Infection Prevention

Of the 14 HCWs (patients I-B–I-E, III-B–III-J, and III-L) who became infected with MERS-CoV after caring for a source case-patient, 13 (93%) were exposed before the patient's diagnosis. PPE use during care was inconsistent among these HCWs (Table 3). The 1 HCW who became infected after caring for a recognized case-patient reported use of gloves and N95 respirator masks during all patient care activities but did not consistently wear a gown and recalled an occasion when patient material contaminated her clothing (Table 3).

Discussion

MERS-CoV in healthcare settings accounts for >40% of all reported infections in Abu Dhabi. We found that healthcare-associated transmission occurred predominantly when HCWs, patients, and visitors were exposed to an infected person before recognition of MERS-CoV and implementation of appropriate infection prevention measures. These findings underscore the importance of early detection and intervention to limit spread of disease.

In the largest healthcare cluster in our investigation, 1 patient appears to have directly infected 12 persons in 1 hospital, resulting in a total of 20 healthcare-associated infections caused by secondary, tertiary, and quaternary transmission. Among ED HCWs, we estimate a 16% attack rate, ≈4 times higher than average household transmission estimates (*4*). Our findings add to previously reported examples of more extensive transmission occurring in healthcare facilities in South Korea, Saudi Arabia, and Jordan (*5,6,11–13*) and suggest that, in the absence of appropriate infection prevention measures, healthcare settings may be particularly efficient for MERS-CoV transmission. As described during an outbreak of severe acute respiratory syndrome, transmission in healthcare settings may be increased by various factors: higher than usual infectiousness of patients because of high viral loads or presence of symptoms that increase shedding; use of procedures that aerosolize infectious respiratory illness; close patient–HCW proximity during medical encounters; and other not-yet-identified factors (*26*). Extensive contact-tracing practices in Abu Dhabi, including testing contacts of case-patients regardless of symptoms, and whole-genome sequencing were essential for fully characterizing the transmission patterns in this cluster.

In our investigation, nearly all healthcare-associated transmission events occurred before MERS-CoV was suspected and diagnosed. After diagnosis, >500 patient-days of hospital care were provided to case-patients in Abu Dhabi; among HCWs providing this care, 1 infection occurred in an ICU nurse who reported not fully adhering to recommended prevention measures while she cared for a case-patient. Although delayed diagnosis contributed to all other transmission events, reasons for delays varied, highlighting challenges faced by the healthcare community: 1 patient was infected early in the outbreak, before high visibility of MERS-CoV and prevention policies; 1 patient sought care from an ED but had no known epidemiologic risk factors for MERS-CoV; 1 hospitalized patient had MERS-CoV symptoms that could be explained by other concurrent conditions; 1 infected HCW with mild illness did not report symptoms and continued working while ill. In the largest cluster, despite strong hospital and public health policies for triaging and isolating patients with respiratory symptoms as part of the MERS-CoV response, the source case-patient was placed under standard precautions, rather than contact and airborne precautions (*27*). Reasons for not implementing protocols in this instance are unknown, but the patient's lack of known risk factors (e.g., exposure to a case-patient) likely contributed to low clinical suspicion. This cluster underscores the importance of maintaining vigilance and adherence to infection prevention policy, particularly in regions where known MERS-CoV infections exist.

The severity of illness associated with MERS-CoV infection among case-patients in our investigation ranged from asymptomatic to severe disease, as has been previously reported (*4,5,10,18*). Severity of symptoms varied by type of case; death occurred among 2 of 3 source case-patients, 1 of 3 infected hospital patients, and no infected HCWs, who typically reported mild or no symptoms. We identified 3 instances in which transmission appears to have occurred from infected HCWs who reported no fever or symptoms of respiratory illness (patients III-B, III-G, and III-I). Although underreporting of symptoms or failure to recognize exposures cannot be ruled out, our findings show that increased understanding of transmission risks for persons with mild disease and improved strategies for early detection of illness are needed (*1,4,7*).

This investigation has several limitations. Although the healthcare clusters we describe are supported epidemiologically and steps were taken to ensure that clusters were conservatively constructed (e.g., excluding healthcare workers with known exposures in the community), other transmission pathways cannot be excluded. Genetic sequencing of a limited number of cases supports the epidemiologic characterization of cases and clusters, but we were unable to sequence and assess the molecular relatedness of all case isolates, a step that previous investigations have shown to be informative (*11,28*). On the basis of the current understanding of the mutation rate of MERS-CoV, the genetic differences observed in case isolates from clusters II and III are consistent with 2 separate introductions (*11*); however, we cannot rule out the possibility that these clusters are related. Furthermore, transmission pathways were generated on the basis of self-reporting or other documentation, and exposures may have been missed or forgotten. Such lapses might explain the difficulty in

Table 2. Nucleotide sequence variations of MERS-CoV full genomes from 8 case-patients in Abu Dhabi, January 1, 2013–May 9, 2014*

	Patients associated with healthcare clusters							
Genome position, nt	III-A	III-B	III-C	III-O	III-Q	III-R	II-A	Patient V†
381	C							T
1,226	T						C	C
2,015	T						C	
3,110	C							T
3,280	T						C	C
3,799	G							A
3,968	C							T
4,625	C							T
5,065	T							C
5,152	A			G				
5,381	C							T
6,189	C							T
7,124	G						T	T
7,610	C						T	
11,631	C							T
11,766	T							C
11,785	Y (T/C)	C	T	T	T	T	C	C
13,331	T						C	
15,592	A							G
16,381	A	C						
18,045	T							C
18,208	T							C
18,966	T	G						
19,072	C						T	
21,382	T		C					
21,531	T							G
21,777	G		A					
22,394	C							T
22,760	C	Y (T/C)						
22,790	T							C
22,913	T						C	
23,549	G							A
23,685	A							C
23,883	G							A
24,518	G							A
24,602	C							T
24,687	T							C
25,364	C							T
26,672	G						T	
27,204	T							A
27,206	C							A
27,208	A							T
27,211	C							A
27,867	G						T	
29,170	G						T	
Total nt differences		2	2	1	0	0	11	30

*Genome sequences compared with those for case III-A, the source case-patient for healthcare-associated cluster III. The variation table was generated on the basis of the full genome sequence described in the Methods section. Blank cells indicate no sequence difference. MERS-CoV, Middle East respiratory syndrome coronavirus.
†Case not associated with healthcare.

ascertaining a source of exposure for 1 healthcare-associated case (patient V). In addition, follow-up serologic testing for MERS-CoV–specific antibodies and repeat PCR testing were not performed on healthcare-associated contacts, so additional cases may have been missed. Last, by restricting our definition of healthcare-associated cases to persons with recognized exposures in healthcare settings, we may underestimate the true number of cases, particularly if sources of infection (i.e., case-patients) went undetected. Because our objective was to characterize transmission patterns among known healthcare-associated cases, we considered the conservative definition to be most appropriate.

In conclusion, large healthcare clusters of MERS-CoV illness contribute to substantial illness and also have potential for secondary consequences, including fear among HCWs and the public. MERS-CoV can clinically appear with mild or nonspecific respiratory symptoms, and patients may seek care without having known risk factors for infection. Maintaining a high index of suspicion in every patient encounter, especially at first points of patient entry such as EDs or primary healthcare settings, is imperative, particularly in regions reporting MERS-CoV cases. Early detection of cases, full adherence to infection prevention recommendations, and recognition of illness among HCWs are necessary factors to prevent further transmission of MERS-CoV in healthcare settings. Supporting healthcare facilities in these efforts remains a priority.

Acknowledgments

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Table 3. Healthcare interactions for 14 healthcare workers who became infected with MERS-CoV after caring for a source case-patient, Abu Dhabi, January 1, 2013–May 9, 2014*

Description of healthcare interaction	Healthcare workers, no. (%)
Timing of interaction	
Before MERS-CoV diagnosis in source case-patient	13 (93)
Type of interaction†	
Patient examination	7 (50)
Procedure with potential aerosol generation‡	5 (36)
Patient transport	3 (21)
Radiograph	1 (7)
Clerical	1 (7)
Unknown	1 (7)
Duration of interaction†	
<10 min	1 (11)
10–30 min	6 (43)
>30 min	2 (22)
Unknown	5 (36)
PPE use during interaction†§	
Any mask	6 (43)
Surgical mask	5 (36)
N95 respirator¶	2 (14)
Gloves	4 (29)
Gown	3 (21)
Gown, gloves, and surgical mask or N95 respirator	3 (21)

*Of the 19 healthcare worker case-patients identified, 14 occurred in persons who provided care for a source case (cases I-B–I-E, III-B–III-J, III-L); these 14 healthcare workers are described here. MERS-CoV, Middle East respiratory syndrome coronavirus; PPE, personal protective equipment.
†Self-reported information on eye protection is not available.
‡Manipulation of cannula or oxygen mask (n = 3), administration of inhaler or nebulizer treatment (n = 2), intubation (n = 1), suctioning before intubation (n = 1); healthcare workers could perform $\geq$1 of these patient care activities.
§Information on eye protection is not available.
¶Of the 2 healthcare workers who reported wearing an N95 respirator, 1 wore N95 inconsistently, and 1 reported wearing gloves and a respirator during all patient care activities but did not consistently wear a gown and recalls an occasion when patient material contaminated her clothing.

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Lassa Virus Seroprevalence in Sibirilia Commune, Bougouni District, Southern Mali

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Lassa virus (LASV) is endemic to several nations in West Africa. In Mali, LASV was unknown until an exported case of Lassa fever was reported in 2009. Since that time, rodent surveys have found evidence of LASV-infected *Mastomys natalensis* rats in several communities in southern Mali, near the border with Côte d'Ivoire. Despite increased awareness, to date only a single case of Lassa fever has been confirmed in Mali. We conducted a survey to determine the prevalence of LASV exposure among persons in 3 villages in southern Mali where the presence of infected rodents has been documented. LASV IgG seroprevalence ranged from 14.5% to 44% per village. No sex bias was noted; however, seropositivity rates increased with participant age. These findings confirm human LASV exposure in Mali and suggest that LASV infection/Lassa fever is a potential public health concern in southern Mali.

Lassa virus (LASV) (family *Arenaviridae*, genus *Arenavirus*) is the etiologic agent of Lassa fever (LF), a viral hemorrhagic fever first documented in 1969 during an outbreak on the Jos Plateau in Nigeria (*1*). In humans, LASV infection is characterized by a variety of clinical manifestations that can range from apparently asymptomatic or mild disease to severe disease consisting of multiorgan failure (*2*,*3*). As much as 80% of infected persons are believed to experience mild disease, whereas 20% exhibit noteworthy and often severe clinical indicators that require medical attention (*4*). An estimated 300,000 LASV infections occur in West Africa each year, resulting in ≈5,000 deaths (*5*). Infection during pregnancy, especially during the third trimester, is particularly severe; estimated maternal mortality rates are 20%, and fetal mortality rates are ≈100% (*6*–*8*). As are most arenaviruses, LASV is maintained in nature in rodent hosts, specifically, the multimammate rat (*Mastomys natalensis*) (*9*). Most commonly, contact with infectious rodents or ingestion/inhalation of virus-laden particles is the source of human infection. Person-to-person transmission is also well documented and can result in outbreaks, especially in nosocomial settings, leading to mortality rates in >50% (*7*).

Historically, LASV has been considered endemic to 2 geographic areas of West Africa: 1) Sierra Leone, Guinea, and Liberia; and 2) Nigeria. However, in recent years, an increased region of LASV endemicity has been suggested, which includes adjoining countries and areas farther north than previously suggested (*10*,*11*). In 2000, a German citizen received a diagnosis of LF after traveling through Ghana, Côte d'Ivoire, and Burkina Faso (*12*). More recently, cases of LF have been identified in Ghana (*13*), and the presence of LASV-infected rodents has been documented in Côte d'Ivoire (*14*).

In a similar situation, LF was unknown in Mali until February 2009, when a young British man was medically evacuated to London after a 10-day history of fever (*15*). The infection was initially diagnosed as *Plasmodium falciparum* malaria, even though the patient did not respond to treatment for malaria. He died on arrival in London, and a postmortem diagnosis of LASV infection was confirmed by molecular techniques. In response to this case, rodent surveys were conducted in the village of Soromba (rural commune of Sibirila, Bougouni district, Mali), where the man was living and working when he became ill. The initial surveys found that 25% of *M. natalensis* rats had molecular evidence of active LASV infection, which was confirmed by virus isolation and sequence analysis (*16*). Similar studies conducted across Mali suggest that LASV is restricted to the southern tip of the country, in several villages near

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the border of Côte d'Ivoire (*17*). On average, 20% of peridomestic *Mastomys* rodents collected in these villages had serologic or molecular evidence of LASV infection, with peak prevalence rates >50%.

Given the infection rates observed in rodents living in close proximity to humans in many villages in southern Mali, it seems likely that humans are frequently exposed to LASV infection and that LF may develop. Nevertheless, despite increased recognition of LF in Mali, to date no outbreaks have occurred, and the 2009 exported case remains the only confirmed human LASV infection contracted in Mali. Reports of a second case of LF associated with the British citizen are unconfirmed. Verbal accounts indicate that shortly after he was evacuated, his housekeeper and cook also fell ill and died. Samples were not collected for testing, in part because malaria was suspected. To better understand the risk for human LASV infection in southern Mali, we conducted a serologic survey of inhabitants of 3 villages within the rural commune of Sibirila to determine the proportion of persons who had been exposed to LASV.

Materials and Methods

Ethics Statement

Ethical approval for research on human subjects was obtained from the independent institutional research boards of the Faculty of Medicine and Pharmacy of the University of Sciences, Techniques and Technologies of Bamako, Mali, and the US National Institutes of Health. Before we conducted these studies, permission was granted from regional health professionals as well as from village elders and chiefs. Research on samples from human subjects was conducted in accordance with the policies and regulations of the National Institutes of Health and in adherence with the principles of the Belmont Report (1979) (http://www.hhs.gov/ohrp/humansubjects/guidance/belmont.html).

Study Setting

On the basis of findings from our previous rodent surveys in southern Mali, we selected the villages of Soromba (10°35′21″N, 07°09′21″W), Bamba (10°22′59″N, 07°09′06″W), and Banzana (10°31′26″N, 07°14′53″W) (*17*) (Figure 1; online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/22/5/15-1814-Techapp1.pdf). Soromba is the most likely exposure site of the only known case of LF in Mali and, along with Bamba, has the highest LASV prevalence documented in peridomestic rodents (*16,17*). Banzana is a nearby village with a low prevalence of LASV-infected rodents. According to a recent national census, the populations of Soromba, Bamba, and Banzana were 855, 1,751, and 4,822 persons, respectively. Samples were collected over 10 consecutive days in February 2015.

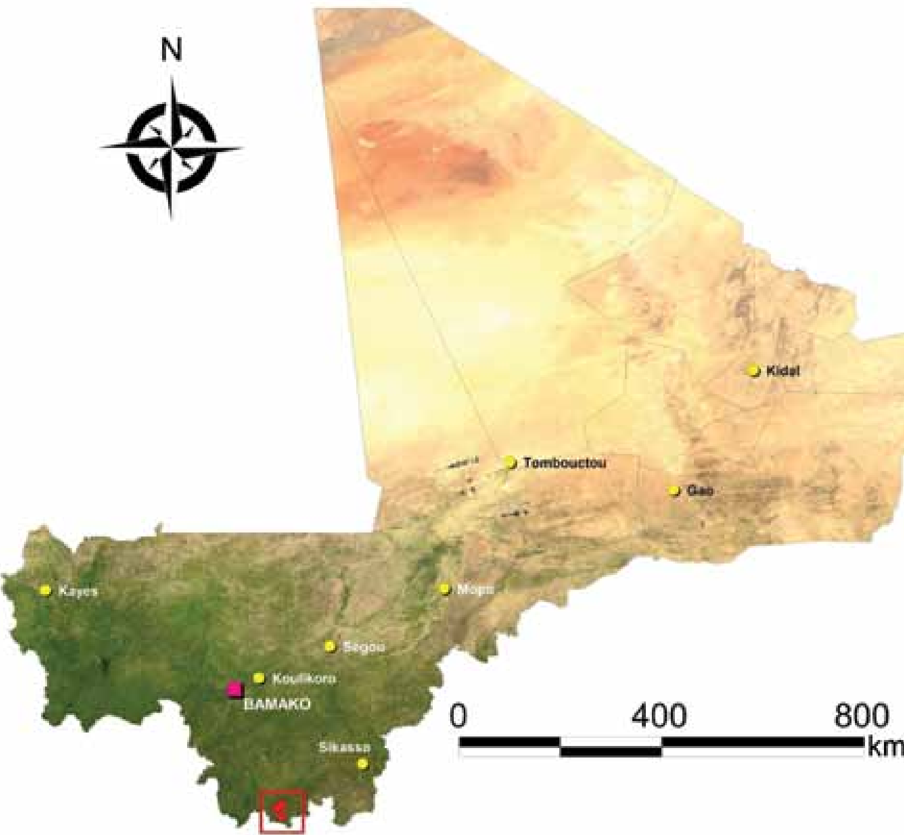

Figure 1. Study sites for assessment of Lassa virus seroprevalence in humans, southern Mali, 2015. The 3 villages of Soromba, Banzana, and Bamba (within square) in Sibirilia commune, Bougouni district, were selected on the basis of previous identification of Lassa virus–infected rodents in peridomestic settings.

Consent and Enrollment

Enrollment criteria for this study were the following: a participant must have been a healthy person ≥6 months of age with no febrile disease reported in the previous month, who (or whose parents) had resided in the community for at least 12 months. Oral and written consent were obtained from all participants in the study before their enrollment. If the participant was illiterate, consent was granted in the presence of a literate witness of his or her choice or a trusted community member assigned by the community. Consent for participants <18 years of age was given by parents or guardians. The study procedures and goals were explained to the participants in the local dialect (Bambara). In addition, all potential participants were given handouts explaining the study, and persons were encouraged to further discuss possible enrollment with trusted members of the community, primarily teachers and nurses. When possible, enrollees were given at least 24 hours to decide whether to voluntarily participate. The goal for this study was to enroll 600 participants, 200 per village.

Biometrics and Sample Collection

Prior to sample collection, enrollees were given a physical examination by a licensed Malian physician. Participants with enlarged spleens, suspected of having an asymptomatic malaria infection, were tested with a rapid diagnostic test, and those whose test results were positive received treatment according to local guidelines. Also, children with suspected vitamin deficiencies were given supplements, and adults with varying medical conditions received treatment as required. Questionnaires were verbally administered to each participant ≥12 years of age; questions were asked about previous febrile diseases with or without hemorrhagic manifestations, rodent sightings/infestations in dwellings, and possible consumption of rodents. After the initial examinations, ≈1 mL of whole blood was collected by fingerstick into an EDTA-treated microtube by a certified Malian laboratory technician.

Sample Processing and Testing

Within 1 h of blood collection, plasma was separated from whole blood by centrifugation, transferred to a cryovial, and frozen on liquid nitrogen. Samples were transported to a climate-controlled laboratory at the University of Bamako in dry shippers within 10 d of collection for serologic testing. An ELISA was used to screen samples for IgM and IgG reactive to a recombinant LASV nucleocapsid antigen derived from LASV Josiah (ReLASV; Corgenix Medical Corporation, Inc., Broomfield, CO, USA) (*18*,*19*). The kits are produced under a Corgenix Quality System (compliant with FDA regulations) (http://www.corgenix.com/news-releases/corgenix-and-viral-hemorrhagic-fever-consortium-release-new-findings-from-sierra-leone-lassa-virus-program/#sthash.P7nnRrCI.dpuf). The kits have been thoroughly evaluated for the detection of LASV antibodies in patients at the LF ward of the Kenema Government Hospital in Sierra Leone (L.M. Branco, M. Boisen, unpub. data) and have previously been used to detect anti-LASV antibodies in rodents collected in southern Mali (*17*). Optimized LASV ELISA methods developed at Kenema Government Hospital were used for testing the samples. In brief, serum specimens were initially screened at a 1:100 dilution in sample diluent. Samples were incubated on the ELISA plate at room temperature (≈25°C) for 30 min, after which they were washed 3 times with wash buffer on a mechanical plate washer. A peroxidase-labeled secondary antibody against human IgG or IgM was then added to each well, and samples were again incubated and washed as above. Peroxidase substrate (3, 3′, 5, 5′-tetramethylbenzidine, 100 µL/well) was added to each well and incubated at room temperature in the dark for ≈10 min after which a stop solution (0.16 M sulfuric acid, 100 µL/well) was added. Color development was assessed on a mechanical plate reader at 450 nm. A baseline for the assay was established by testing 374 serum samples collected from citizens of Mali who lived well outside the known region where LASV was endemic. Serum samples collected in the current study were considered serologically positive if they yielded optical densities (OD_{450}) >2 SDs above the baseline value (≥0.8). All reactive samples were titrated by 4-fold dilutions to determine the final titer. Serologic testing was done on blinded samples.

Statistical Analysis

Univariate generalized log-binomial regression models were used to estimate risk ratios (RRs) and 95% CIs and to identify factors that were significantly ($p<0.05$) associated with IgG seropositivity. Variables significant in univariate models were evaluated in multivariate models to assess for potential confounding effects. Univariate logistic regression models were used to identify significant factors associated with the expectedly rarer outcome of IgM seropositivity and estimate odds ratios and associated CIs. All regression analyses were conducted by using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA).

Results

Study Demographics

A total of 600 participants were enrolled in this study, 200 per village, which represents 23.4%, 11.4%, and 4.1% of the populations of Soromba, Bamba, and Banzana, respectively. The average age of subjects enrolled was 21 years (range 7 months–83 years) (Figure 2). The sex ratio was slightly skewed toward female participants; 315 (52.5%) enrollees were female, versus 285 (47.5%) male (sex ratio 1.1) (Figure 2). The demographics of the study

populations were essentially the same from each village and were representative of the population of Sibirila, which is primarily young (65% of inhabitants are <30 years old), with more female than male inhabitants. All study participants that were polled reported seeing rodents in their dwellings frequently and had an extensive history of febrile diseases without hemorrhagic manifestations.

Seroprevalence Rates

LASV IgM was detected in 4 samples, for an overall prevalence rate of 0.67%. All 4 IgM-positive samples were from female participants, ages 3, 11, 12, and 22 years (female-specific ratio of 4/315 [1.3%]). Three of the 4 samples reactive for IgM were also IgG positive. IgM-positive samples came from all 3 sites: 2 from Bamba, 1 from Banzana, and 1 from Soromba. The overall IgG seroprevalence for LASV across the 3 sites was 33.2% (199/600 samples) (Table 1). Overall village-specific LASV seroprevalence correlated with previous results of rodent surveys (*17*). The IgG seroprevalences for Bamba (44.0%; 95% CI 37.0%–51.2%) and Soromba (41.0%; 95% CI 34.1%–48.2%) were comparable and considerably higher than that for Banzana (14.5%; 95% CI 9.9%–20.2%) (Table 1).

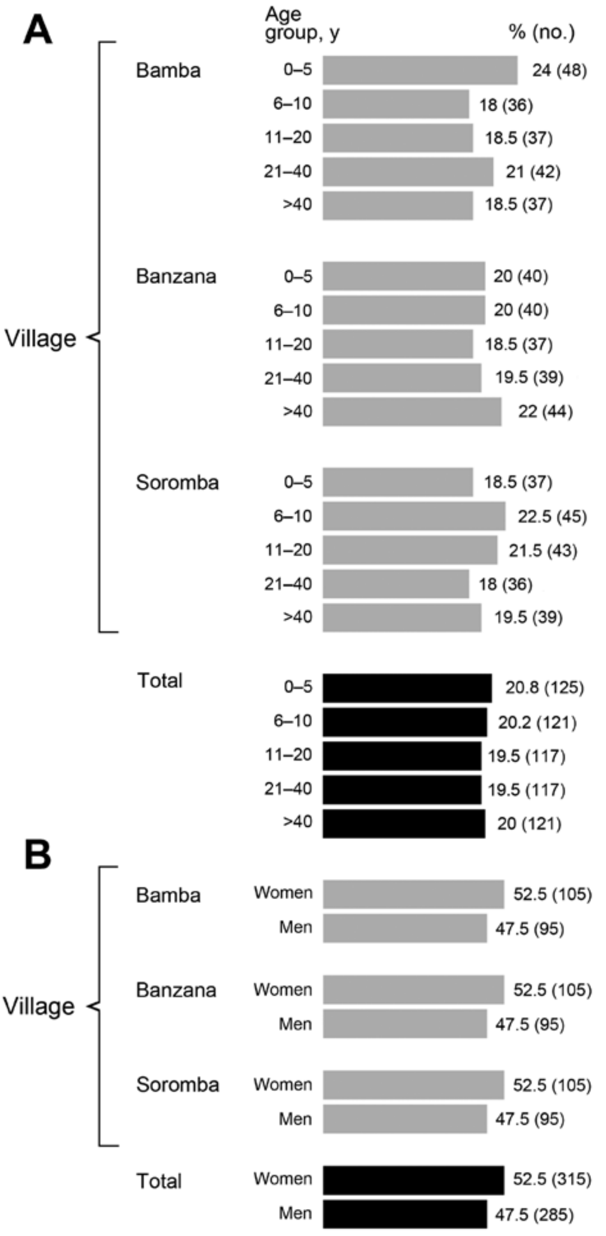

Figure 2. Demographic characteristics of study population in assessment of Lassa virus seroprevalence, southern Mali, 2015. A) Age; B) sex.

Persons that were IgG positive were more likely to be older than those who were IgG negative (each 10+ years of age: RR 1.08; CI 1.02–1.14; p = 0.005) and were also more likely to be from Soromba than from Banzana (RR 2.8; CI 1.9–4.1; p<0.0001) but were not more likely to be from Bamba (p = 0.5). No differences in seropositivity were found by sex (p = 0.3) (Table 2). When analyzing IgG positivity as an outcome in a multivariate model, all significant variables from the univariate models (village [Banzana], age, and IgM OD) remained significant and at nearly the same magnitude of effect, suggesting that they are not confounding each other. Endpoint ELISA titers for most samples were relatively low, possibly because a nonhomologous recombinant LASV nucleocapsid antigen was used in the immunoassays (*20*). Three samples demonstrated titers of ≥6,400, and an additional 37 had titers of 1,600. The remaining samples had titers of 100 (n = 77) or 400 (n = 82).

Discussion

LASV has most likely been present in southern Mali for several hundred years (*21*,*22*). However, until 2009, when an imported case of LF in a patient with travel history exclusive to Mali was diagnosed in the United Kingdom, LASV as well as LF were undocumented (*15*). Since then, despite increased awareness of the disease and ecologic studies defining the distribution of infected rodent reservoirs in the southern portions of the country, no additional case of LF has been observed or suspected.

The apparent underrecognition of LASV infection in this region is likely multifactorial. The wide range of clinical features and nondescript symptoms that appear early in LASV infection impedes a diagnosis based strictly on clinical manifestations, even for experienced physicians (*23*–*25*). Studies in disease-endemic regions suggest that ≈20% of LASV infections lead to advanced and clinically severe disease manifestations. However, the classic indicators of LF may have easily been attributed to other infectious diseases, particularly malaria (as was the situation with the LF case imported to the United Kingdom), typhoid fever, or a variety of other etiologic agents known to have a high

Table 1. Lassa virus IgG seroprevalence by study site, southern Mali, 2015

Village	Male participants, no. positive/no. total (%)	Female participants, no. positive/no. total (%)	Combined seroprevalence, no. positive/no. total	Combined seroprevalence, % (95% CI)
Bamba	35/95 (37.2)	53/105 (50.5)	88/200	44.0 (37.0–51.2)
Banzana	14/95 (14.7)	15/105 (14.3)	29/200	14.5 (9.9–20.2)
Soromba	39/95 (41.1)	43/105 (41.0)	82/200	41 (34.1–48.2)
Total	88/285 (30.9)	111/315 (35.2)	199/600	33.2 (29.4–37.1)

incidence in West Africa (*26–28*). In addition, the overall lack of confirmed cases in several West Africa countries where infected rodents and sporadic cases have been documented may be due to atypical or even attenuated clinical manifestations of these viruses, as has been suggested in recent nonhuman primate studies (*29*). Conceivably, the 80/20 ratio for LASV infection severity in many of the countries outside of the historical regions of endemicity could be even greater. Nevertheless, the absence of identifiable cases in this region is conspicuous, given that up to 50% of peridomestic rodents captured in some of these villages exhibit evidence of LASV infection (*17*).

To date, few LASV seroprevalence studies have been conducted outside of populations that reside in regions to which LASV is hyperendemic. With LASV IgG detectable in 33.2% of this study population, our results demonstrate that a high proportion of inhabitants in these 3 villages have been exposed to LASV and, by extension, indicate that a wider population in this region may also have been exposed. Village-specific prevalence rates were in accordance with the infection rates observed in rodents previously collected in this area. The IgG seroprevalences for Soromba and Bamba were 3-fold higher than that for Banzana, which is similar to infection rates observed in *Mastomys* rodents collected from these villages (*16*,*17*).

IgG seropositivity was positively associated with age, which is not surprising given that the primary source for most infections would be infectious rodent reservoirs. The older a person is, the more likely they are to have had close contact with these animals. A substantial number of young children also demonstrated serologic evidence of previous exposure, which, because they were >7 months of age, are not likely to be false-positive results associated with maternal transfer of antibodies. Although seropositivity was higher in female participants, these results were not significant, suggesting that both sexes are equally infected in this region.

The prevalence reported here is, on average, slightly higher than (although similar to) results of previous human serosurveys conducted in disease-endemic and non–disease-endemic regions (*30*). In Liberia, Sierra Leone, Guinea, and Nigeria, LASV prevalence rates of 2%–52% by immunofluorescence assay and 10%–55% by ELISA have been documented (*20*,*23*,*31–35*). In countries where infected rodents, cases of LF, or both have only recently been documented, such as Benin, Ghana, and Côte d'Ivoire, seroprevalence rates among humans of 9.9%, 3.8%, and 20%, respectively, have been found by ELISA (*20*).

Overall, the endpoint ELISA titers we report are low. However, these values are likely affected by the use of a nonregional LASV antigen from the lineage IV LASV strain Josiah. Although an ELISA based on a homologous LASV antigen may have been more appropriate, the kits we used (ReLASV), based on LASV Josiah from Sierra Leone, provided 2 strong advantages: 1) the standardized production and quality assessment ensures reliable and reproducible assays for current and future studies; and 2) the kits have been thoroughly evaluated in LASV-endemic areas for detection of LASV-reactive antibodies (a factors that strengthens the data presented here). A caveat regarding these kits is the potential for reduced endpoint titers. Emmerich et al. demonstrated reduced geometric mean titers in seropositive samples collected in Côte d'Ivoire (where LASV strain AV circulates) when diagnostic assays used antigen from strain Josiah rather than from strain AV (*20*). Full-genomic analysis demonstrates that LASV strain AV is genetically closest to the LASV strains from Mali, and, combined, may represent an emerging fifth lineage of LASV (*21*).

Given these findings, the endpoint titers we found may be ≈2- to 4-fold lower than one might expect had the diagnostic antigen based on the LASV strains from Mali been used. Nonetheless, even if we considered the 77 samples with a serologic titer of 100 as equivocal, the overall seroprevalence in this study would still remain high (20.3%).

Table 2. Lassa virus IgG seroprevalence by age group, in 3 villages, southern Mali, 2015

Age group, y	Village			Total no. positive/no. tested (%)
	Bamba, no. postive/no. tested (%)	Banzana, no. positive/no. tested (%)	Soromba, no. positive/no. tested (%)	
0–5	18/48 (37.5)	2/40 (*5*)	12/37 (32.4)	32/125 (25.6)
6–10	15/36 (41.7)	5/40 (12.5)	16/45 (35.6)	36/121 (29.8)
11–20	12/37 (32.4)	4/37 (10.8)	20/43 (46.5)	36/117 (30.8)
21–40	24/42 (57.1)	9/39 (23.1)	12/36 (33.3)	45/117 (38.5)
>40	19/37 (51.3)	9/44 (20.5)	22/39 (56.4)	50/120 (41.7)

Although the possibility exists that a certain proportion of persons with positive test results were exposed to a serologically cross-reactive arenavirus or were exposed during travel, the isolation of LASV from infected rodent reservoirs in many of these areas, combined with the observation that relatively few of these persons travel beyond their immediate geographic locations, argues against this.

In this study, we were unable to address possible risk factors associated with LASV seropositivity in Mali. A questionnaire was administered to study participants, but the data gained were limited. All participants reported seeing rodents frequently in their homes, with no apparent seasonality. No participants reported consuming rodents, although study staff observed adolescent boys cooking rodents over a fire on at least 1 trip to southern Mali (N. Sogoba, D. Safronetz, unpub. data). Social stigma may prevent persons from admitting to this practice.

Furthermore, we were unable to provide insight into the clinical picture of LASV infection in this region. Not surprisingly, all participants in our study reported several episodes of febrile disease, although none with hemorrhagic manifestations or long-term sequelae, such as hearing loss, which is associated with LF. Because most of these clinically notable cases are likely attributed to malaria, the precise etiology of febrile diseases across Mali, in particular, in rural southern Mali, remains largely undefined. The Ebola virus outbreak in West Africa demonstrates why this diagnostic deficiency needs to be rapidly corrected.

In conclusion, the high seroprevalence rate we document highlights the need for increased surveillance for LASV in southern Mali. Further, prospective studies are required to define the clinical manifestations of LASV infection and LF in this region, as are follow-up studies to our work to determine incidence rates. Overall, these findings confirm that exposure to LASV is occurring in Mali, an area historically considered low risk for LF, and suggest that the annual incidence rate of LASV infection across West Africa may be higher than previously thought.

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Tragically, Darin Oottamasathien, a co-author who assisted in the development of the ReLASV ELISAs, lost her life. We honor her memory.

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Nipah Virus Transmission from Bats to Humans Associated with Drinking Traditional Liquor Made from Date Palm Sap, Bangladesh, 2011–2014

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Nipah virus (NiV) is a paramyxovirus, and *Pteropus* spp. bats are the natural reservoir. From December 2010 through March 2014, hospital-based encephalitis surveillance in Bangladesh identified 18 clusters of NiV infection. The source of infection for case-patients in 3 clusters in 2 districts was unknown. A team of epidemiologists and anthropologists investigated these 3 clusters comprising 14 case-patients, 8 of whom died. Among the 14 case-patients, 8 drank fermented date palm sap (*tari*) regularly before their illness, and 6 provided care to a person infected with NiV. The process of preparing date palm trees for *tari* production was similar to the process of collecting date palm sap for fresh consumption. Bat excreta was reportedly found inside pots used to make *tari*. These findings suggest that drinking *tari* is a potential pathway of NiV transmission. Interventions that prevent bat access to date palm sap might prevent *tari*-associated NiV infection.

Nipah virus (NiV) is a bat-borne emerging infection, and *Pteropus* spp. bats are the wildlife reservoir (*1*). NiV was discovered in an outbreak in Malaysia in 1998 that affected 283 persons and caused 109 deaths (case-fatality rate 39%) (*2*). Subsequently, outbreaks of NiV infection have occurred nearly every year in Bangladesh and occasionally in India (*1*,*3*–*5*). A total of 33 outbreaks of NiV encephalitis were reported in Bangladesh and India during 2001–2014, and epidemiologic investigations implicated batborne and human-to-human transmission (*6*,*7*). During 2004–2012, a total of 157 NiV infections were reported in Bangladesh, and 22% of these occurred through human-to-human transmission (*8*).

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Investigations of NiV-associated outbreaks in Bangladesh identified consumption of fresh date palm sap as the primary route of bat-to-human transmission (*1*,*9*). In Bengali culture, sap harvested from the date palm tree is commonly used for fresh consumption and fermentation (*10*,*11*). Moreover, in Asia, Australia, and Africa, fermented date palm sap is used to make alcoholic drinks, known as toddy, *tari*, or palm wine (*12*,*13*). In Bangladesh, date palm sap is typically collected in clay pots that are attached to the tree. A top section of the date palm tree bark is shaved, allowing the sap to ooze overnight into the collection pot (*11*). A previous NiV study reported that *Pteropus* spp. bats frequently feed on the shaved bark and often contaminate the sap with saliva, urine, and excreta (*14*). *Pteropus* spp. bats are also known to occasionally shed NiV in their secretions and excretions (*15*,*16*).

Since 2006, the Institute of Epidemiology, Disease Control, and Research (IEDCR) in Dhaka, Bangladesh, under the Ministry of Health and Family Welfare of Bangladesh, has collaborated with the International Centre for Diarrhoeal Diseases Research, Bangladesh (icddr,b), Dhaka, on hospital-based encephalitis surveillance in the areas where NiV-associated outbreaks have been reported (*3*). From December 2010 through March 2014, the surveillance identified 18 clusters of NiV infection; in 15 of these clusters, the index case-patients had exposure to fresh date palm sap before illness onset. For the remaining 3 clusters, the index case-patients had no known contact with date palm saps, bats, or sick animals other than bats. Recognizing the potential for new pathways of transmission, we investigated

other possible exposures to NiV by applying epidemiologic and anthropological approaches in our study of these 3 NiV disease clusters. We used the epidemiologic study to explain the proximate individual-level factors linked to the disease outbreak (*17*) and an anthropological approach to explicate local perceptions, behaviors, and practices that might have contributed to the disease occurrence (*18*). Therefore, the objectives of our investigation were to describe the clinical signs and symptoms of the case-patients and determine the possible route of transmission for these clusters.

Methods

The team conducted this study during 2011–2014 in Rajshahi and Rangpur Districts, Bangladesh. Surveillance physicians from Rangpur Medical College Hospital and Rajshahi Medical College Hospital identified suspected case-patients (Table), recorded clinical histories and home addresses, and collected whole blood samples from each. The serum was separated from each sample, stored in liquid nitrogen at the hospital, and then transported to IEDCR (*3*). The laboratory team at IEDCR tested serum samples for NiV IgM and IgG by using IgM-capture and indirect IgG enzyme immunoassays (*19*,*21*).

On the basis of type of exposure, we categorized suspected case-patients as primary or secondary case-patients, and these two groups were further categorized as case-patients with probable or laboratory-confirmed NiV infection (Table). To identify a potential NiV infection cluster, surveillance physicians asked each of the admitted suspected case-patients and their caregivers present in the hospital about other sick persons or persons in their communities who had recently died with similar symptoms. We defined a NiV infection cluster as ≥2 suspected meningoencephalitis case-patients living within 30 minutes' walking distance from each other who had onset of similar illnesses within 3 weeks of one another or had epidemiologic linkages to one another (*22*).

Table. Case definitions for Nipah virus (NiV) infections that occurred in 3 clusters, Rangpur and Rajshahi Districts, Bangladesh, 2011, 2012, and 2014

Type of case	Case definition
Suspected	Fever or history of fever with axillary temperature >38.5°C, altered mental status, new onset of seizures, or a new neurologic deficit in a patient from an adult or pediatric ward of an NiV surveillance hospital during the NiV season (*3*).
Probable	Illness meeting the case definition for suspected NiV infection in a person who lived in the same village as a person with laboratory-confirmed NiV infection but who died before specimens could be collected for diagnosis (*19*).
Laboratory-confirmed	Acute onset of fever and subsequent altered mental status or other neurologic deficits during the outbreak period and having NiV IgM or IgG antibodies in serum (*19*).
Primary	A case in which illness occurred in the absence of contact with a symptomatic case-patient.
Secondary	Illness in a person whose only known exposure was to a case-patient and whose illness occurred within 5–15 d after that contact (*20*).

After identifying laboratory-confirmed NiV infection cases (Table), the team reviewed hospital records, took preliminary information from the surveillance physicians, and visited case-patients from each cluster within a month of case confirmation. We limited this study to clusters in which no case-patients had a history of drinking fresh date palm sap. In the community, the team used a structured questionnaire to interview surviving case-patients and the friends, relatives, and neighbors of deceased case-patients as proxy respondents. The team collected information related to exposure and signs and symptoms of illness to determine routes of NiV transmission for each case-patient.

The team visited the households of case-patients and used culturally appropriate approaches to build rapport and trust with the community (*23*). The team conducted in-depth interviews and group discussions with surviving case-patients and the family caregivers, friends, and neighbors of the deceased to explore the exposure histories of each case-patient by using an open-ended interview guide. Open-ended questions allowed the interviewers to obtain new insights about the outbreak. Good rapport with community members helped the team collect information on potentially sensitive issues, such as alcohol consumption (*24*), which is prohibited among the majority Muslim population of Bangladesh. The preliminary data collected during this study suggested that the case-patients might have consumed *tari* before their illness onset. Therefore, the team also interviewed 5 date palm sap harvesters and conducted 3 group discussions with community members to learn about *tari* production, consumption, and selling practices in the affected communities. The team also collected and analyzed whole blood samples from surviving case-patients and from nonpatients (defined as persons in the community who drank *tari* with the case-patients but did not experience any symptoms) by using the same methods described above.

Data Analysis

We used descriptive statistics to characterize the demographic and clinical characteristics of the case-patients. The team expanded the observation and interview field notes and summarized them. The primary author (M.S.I.) read summaries of the interviews and identified themes. These themes were shared among investigators for review and consensus. The primary author then categorized the data

according to the selected themes, consistent with methods previously described (*25*).

Ethical Considerations

The team obtained verbal informed consent from study participants. The surveillance and outbreak investigation study protocol was reviewed and approved by the ICDDR,B Ethical Review Committee.

Results

Three clusters were identified, consisting of 14 case-patients (9 with confirmed NiV infection, 5 with probable NiV infection). Eight of the 14 case-patients were primary case-patients (3 with confirmed NiV infection, 5 with probable NiV infection). All 6 of the secondary case-patients had confirmed NiV infection. Among the 14 case-patients, 7 had illness onset during January–March 2011 in Rangpur District (first cluster), 3 had illness onset in February 2012 in Rajshahi District (second cluster), and 4 had illness onset during January–February 2014 in Rangpur District (third cluster). Eight drank *tari* before their illness onset, whereas 6 only had exposure to other case-patients. None of the primary case-patients had any history of drinking fresh date palm sap or exposure to sick humans or animals. All of the illnesses began with fever. All 8 of the primary case-patients had altered mental status followed by loss of consciousness and death. The median duration from illness onset to death was 6 days. All 6 secondary case-patients survived. The median age of all 14 case-patients was 32 years. All the primary case-patients were male, and all the secondary case-patients were female. None of the non–case-patients had NiV IgM or IgG detected in their serum samples.

In the 2011 Rangpur cluster, case-patients A, B, C, and G drank *tari* regularly in the evenings, and the sap to make *tari* was collected from a single village (Figure). Case-patients A and G were family friends and lived in the same village. Case-patients B and C were also related and lived in the same village. Case-patients D, E, and F were family caregivers of case-patient C and provided close-contact care during his illness. All 3 family caregivers experienced illness within 2 weeks of case-patient C's illness onset.

In the 2012 Rajshahi cluster, case-patient H was a *tari* producer and harvester of date palm sap. Case-patient I was a neighbor and cousin of case-patient H, and both drank *tari* regularly in the evenings. They drank *tari* together from the same pot 6 days before their illness onsets. Case-patient J was a family caregiver of patient H and provided close-contact care during his illness. She experienced fever 11 days after case-patient H's illness onset.

In the 2014 Rangpur cluster, the respondents reported that case-patients K and L were family friends and had drunk *tari* every day in the evenings before their illness onsets (Figure). Case-patient L became ill after 5 days after case-patient K, and case-patient L did not have any contact with case-patient K while case-patient K was ill. The *tari* they drank was collected from the same village where the 2011 Rangpur cluster was identified. Case-patients M and N shared the same bed with case-patient L at home; case-patient N was a family caregiver to case-patient L, providing close-contact care for him at home and in the hospital. Case-patient M had onset of fever 12 days later, and case-patient N had onset of fever 14 days after case-patient L's illness onset.

Tari Production, Processing, and Selling Patterns in Rangpur and Rajshahi

The area of Rangpur where the case-patients lived was well known for its *tari* production and was the largest *tari*-producing area in the district. An estimated 500 date palm trees grow in the area, which produces and supplies *tari* throughout the district. *Tari* retailers from different subdistricts of Rangpur also come to this area to buy *tari* at a wholesale price. In the affected villages, the *tari* producers reported that they had been leasing all the date palm trees for several years. Villagers reported they did not have regular access to fresh date palm sap because almost all the date palm sap from the village was made into *tari*.

In the NiV-affected area of Rajshahi, the *tari* is produced in small quantities. An estimated 15 date palm trees

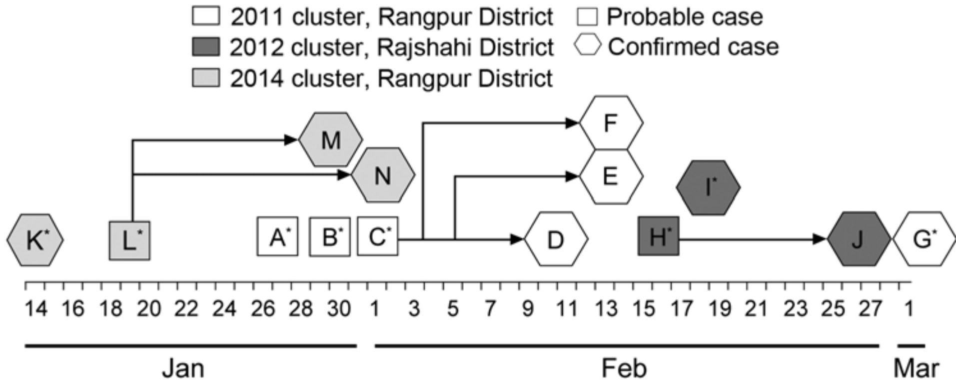

Figure. Timeline of illness onset in persons with primary and secondary cases of Nipah virus infection that occurred in 3 clusters, Rangpur and Rajshahi Districts, Bangladesh, 2011, 2012, and 2014. Asterisks indicate primary cases; cases without an asterisk are secondary cases.

grow in the affected community of Rajshahi. The *tari* producer leases these trees only during winter to produce *tari*.

The *tari* production process was identical in each of the affected communities. The process of preparing the date palm trees for *tari* production was similar to the process for collecting date palm sap for fresh consumption (*11*). To prepare the tree for tapping, the sap harvesters cut the old leaves close to the top of the trees with a knife to expose the tender part of the tree. To tap the tree, the harvesters shave a V-shaped cut at the top of the tree and set a bamboo spigot at the end of the cut. After 3–5 days, the V cut is shaved again, and an earthen pot is hung under the spigot to catch the sap that oozes out. For fresh date palm sap, the harvesters need to clean and dry the sap collection pots after each episode of sap collection. However, *tari* harvesters from both affected communities reported that they use the same earthen pot for sap collection for several days without cleaning it so that yeast can form at the bottom of the earthen pot. Yeast aids the fermentation of the fresh date palm sap in the pot.

In Rangpur, the date palm sap is harvested for *tari* year round. The harvesters reported that date palm sap can be collected from each tree for 3–4 days a week for 4 months at a time, and then the tree is left to recover for the next 7–8 months. The harvesters stagger the tapping of trees so there is a continuous supply of date palm sap to make *tari* throughout the year. The harvesters reported that they harvest more sap during winter than during other seasons because of higher demand for *tari* from consumers. Moreover, they reported that the sap flows more freely from each tree during the winter. In the affected communities, the harvesters reported that every day from 8 a.m. until noon, they collect the sap from the hanging earthen pots and accumulate the collected sap in other earthen pots or containers and leave the hanging pots on the trees. In Rangpur, the harvesters bring *tari* to their house and immediately sell it to retailers and consumers from morning until late at night. Occasionally, consumers take *tari* away with them in plastic bottles. In the affected area of Rajshahi, after being removed from the trees, the *tari* pots are kept in a betel leaf garden, and the harvesters sell *tari* from there.

The *tari* sellers and harvesters reported that *tari* consumers are men and included truck and bus drivers, day laborers, rickshaw pullers, and local farmers. They also reported that *tari* is less expensive than other illegal alcohol available for sale and that making *tari* is less laborious than collecting fresh date palm sap because harvesters do not need to clean and dry the earthen pots after each collection. They added that making *tari* is more profitable than selling fresh date palm sap. During our visit to communities near to the affected areas in Rangpur, the price of fresh date palm sap was $0.20 per liter, and the price of *tari* was $0.50 per liter. The price differential provides an incentive for making *tari*.

We observed bat roosts in the affected community of Rangpur, and date palm sap harvesters reported that they frequently observe bats flying near the date palm trees. The harvesters reported that they often find bat excreta in and on the sap pots. The harvesters reported filtering *tari* with a net or cloth before selling it to remove the excreta. In Rajshahi, the villagers reported that there is no bat roost in their community. However, they reported seeing bats visiting date palm trees at night. None of the harvesters in Rajshahi reported filtering *tari* before selling it to consumers.

Discussion

The laboratory, clinical, and qualitative findings in this study suggest that the 14 case-patients in the 3 clusters we investigated were infected with NiV. The primary NiV case-patients identified in the clusters drank *tari* regularly in the evenings before their illness onsets, and none of them had a history of fresh date palm sap consumption or any exposure to other NiV case-patients, which were the main transmission pathways for NiV infection identified in previous outbreak investigations in Bangladesh (*9*,*26*). Moreover, none of the case-patients had exposure to sick animals, another possible pathway for NiV transmission reported in studies conducted in Malaysia and Singapore (*27*).

Tari is a date palm sap product. Because *tari* fermentation was a continuous process and date palm sap was fermented inside the *tari* pots while they were hanging in the trees, some date palm sap added to the *tari* might technically be fresh sap. However, the primary case-patients probably did not consume fresh date palm sap because *tari* was collected from 8 a.m. to noon but the primary case-patients drank *tari* only in the evening, which suggests that all the sap they consumed was at least partially fermented by the time of consumption. Findings from this investigation suggest that drinking *tari* is a potential source of NiV infection in Bangladesh. Investigators in India had similar findings during an outbreak reported in 2007 in West Bengal near the border with Bangladesh (*4*). They reported that drinking fermented date palm sap possibly contaminated with bats excreta and secretions was the source of NiV infection for the index patient, which further supports our assertion that NiV infection from drinking *tari* is plausible (*4*). Moreover, 2 clusters in this study were traced back to the same *tari* production village in Rangpur, further strengthening the conclusion that *tari* was the mode of transmission.

Previous studies have shown that fruit bats frequently lick the date palm sap and occasionally urinate inside collection pots (*14*). In our investigation, the reported evidence of bats visiting date palm trees, the presence of bat excreta inside *tari* pots, the reported use of the same pot for several days without cleaning, and the accumulation of sap from multiple pots into 1 pot suggest that sap is probably contaminated with bat urine or saliva during collection and

fermentation. NiV can survive up to 4 days in bat urine and at least 1 day in sap contaminated with bat urine when kept at an average temperature of 19°C (*28*). A study to determine viability of NiV in artificial palm sap contaminated with NiV (strain Bangladesh/200401066) found no statistically significant reduction in NiV titers for at least 7 days when kept at a temperature of 22°C (*29*). Generally, enveloped and thus lipophilic viruses like NiV are susceptible to alcohol. A 60%–70% alcohol solution is recommended for sterilizing contaminated objects (*30*). A study conducted in India showed that *tari* derived naturally from fermenting date palm sap contains 5%–8% alcohol and has a pH of 4.5–6.0 (*12*). This 5%–8% alcohol concentration might not have been high enough or sufficiently distributed throughout the *tari* to sterilize the NiV, thus allowing persistence of viable virus and transmission of NiV to *tari* consumers during the winter months, when the ambient temperature ranges from 15°C to 28°C (*21*).

In Bangladesh, family caregivers commonly provide close-contact care to hospitalized patients (*31*). Infected patients often shed the virus through body secretions and excretions and can contaminate foods and surfaces, including bed rails, bed sheets, and towels (*32*,*33*). Close contact is the most likely route through which family caregivers became infected (*26*). Family caregivers identified in this study had direct contact with primary case-patients and their body secretions.

The findings of our study are subject to limitations. First, some participants may have been reluctant to report production, consumption, and selling of *tari* because these are illegal activities in Bangladesh. Initially, some of the date palm sap harvesters and *tari* producers were reluctant to share information with us. However, the social scientists on the investigation team built rapport and trust with the respondents, which encouraged the respondents to share sensitive information (*23*). Thus, the hesitance to disclose behaviors related to *tari* probably did not have a substantial effect on our findings. Second, no control group was available. Without a comparison group, we were unable to determine if our primary case-patients were more likely than other persons residing in these villages to drink *tari*. However, given the absence of evidence that the primary case-patients had other contact with bats, sick animals, or persons with NiV infection, consumption of *tari* appears to be the most likely transmission route. Third, we interviewed family members and friends of the deceased case-patients as proxy respondents to ascertain case exposures. However, during outbreaks of fatal diseases such as NiV infection (with a case-fatality rate >70%), there is no alternative to this approach (*20*). Since 2003, we have interviewed proxy respondents for case-exposures in every NiV-associated outbreak investigation conducted in Bangladesh (*3*,*9*,*19*). Fourth, because of the delays in investigation of the 2011 cluster, our definition for a confirmed cases of NiV infection was based on the presence of NiV IgM or IgG in serum samples. Confirming a case based on the presence of IgG is reasonable, however, because all the family caregivers from the 2011 Rangpur cluster had illness onset within 2 weeks after contact with a case-patient (*19*).

Because harvesting date palm sap for *tari* production is similar to harvesting it for consumption of fresh date palm sap, the intervention of using bamboo skirts to cover the shaved part of the date palm tree and the sap collection pots to prevent bat contact and possible NiV introduction is worth exploring. The use of bamboo skirts is already a successful, affordable, and culturally acceptable method to prevent bat access to date palm sap, and this strategy could also be used to prevent NiV transmission from *tari* consumption (*34*,*35*). In addition, *tari* harvesters from ethnic minority communities have limited access to mass media because of their ethnic, religious, and linguistic minority status in Bangladesh. Efforts should be made to raise their awareness about strategies that interrupt bat access to date palm sap. At this time, we are not aware of any studies that have tested the survival of NiV in *tari*. As a next step, we recommend testing NiV survival in *tari* at different levels of alcohol concentration.

All 3 of the clusters of NiV infection that we investigated were linked to drinking *tari*. Drinking *tari* might also be a route of exposure for other batborne viruses. A total of 55 newly described viruses from 7 virus families were recently identified in urine and saliva from *Pteropus* spp. bats in Bangladesh (*36*), suggesting that these bats could also contaminate *tari* with other viruses that could cause disease in humans. Date palm sap is harvested for fermentation in many areas where *Pteropus* spp. bats and other fruit bats are native, including Australia, Asia, and Africa (*37–40*). Consumers of fermented drinks and other date palm products that are harvested using similar processes as in Bangladesh might be at risk for NiV infection and other batborne diseases (*12*,*13*,*36*).

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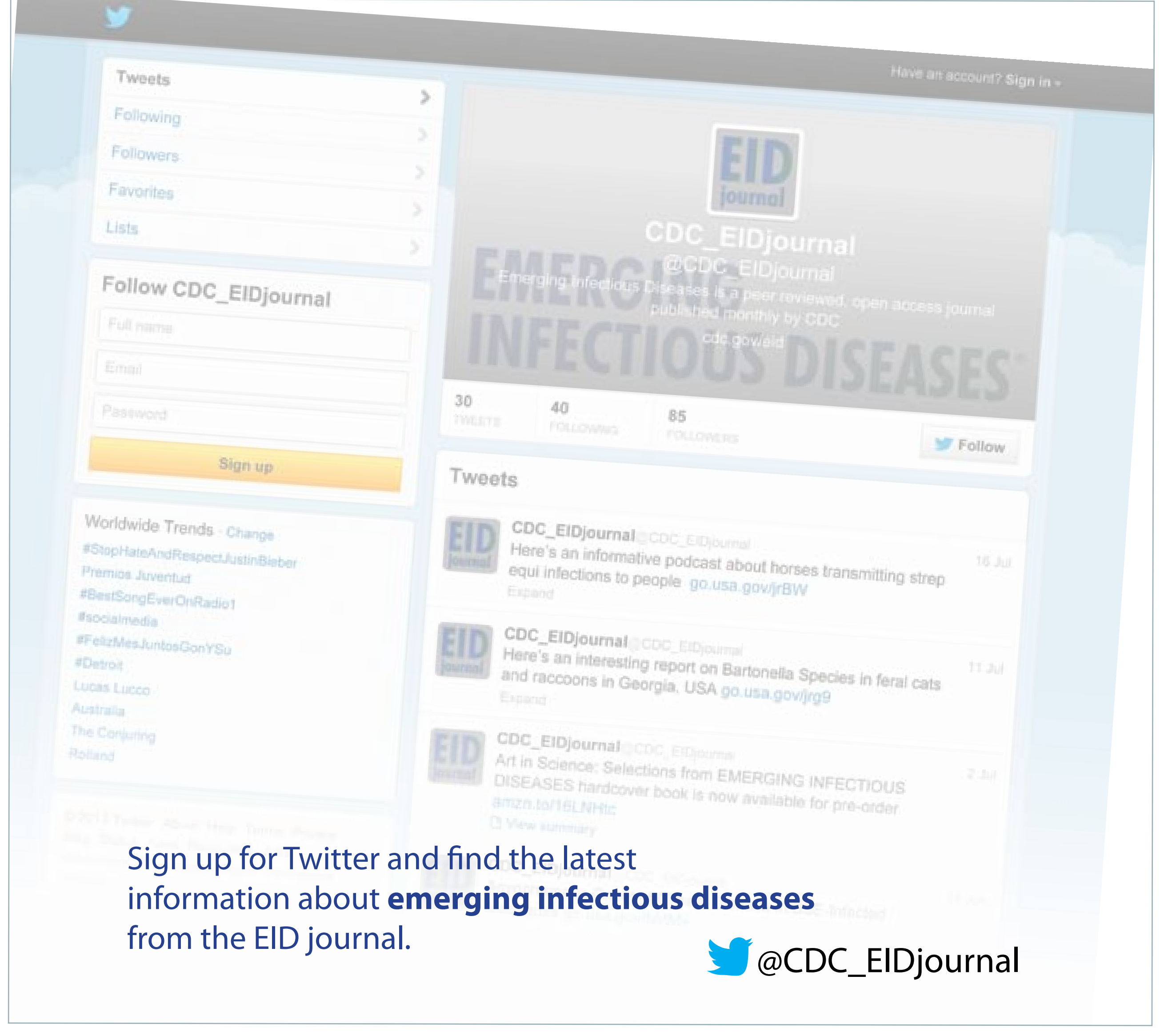

Evaluation of Viremia Frequencies of a Novel Human Pegivirus by Using Bioinformatic Screening and PCR

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Next-generation sequencing has critical applications in virus discovery, diagnostics, and environmental surveillance. We used metagenomic sequence libraries for retrospective screening of plasma samples for the recently discovered human hepegivirus 1 (HHpgV-1). From a cohort of 150 hepatitis C virus (HCV)–positive case-patients, we identified 2 persons with HHpgV-1 viremia and a high frequency of human pegivirus (HPgV) viremia (14%). Detection of HHpgV-1 and HPgV was concordant with parallel PCR-based screening using conserved primers matching groups 1 (HPgV) and 2 (HHPgV-1) nonstructural 3 region sequences. PCR identified 1 HHPgV-1–positive person with viremia from a group of 195 persons with hemophilia who had been exposed to nonvirally inactivated factor VII/IX; 18 (9%) were HPgV-positive. Relative to HCV and HPgV, active infections with HHpgV-1 were infrequently detected in blood, even in groups that had substantial parenteral exposure. Our findings are consistent with lower transmissibility or higher rates of virus clearance for HHpgV-1 than for other bloodborne human flaviviruses.

The development of next-generation sequencing methods and related molecular tools has greatly increased the pace of virus discovery (*1*,*2*), and these methods have become widely used for the investigation of novel zoonotic infections. Examples in which next-generation sequencing methods have identified novel viral agents associated with disease outbreaks include severe fever with thrombocytopenia virus (SFTV) in China (*3*), a bunyavirus in the United States (*4*), and a novel rhabdovirus in Central Africa (*5*). Using such methods, 2 authors of this study (A.K. and P.S.) recently described a novel flavivirus, distantly related to human pegivirus (HPgV, formerly described as GB virus C or hepatitis G virus) but with several genome attributes, such as a type IV internal ribosomal entry site (IRES), possession of a core-like protein, and a heavily glycosylated envelope protein that show greater affinity with hepatitis C virus (HCV) and other members of the genus *Hepacivirus* (*6*). The virus, named human hepegivirus 1 (HHpgV-1) to reflect these mosaic characteristics, was detected in 2 blood recipients and as a persistent infection in 2 persons with hemophilia exposed previously to nonvirally inactivated factor VIII/IX concentrates.

Following the example of the previous use of metagenomic libraries to detect human pathogens (*7–9*), we developed a bioinformatics-based method to screen existing libraries for HHpgV-1 and HPgV sequences from previously tested persons in the United Kingdom, enabling viremia frequencies in different risk groups to be estimated. Samples used to generate libraries with and without HHpgV-1 sequences were retrieved and used to validate the specificity and sensitivity of a newly developed reverse transcription PCR (RT-PCR)–based method for sample screening. This method was subsequently used to screen samples from patients extensively treated with nonvirally inactivated factor VIII or IX concentrates and controls.

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Methods

Samples

We obtained samples from 195 persons with hemophilia from the Hemophilia Growth and Development Study (HGDS) cohort (*10*). We obtained metagenomic datasets used for bioinformatic screening from OxBRC Prospective Cohort Study in Hepatitis C (ethics reference 09/H0604/20), the Short Pulse Antiretroviral Therapy at seroConversion cohort (*11*), Thames Valley HIV Cohort Study (*12*), and a cohort in the Democratic Republic of

the Congo (DRC) (*13*). All datasets were derived by using nontargeted viral RNaseq from total plasma RNA and the Illumina Hiseq sequencing platform (Illumina Inc., San Diego, CA, USA).

Screening Assays for Group 1 and Group 2 Pegiviruses

RNA was extracted from 200 mL of pooled or 20 mL of individual plasma by using the RNeasy Kit (QIAGEN, Hilden, Germany) and recovered in 30 mL of nuclease-free water. First-strand cDNA was synthesized from 6 mL of recovered RNA by using Superscript III reverse transcription (Life Technologies, Carlsbad, CA, USA) with random hexamer primers. Nested PCRs were performed by using GoTaq DNA polymerase (Promega, Madison, WI, USA) and the primers described in Table 1. First round reactions were obtained by using 2 mL of cDNA as a template under these conditions: 40 cycles of 18 seconds at 94°C, 21 seconds at 50°C and 60 seconds at 72°C, and a final extension step of 5 minutes at 72°C. Second round reactions were done by using 2 mL of first round template under identical conditions.

Metagenomic Sequencing and Bioinformatics

Metagenomic datasets used in this study had previously been sequenced on the Illumina platform from sequencing libraries synthesized with either the NEBNext mRNA Sample Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA), or the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs) with modifications to the manufacturer's protocols (*10*). Datasets in .bam format were depleted of human reads by using the bowtie method to map them to the HG19 human genome and convert them to fasta files by using custom awk scripts, then were piped to the blastn program (BLAST+ version 2.2.25; http://blast.ncbi.nlm.nih. gov/Blast.cgi) by using a nucleotide database of all GenBank viral reference genomes and the initial HHpgV-1 variant, AK-790 (GenBank accession no. KT439329). All hits with E values <0.01 were accepted. Datasets containing HHPgV-1 or HPgV-1 sequence data were subjected to a custom-made assembly pipeline by which reads were trimmed of low PHRED quality bases (QUASR, Sourceforge, http://wwwsourceforge.net) and adaptor sequences, before virus reads (identified by using blastn) were assembled by using Vicuna (*14*) and VFAT software (http://www.broadinstitute.org/scientific-community/science/projects/viral-genomics/v-fat).

RNA folding energies and ratios of non-synonymous to synonymous nucleotide substitutions (dN/dS) were calculated for consensus whole-genome sequences by using SSE version 1.2 (*15*). Complete genome sequences of HHpgV-1 and HPgV obtained in this study have been submitted to GenBank (accession nos. LT009476–LT009494).

Results

Library Screening in Silico

We used the assembled sequence of AK-790 as a reference to screen libraries of metagenomic sequence reads derived from plasma samples from 120 HCV-infected persons (primarily infected through injected drugs), 36 persons infected with HIV-1 from sexual contact, and 30 persons who were co-infected with HCV and HIV-1 (Table 2). From these samples, a total of 3 sequence libraries contained HHpgV-1 sequences. However, only 2 reads were detected in sample D1212, and these were identical in sequence to those of D1220. Because of the possibility of extraneous contamination of either the sample position in the sequencer or of the sequence dataset (e.g., through misidentified tags), we provisionally considered D1212 to be HHpgV-1 negative.

By using the sequence reads, we obtained near-complete genome sequences of HHpgV-1 from the 2 positive samples (Table 2); their divergence, and other sequence characteristics were compared with those of the AK-790 prototype sequence (Figure 1; Table 3). Sequences were >99% complete with 5′ and 3′ ends approximately co-terminus with the HHpgV-1 prototype sequence (D1255 and D1220 lacked 12 and 23 bases at the 5′ end, respectively, and

Table 1. Primer sequences used for PCR screening of HPgV groups

Orientation	Position†	Sequence, 5′ → 3′
HHpgV-1/group 2 primers		
Sense, outer	4,488	CGTSGTSMTYTGYGACGAGTGCCA
Antisense, outer	5,021	CCRCGCCGCTGCATVCGSAAYGC
Sense, inner	4,723	CAYGYDATCTTYTGYCACTCGAAGG
Antisense, inner	4,900	CRAAGTTBCCDGTGTAGCCDGTGGA
HPgV/group 1 primers		
Orientation	Position‡	
Sense, outer	3,931	GSGCNATGGGNCCNTAYATGGA
Antisense, outer	4,546	GTNACYTCVACNACCTCCTCYACCA
Sense, inner	4,092	GTGGTNATHTGYGAYGAGTGYCA
Antisense, inner	4,357	TCRCACTCMRCCTTKGARTGRCARAA

*HHpgV, human hepegivirus; HPgV, human pegivirus.
†Position of 5′ base in the AK-790 genome (GenBank accession no. KT439329).
‡Position of 5′ base in the HPgV Iowa genome (GenBank accession no. AF121950).

Table 2. Detection frequencies of HPgV by sequence library screening and PCR*

Diagnostic method	Diagnoses and characteristics	No. tested	No. (%) HHpgV-1+	No. (%) HPgV+
Bioinformatics				
	HIV+	36	0	3 (8.3)
	HIV+/HCV+/PWID	30	0	3 (10.0)
	HCV+/PWID	120	2 (1.7)	14 (11.6)
PCR				
	Hemophilia†	195	1 (0.5)	18 (9.2)
	Control group‡	50	0	1 (2.0)

*HPgV, human pegivirus; HHpgV, human hepegivirus; +, positive;HCV, hepatitis C virus; PWID, persons who inject drugs.
†Previously exposed to virally inactivated factor VIII/IX; all seropositive for HCV.
‡No history of parenteral exposure; HIV-1 and HCV negative.

D1255 had a 23 base extension at the 3′ end). Sequences were ≈5% divergent from each other and from AK-790 over the length of the genome; most were at synonymous sites that left protein encoding unchanged (dN/dS 0.170–0.193). Sequences were also phylogenetically distinct from the larger dataset of variants sequenced in the nonstructural 3 (NS3) region (Figure 2; *6*,*16*). Genomes of both HHpgV-1 variants showed bioinformatic evidence for genome scale–ordered RNA structure (*17*), with mean folding energy differences in the coding region of 7.6% and 8.3% for

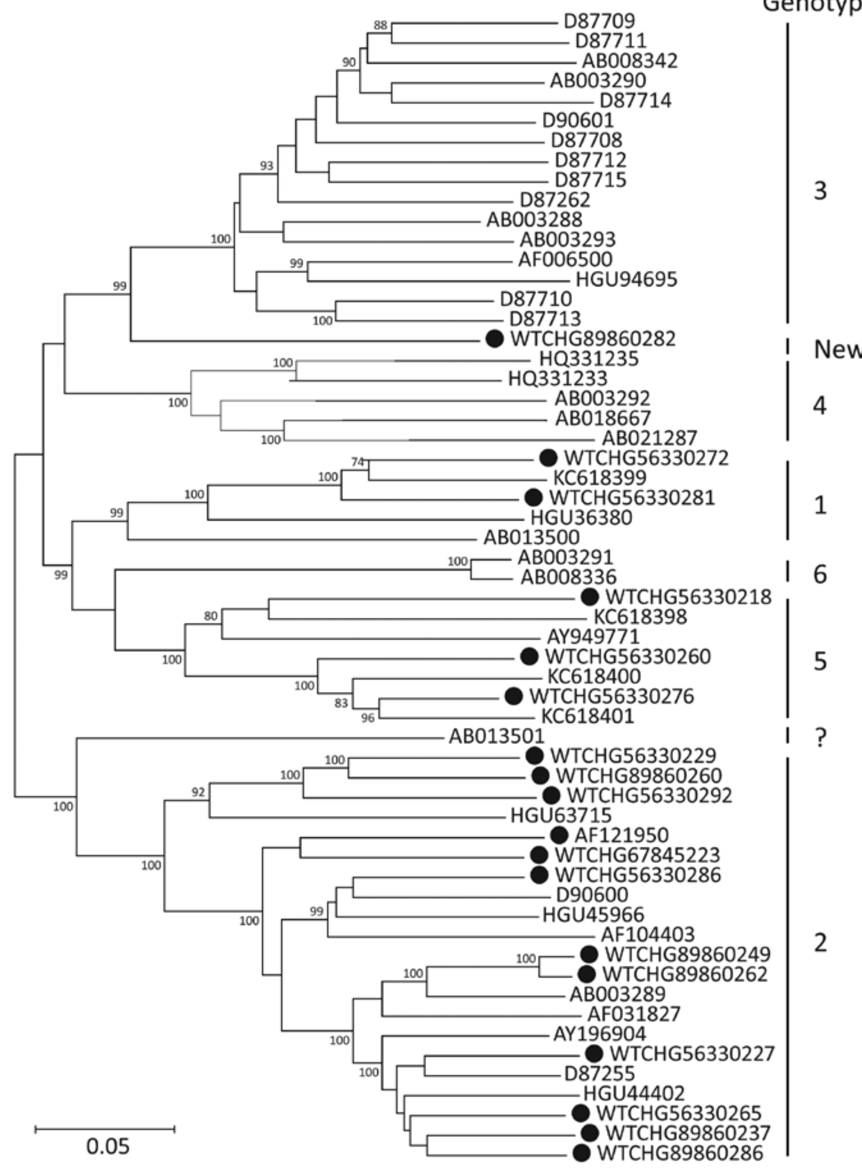

Figure 1. Maximum-likelihood phylogenetic analysis of complete genome sequences of human pegivirus assembled in this study (black circles) compared with available human hepegivirus (HPgV) sequences of genotypes 1–6 published in GenBank (accession numbers shown). The tree was constructed by using the maximum-likelihood algorithm implemented in the MEGA6 software package (*16*). For this dataset, the optimum maximum model was general time reversible model (*18*) with a gamma distribution (5 rates) and invariant sites. Phylogenetic analysis of each dataset used 100 bootstrap resamplings to infer the robustness of groupings. Genotypes previously assigned to HPgV sequences are shown on the right with the exception of sequence AB013501 (from the United Kingdom, shown with genotype “?”). Scale bar indicates nucleotide substitutions per site.

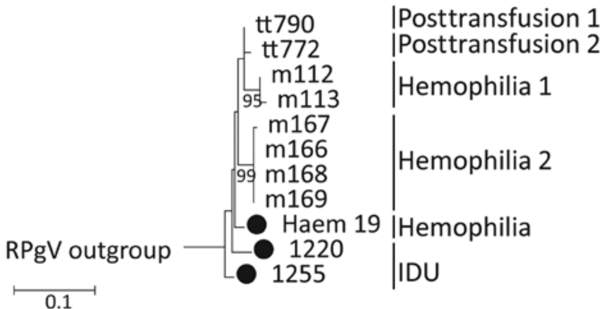

Figure 2. Maximum-likelihood (ML) phylogenetic analysis of human pegivirus sequences. NS3 region sequences (positions 4609–4880 as numbered in the AK-790 reference sequence, denoted here as tt790) were selected to overlap with sequences from PCR-derived amplicons generated in this study (black circles) and partial NS3 region sequences reported previously (*6*). The tree was constructed by using the maximum likelihood algorithm implemented in the MEGA6 software package (*16*). The optimum ML model (lowest Bayesian information criterion score and typically greatest ML value) was Kimura 2 parameter and invariant sites. Phylogenetic analysis of each dataset used 100 bootstrap re-samplings to infer the robustness of groupings. The tree was rooted with a rat pegivirus sequence (GenBank accession no. KC815311, not shown). IDU, injection drug use; NS, nonstructural. Scale bar indicates nucleotide substitutions per site.

samples D1255 and D1220, respectively, which is similar to that calculated for AK-790 (7.6%) but lower than that calculated for HPgV (Table 3) and other pegiviruses (*6*).

The 2 HHPgV-1 samples originated from HCV-infected men enrolled in the OxBRC Prospective Cohort Study in Hepatitis C; samples were collected before initiation of antiviral treatment with telaprevir, pegylated-interferon, and ribavirin. Sample D1255 was collected from a person with a history of injection drug use, 50 years of age, 12 weeks before successful eradication of HCV genotype 1b virus; at the time of the study, he had remained under observation for a segment IV liver lesion, cirrhosis, and raised alphafetoprotein. Sample D1220 was collected from a patient 58 years of age who had genotype 1a HCV infection and died from decompensated liver failure 4 weeks into treatment. Both patients denied receiving blood or donor blood–derived products at the time of enrollment, and neither patient received blood products before sampling, according to hospital medical records and local transfusion service records.

As a control for the bioinformatic screening method, the same samples were screened for human pegivirus (HPgV) by using 60 whole pegivirus genomes from the US National Center for Biotechnology Information nucleotide database (http://www.ncbi.nlm.nih.gov/nuccore) as references for blastn filtering (Table 2). A total of 20 sample libraries contained at least 10 HPgV-matching reads and showed a median of 8,185 reads (IQR 5207–15,863), equating 17,000 to 19 million total sequenced bases (Table 3). Near-complete genome sequences could be assembled from 17 of the libraries, and reduced genome coverage obtained from the 3 samples with sequence read totals <100,000. The sequence characteristics of the HPgV sequences were typical for members of this virus group: moderate sequence divergence between each other and to the HPgV reference sequence (9%–13%), extremely low dN/dS ratios (0.022–0.049) and evidence for extensive internal RNA secondary structure (mean folding energy differences of 10.5%–13.2%; Table 3). Persons infected with HPgV originating from the UK were infected with genotype 2, and those from South Africa harbored genotypes 1 and 5 (Figure 1; *18*). The exception was patient 89860282, a UK resident man enrolled in the Short Pulse Antiretroviral Therapy at seroConversion trial who was infected with a candidate novel genotype pegivirus in addition to a clade B HIV-1.

In addition to being detected at a lower frequency than HPgV in groups that listed sexual contact and injected drug use as potential risk factors for infection (Table 2), read totals for HHpgV-1 were >2 SDs below mean totals for HPgV. This finding is consistent with a lower degree of viremia.

Validation of PCR for HHpgV-1

We compared sequences of the 2 HHpgV-1 samples with AK-790 and other group 2 pegiviruses (*6*) to identify conserved regions that might serve as binding sites for primers suitable for HHpgV-1 screening. A PCR based on primers hybridizing to a conserved region of group 2 pegiviruses (Table 1; *6*) and those previously described (AK1/AK2) were validated by using the original samples identified as positive on bioinformatic screening, the suspected false-positive sample (D1212), and a selection of samples in which HHpgV-1 sequences were not detected (Table 4). To cross-validate the assay for HPgV detection, primers were designed based on regions conserved in the NS3 region of group 1 pegiviruses (human, primate, and bat pegiviruses; Table 1).

For groups 1 and 2 primers, PCR detection showed high concordance with bioinformatic screening (Table 4). By using group 2 primers, both samples that contained high numbers of HHpgV-1 reads on bioinformatic screening tested positive and the suspected negative sample (D1212) with only 3 reads was negative, as were the 20 controls in libraries that contained no HHpgV-1 sequences. Similar concordance between HPgV detection by PCR with library screening was observed in parallel (both methods identified 10 positive samples and 13 negative). The observed concordance between PCR- and bioinformatic-based screening methods for both virus groups validates both approaches for the wider screening for both virus groups in epidemiologic analyses.

Table 3. Read depth and divergence of HPgV sequences obtained by using metagenomic screening*

Virus, ID no.	Case-patient origin	Type†	Length	Coverage, %	Reads	Div, %‡	dN/dS	MFED, %§
HHpgV-1, n = 2								
D1220	UK	NA	9,550	99.8	247,798	4.7	0.193	7.6
D1255	UK	NA	9,503	99.6	101,951	5.1	0.170	8.3
HPgV, n = 20								
89859249	UK	2	9,383	99.9	19,231,581	10.0	0.031	11.4
89859262	UK	2	9,388	99.8	6,766,497	9.9	0.029	12.4
56330218	South Africa	5	9,364	99.7	4,242,732	13.6	0.043	11.6
56330227	Australia	2	9,366	99.7	2,411,508	9.9	0.031	12.0
56330281	DRC	1	9,362	99.7	2,297,064	13.5	0.048	13.2
67845223	UK	2	9,382	99.9	1,332,130	9.4	0.023	11.5
56330260	UK	5	9,365	99.7	1,306,864	13.7	0.043	12.1
56330265	UK	2	9,425	99.7	1,178,508	9.5	0.035	12.5
56330292	NA	2	9,356	99.6	1,021,219	11.5	0.035	11.9
89860237	UK	2	9,367	99.7	953,683	9.5	0.035	11.0
56330272	DRC	1	9,340	99.4	707,495	13.6	0.052	12.0
56330229	UK	2	9,366	99.7	698,622	11.5	0.036	12.6
56330276	DRC	5	9,337	99.4	612,424	13.3	0.042	12.3
89860282	UK	Novel	8,443	89.9	574,652	13.2	0.049	10.5
89860286	UK	2	8,923	95.0	513,420	9.6	0.035	10.3
89860260	UK	2	8,983	95.6	512,515	11.6	0.041	10.5
56330286	NA	2	9,355	99.6	454,106	9.6	0.022	12.5
56330228	Australia	ND	436	4.6	68,351	8.6	0.019	ND
56330250	UK	ND	423	4.5	38,083	11.6	0.043	ND
89860212	UK	ND	1,117	11.9	17,226	8.6	0.068	ND

*HPgV, human pegivirus; ID, identification; Div, sequence divergence; dN/dS: ratio of nonsynonymous (dN) to synonymous (dS) substitutions; MFED, mean folding energy difference; HHpgV, human hepegivirus; UK, United Kingdom; NA, not applicable (no genotypes of HHpgV-1 are currently assigned); DRC, Democratic Republic of Congo; ND, not done (insufficient sequence length).
†Genotype based on phylogenetic analysis of complete genome sequences (Figure 1).
‡Comparison with AK790 prototype sequence (HHPgV-1) or AF121950 (HPgV; genotype 2).
§Difference in minimum folding energy of sequences compared with those of sequence order-randomized controls (MFED) (*16*).

HHpgV-1 Detection in Case-Patients Transfused Multiple Times

We used pegivirus groups 1 and 2 PCRs to screen plasma samples from persons with hemophilia exposed to non–virally inactivated factor VII/IX concentrates and non–parenterally exposed controls (Table 2). Persons with hemophilia showed increased frequencies of HPgV viremia when compared with controls (18 of 195 compared with 1 of 50, respectively; Table 2), although this difference did not achieve statistical significance ($p \approx 0.069$ by Fisher exact test). One sample from a person with hemophilia was positive for HHpgV-1 by using group 2 primers; the amplicon sequence was 2.4%–4.8% divergent from AK-790, D1255, and D1220 between positions 4498–4896 in the AK-790 genome, with substitutions predominantly at synonymous sites. The sequence was phylogenetically distinct from the larger dataset of HHpgV-1 sequenced in the NS3 region (Figure 2; *17*).

Discussion

This study used a combined approach of bioinformatic screening of metagenomic sequence libraries and pegivirus group-specific PCRs to investigate the frequency and risk group associations of HHpgV-1 infections. The cross-validation of these 2 screening methods provides reassurance that the methods used for detection of the 2 pegivirus groups were both sensitive and specific, notwithstanding the relatively infrequent detection of HHpgV-1 in the study populations.

The 2 different screening approaches clearly have their own advantages and disadvantages. Bioinformatic screening methods are able to detect a much broader range of genetic variants of a target virus that would require separate PCRs for their detection. As an example, HHpgV-1 was originally detected by bioinformatic screening of metagenomic libraries by using HPgV as the reference sequence (*6*), but the design of primers capable of detecting all pegiviruses is problematic and in practice may require separate assays for group 1 and 2 variants as used in this study. Metagenomic virtual screening could be easily extended by the use of multiple reference sequences representing a much wider range of viruses than would be practical for PCR, for which multiple assays would have to be developed, validated, and applied in complex, multiplexed formats. Library screening for human pathogens has been proposed as alternative to multiplex PCR for this purpose (*7–9*). Another advantage of bioinformatic screening is that it is usually possible to assemble near-complete genome sequences of the viruses being screened, which provides invaluable information for studies of its molecular epidemiology, transmission, and evolution. Both HHPgV-1 and 17 of the 20 HPgV variants detected by bioinformatic screening could be assembled in near-complete genome sequences (Table 3). The HPgV

Table 4. Validation of HPgV detection in plasma samples by using PCR*

No. samples	Metagenomic screen results†		Primers, no. positive		
	HHpgV-1	HPgV	Group 2	ak1/ak2‡	Group 1
2	+	–	2	2	0
1	+/–	–	0	0	0
10	–	+	0	0	10
10	–	–	0	0	0

*HPgV, human pegivirus; HHpgV, human hepegivirus; +, positive; –, negative.
†Virus status as determined by bioinformatic screening.
‡Nonstructural 3 genes as previously described (*6*).

sequences represent a substantial increase on the number of complete genome sequences obtained to date and have identified further examples of rarely reported genotype 1 and 5 sequences, along with a putative new HPgV type (sample 89860282). In contrast, PCR amplicons are generally short and far less informative for strain identification or phylogenetic analysis, particularly if derived from highly conserved regions of the genome, as was the case for the HHpgV-1 and HPgV PCRs used in this study.

The specificity and sensitivity of bioinformatic screening is critically dependent on library quality; sequences derived from plasma samples or other largely acellular samples (cerebrospinal fluid, nasopharyngeal aspirates, urine) vary considerably in the numbers of contaminating host genomic sequences that may influence the effectiveness of screening for viral sequences in an unpredictable way (*19*). As demonstrated in this study, metagenomic libraries may be variably affected by contaminating sequences originating from other samples in the sequencing run, or may be bioinformatically contaminated from errors in reading identification tags for multiplexed sequencing reactions (*20*). In contrast, PCR-based screenings are capable of single copy target sensitivity in a wide range of sample types, and appropriate laboratory and assay design can entirely avoid false-positive results arising from sample or reagent contamination (*21*).

In this study, results from the 2 detection approaches for HHpgV-1 viremia were consistent and demonstrated the rarity of viremia with this virus in groups most at risk for parenterally and sexually transmitted virus infections. For example, only 1 person in the HGDS cohort showed viremia for HHpgV-1, despite previous extensive treatment with nonvirally inactivated factor VIII or IX concentrates (*10*). Exposure to bloodborne pathogens is attested by their universal seropositivity for HCV and high rate of HIV-1 infection (50% in those selected for this study). Despite the evidence for parenteral transmission of HHpgV-1 in the original study (*6*), our finding of a low frequency of detectable infection in this risk group is consistent with the originally reported low rate of viremia among persons with hemophilia (2/106 [*6*]). HHpgV-1 was similarly detected at low frequency in HCV-positive persons who inject drugs (2/120; Table 2), although persons in this risk group were almost universally infected with HCV from needle sharing.

In interpreting these results, we can rule out a poor sequence library or physical sample quality as the cause for nondetection of HHpgV-1. Viremia frequencies of the other pegivirus, HPgV, were comparable to those previously described in these risk groups, with elevated frequencies in those with histories of sexual exposure (8% in the HIV-positive persons in this study) previously reported to have high rates of HPgV active infection (*22–25*). Elevated frequencies are similarly reported in persons who inject drugs (*26*,*27*), which is consistent with the increased detection frequencies in this study (11%).

The findings of relatively low frequencies of HHpgV-1 viremia in the groups screened in this study suggest that it circulates less in human populations than HPgV, HCV, or HIV-1 or that infections are associated with a higher rate of clearance than for these other bloodborne viruses. Persistence over months or years was observed in 2 persons with hemophilia in the original study, although both blood recipients infected with HHpgV-1 cleared viremia within 241 and 281 days posttransfusion (*6*). The propensity of HHpgV-1 to persist for long periods, at least in some persons, is shared with many hepaciviruses and pegiviruses. Analysis of the coding regions of the 2 HHpgV-1 variants detected in this study revealed evidence for large-scale RNA secondary structure (mean folding energy differences of 7.6% and 8.1%), similar to that of the originally described HHpgV-1 sequence (*6*). This finding is within the range of values previously associated with host persistence in a wide range of positive-stranded mammalian RNA viruses, including HCV and HPgV in humans (*16*,*28*). Nevertheless, given the extensive exposure of the HGDS cohort investigated in this study to bloodborne viruses, the absence of detectable HHpgV-1 viremia in all but 1 of the persons in this study is more consistent with a higher rate of virus clearance for HCV and HPgV in this group rather than a lack of exposure.

Our findings potentially mirror those of our previous investigation of the parenterally transmitted parvovirus PARV4 in the HGDS cohort (*29*), where exposure is also closely associated with HCV and HIV through shared routes of transmission (*30*). Although all 195 study subjects were PCR-negative for PARV4 DNA at the end of the study period, 44% were seropositive for anti-PARV4 antibodies, and a process of acute infection followed by clearance was documented for a large number from whom serial samples were available over the period of infection. Without a serology assay for HHpgV-1, it is problematic to determine whether the lack of viremia detection in the HGDS cohort and other groups arose though lack of exposure or high rates of clearance of viremia.

In summary, this study used 2 complementary and cross-validated screening approaches to document frequencies of active infection of several different study groups with HHpgV-1 compared with other parenterally and sexually transmitted flaviviruses. Complementary screening of these risk groups for past exposure by using serology assays is required to understand more about its epidemiology, transmission routes, and host interactions.

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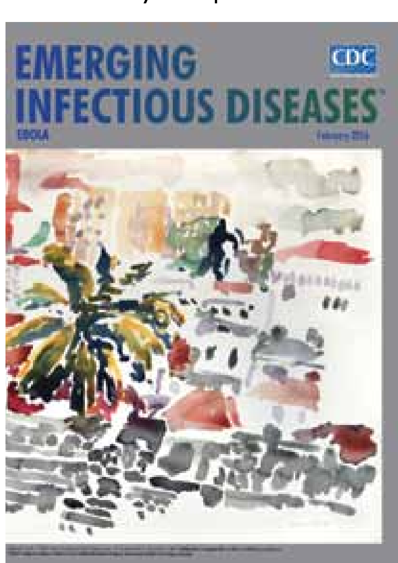

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Shiga Toxin 1–Producing *Shigella sonnei* Infections, California, United States, 2014–2015

Katherine Lamba, Jennifer A. Nelson, Akiko C. Kimura, Alyssa Poe, Joan Collins, Annie S. Kao, Laura Cruz, Gregory Inami, Julie Vaishampayan, Alvaro Garza, Vishnu Chaturvedi, Duc J. Vugia

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Learning Objectives

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

- Distinguish the epidemiologic features of recent emergence of Shiga toxin 1–producing *Shigella sonnei* in the United States, based on a surveillance study
- Identify clinical characteristics of recent Shiga toxin 1–producing *Shigella sonnei* infections in the United States
- Determine antibiotic resistance, treatment, and outcomes of recent Shiga toxin 1–producing *Shigella sonnei* infections in the United States

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Shiga toxins (Stx) are primarily associated with Shiga toxin–producing *Escherichia coli* and *Shigella dysenteriae* serotype 1. Stx production by other shigellae is uncommon, but in 2014, Stx1-producing *S. sonnei* infections were detected in California. Surveillance was enhanced to test *S. sonnei* isolates for the presence and expression of *stx* genes, perform DNA subtyping, describe clinical and epidemiologic characteristics of case-patients, and investigate for sources

of infection. During June 2014–April 2015, we identified 56 cases of Stx1-producing *S. sonnei*, in 2 clusters. All isolates encoded stx_1 and produced active Stx1. Multiple pulsed-field gel electrophoresis patterns were identified. Bloody diarrhea was reported by 71% of case-patients; none had hemolytic uremic syndrome. Some initial cases were epidemiologically linked to travel to Mexico, but subsequent infections were transmitted domestically. Continued surveillance of Stx1-producing *S. sonnei* in California is necessary to characterize its features and plan for reduction of its spread in the United States.

Shiga toxins (Stxs) are cytotoxins that mediate severe gastrointestinal disease caused by Shiga toxin–producing *Escherichia coli* (STEC) and *Shigella dysenteriae* serotype 1 (*1*). *S. dysenteriae* 1 produces the prototype Shiga toxin (Stx), and STEC can produce 2 groups of Stxs: Stx1 and Stx2. Vascular damage caused by Stxs in the colon, kidneys, and central nervous system may result in hemorrhagic colitis, or more severe conditions such as hemolytic uremic syndrome (HUS) (*2*). Stx2 has been shown to be more virulent than Stx1 (*3*), and adverse clinical outcomes such as HUS are more frequently associated with Stx2-producing strains of STEC than Stx1-producing strains (*4*). Antimicrobial drug treatment for STEC infections and late or inappropriate antimicrobial drug treatment for *S. dysenteriae* 1 infections have been associated with an increased risk for HUS (*5*,*6*).

Although Shiga toxins have been associated with STEC and *S. dysenteriae* 1, infections caused by other types of Stx-producing *Shigella* spp. have been recognized in recent years. Sporadic infections with Stx1-producing *S. dysenteriae* serotype 4 and *S. flexneri* in persons with a history of travel to the Caribbean island of Hispañiola have been characterized in the United States and Canada (*7*–*9*). A recent survey of *Shigella* isolates from persons with a history of travel to the Caribbean found that 21% of isolates encoded and produced Stx; positive strains were *S. flexneri* 2a, *S. flexneri* Y, and *S. dysenteriae* 4 (*10*). The same Stx-converting bacteriophage was identified among these isolates, suggesting the emergence of Stx-producing shigellae in this region was caused by spread of the phage to multiple *Shigella* species and serotypes. One case of infection with Stx1-producing *S. sonnei* in a patient from Germany who had a history of travel to Ukraine (*11*) and one instance of isolation of stx_{2a}-encoding *S. sonnei* from a patient from Finland who had a history of travel to Morocco have been described (*12*). Although these novel strains of Stx-producing shigellae have been reported recently, data regarding the clinical characteristics and epidemiology of these infections remain limited.

In the United States, infections with Stx-producing organisms are primarily caused by STEC; *S. dysenteriae* 1 infections are rare. Of laboratory-confirmed cases of shigellosis reported in the United States, ≈75% are caused by *S. sonnei* (*13*). *S. sonnei* infections are typically less severe than infections with *S. dysenteriae* 1 and are characterized by diarrhea, which may be bloody and accompanied by fever, nausea, and abdominal cramps. Illnesses are usually self-limited and resolve within 5–7 days of onset. Extraintestinal complications such as bacteremia and urogenital infections are rare but have been documented (*14*–*17*). Although antimicrobial drug treatment is generally unnecessary for patients with uncomplicated *S. sonnei* infections, antimicrobial drugs are often used to limit the duration of illness and communicability and to reduce illness severity (*18*).

In August 2014, Stx1-positive fecal samples from 2 patients tested by using enzyme immunoassay were reported to the County of San Diego Health and Human Services Agency (COSD HHSA; San Diego, CA, USA); the same clinical specimens showed positive *S. sonnei* culture results. COSD HHSA initiated an investigation and notified the California Department of Public Health (CDPH). Surveillance was enhanced retrospectively and prospectively in California to confirm Stx-producing *S. sonnei* isolates, identify additional cases, describe the clinical and epidemiologic characteristics of infected persons, monitor for severe clinical outcomes such as HUS, and to investigate for potential sources of infection. We subsequently identified 2 clusters of Stx-producing *S. sonnei*; the second cluster continued into late 2015. We report our findings on the initial 56 confirmed cases of Stx1-producing *S. sonnei* identified during June 2014–April 2015.

Methods

Case Finding

We defined a suspected case as detection of *S. sonnei* and Stx or *stx* genes in a clinical specimen collected during June 2014–April 2015 from a resident of California. We implemented several methods to identify suspected cases for laboratory confirmation and epidemiologic investigation. CDPH notified California local health departments of unusual detections and requested that suspected cases be reported. In addition, regular reviews of shigellosis and STEC electronic surveillance data were conducted to identify suspected cases. Some local health departments communicated with healthcare providers in their administrative areas to request reporting of suspected cases. After the detection of a cluster of suspected cases in San Joaquin County in February 2015, San Joaquin County Public Health Services prospectively enhanced laboratory surveillance and requested that local laboratories submit all *Shigella* isolates for Stx testing. Stx-positive broths and *S. sonnei* isolates for all suspected cases were forwarded to

the CDPH Microbial Diseases Laboratory to confirm the presence and expression of *stx* genes in *S. sonnei*.

Laboratory Testing

All isolates from patients with suspected cases were identified by using a commercial rapid identification system (VITEK 2 Compact, bioMérieux, Bio Marcy l'Etoile, France) or *Shigella* antisera. Isolates were further characterized by using a multiplex real-time PCR for stx_1 and stx_2 genes according to a scheme for STEC testing described by Probert et al (*19*). The Vero cell neutralization assay was used to detect active Stx. Pulsed-field gel electrophoresis (PFGE) was performed by using PulseNet protocols (*20*).

Case Definitions

We defined a confirmed case as isolation of *S. sonnei* positive for *stx* genes and active Stx production from a clinical specimen collected during June 2014–April 2015 from a resident of California. A secondary case was defined as a confirmed case in a person with illness onset >12 hours but within 2 weeks after household or other close contact with a person with a diarrheal illness. Cases in persons for whom data were available regarding ill contacts that did not meet the secondary case criteria were considered primary cases. Cases of undiagnosed diarrheal illness in contacts of suspected or confirmed case-patients were excluded from the investigation.

Data Collection

After receiving a report of Stx-positive feces/STEC/HUS or a diagnosis of shigellosis in a patient from a healthcare provider or laboratory, California local health department personnel routinely interview patients by using a standardized shigellosis or STEC/HUS case report form. We re-interviewed patients who had a suspected case of Stx-producing *S. sonnei* with a standardized investigation questionnaire that collected information on demographics, symptoms, treatment, clinical outcomes, risk factors for shigellosis, and exposure history. Suspected case-patients were queried regarding exposures during the 7 days before symptom onset. We extracted data from the local health department standardized shigellosis or STEC case report form if case-patients were not reached for re-interview with the investigation questionnaire and retrieved clinical data from medical records for case-patients lost to follow-up.

Data Analysis

Illness onset date was unavailable for 1 case-patient and was estimated as the date 3 days before specimen collection. For case-patients symptomatic at the time of the last interview, we calculated duration of symptoms by subtracting illness onset date from interview date. Data were summarized with descriptive statistics; we used the χ^2 test, Fisher's exact test, and Student *t*-test to compare characteristics of case-patients in clusters 1 and 2 and by age category (<5 vs. $\geq$5 years of age). We used the binomial test to compare the number of Hispanic case-patients in each cluster to population proportions based on 2014 American Community Survey data (*21*). A p value of $\leq$0.05 was considered significant. We used SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA) for analyses.

Results

During June 2014–April 2015, we identified a total of 56 cases of Stx1-producing *S. sonnei* in 2 epidemiologically distinct clusters in California. Cluster 1 consisted of 25 cases in persons epidemiologically linked to southern California with illness onset dates during June 26–December 18, 2014 (Figure 1). Cluster 2 consisted of 31 cases in residents of San Joaquin County in northern California with illness onset dates during January 28–April 28, 2015. The median age of all case-patients was 19.5 years (range 1–71 years); 54% were male. Eleven (20%) case-patients were children <5 years of age.

Laboratory Testing

The 56 isolates tested were confirmed to be *S. sonnei* by the rapid identification system or by agglutination in group D antiserum. All isolates tested by the multiplex PCR were positive for the stx_1 and negative for the stx_2 gene and showed active Stx production by neutralization with Stx1 antiserum in the Vero cell assay. We constructed a dendrogram of predominant and selected PFGE patterns after *Xba*I digestion (Figure 2).

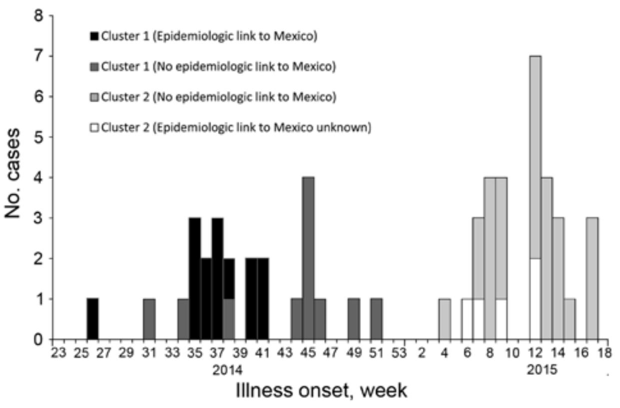

Figure 1. Epidemic curve of Shiga toxin 1–producing *Shigella sonnei* cases in California (N = 56), by week of illness onset, cluster, and epidemiologic link to Mexico, June 2014–April 2015. Cluster 1, southern California, June–December 2014 (n = 25); cluster 2, San Joaquin County, northern California, January–April 2015 (n = 31). Illness onset week designated using Centers for Disease Control and Prevention Morbidity and Mortality Weekly Report week.

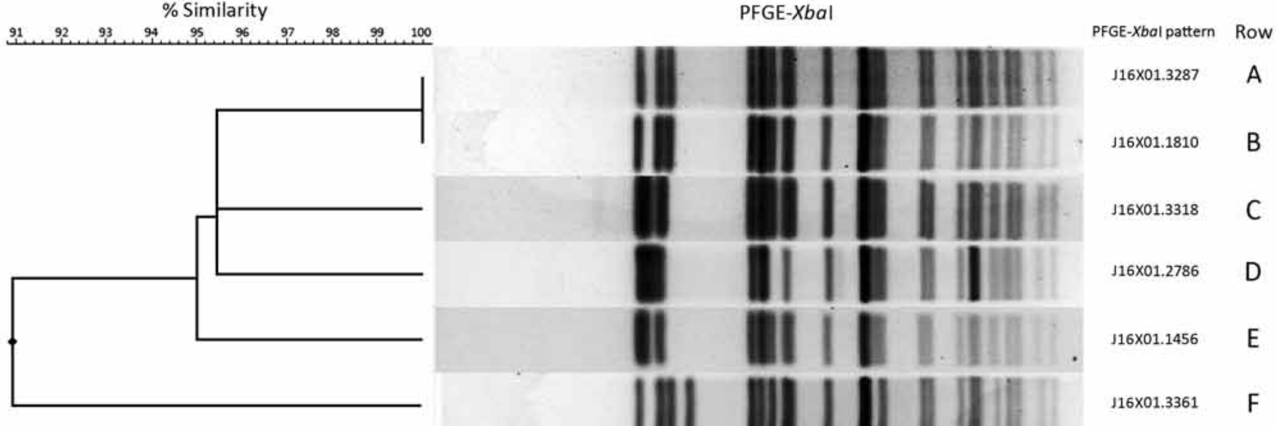

Figure 2. Dendrogram and selected pulsed-field gel electrophoresis (PFGE) patterns of *Xba*I-digested Shiga toxin 1–producing *Shigella sonnei* isolates from California. Predominant *Xba*I patterns identified in cluster 1 are shown in rows A and B and outlier on row F. Predominant patterns identified in cluster 2 are shown in rows C and E and outlier on row D. ID no., identification number.

Clinical Summary

Overall clinical characteristics of the 56 cases (Table 1) did not differ significantly by cluster (data not shown). Case-patients were interviewed by using the investigation questionnaire a median of 67 and 11.5 days, respectively, after illness onset in clusters 1 and 2. Symptoms most commonly reported were diarrhea (100%), abdominal cramps (87%), fever (72%), and bloody diarrhea (71%). Twelve (21%) case-patients were hospitalized and 27 (53%) of 51 sought medical care at an emergency department. Case-patients <5 years of age were more likely to have fever (100% vs. 67%, p = 0.054) and bloody diarrhea (80% vs. 68%; not statistically significant) than were case-patients ≥5 years of age. The median duration of symptoms was 5 days (interquartile range 3–7 days), and median length of hospitalization was 3 days (interquartile range 1.5–4 days). Six case-patients in cluster 2 were symptomatic at the time of interview; illness duration ranged from 5–12 days. No cases of HUS were identified, and no patients died.

S. sonnei was isolated from fecal specimens from 54 case-patients, from a blood specimen from 1 case-patient, and from a urine specimen from 1 case-patient. The case-patient who had bacteremia was a woman, 57 years of age, who had a history of substantial underlying medical conditions, including cirrhosis of the liver secondary to hepatitis C; she was hospitalized for hepatic encephalopathy. Her symptoms included abdominal pain and diarrhea, and computed tomography scan results of the abdomen indicated colitis and mesenteric lymphadenopathy; however, fecal culture was negative for *Shigella* and *E. coli* O157. The patient was treated with levofloxacin for her infection and discharged after 6 days. The case-patient from whom *S. sonnei* was isolated from a urine specimen was a 50-year-old woman with a diarrheal illness and clinical urinary tract infection (dysuria, pyuria >10 leukocytes/high power field). Urine culture for this patient also yielded *E. coli* and *Enterococcus faecalis*. *S. sonnei* was not isolated from her fecal sample.

Among the 29 isolates for which antimicrobial drug susceptibility testing results were available from clinical laboratories, we documented several antibiogram profiles. The most commonly identified profile (n = 16, 55%) showed resistance to trimethoprim/sulfamethoxazole and susceptibility to ciprofloxacin and ampicillin. Four (14%) isolates were resistant to trimethoprim/sulfamethoxazole and ampicillin but susceptible to ciprofloxacin. Of 10 isolates from case-patients in cluster 2, six (60%) were resistant to ampicillin; none were resistant in cluster 1.

Of 53 case-patients with data available, 43 (81%) were treated with antimicrobial drugs. Most (n = 33, 77%) case-patients treated with antimicrobial drugs were treated with 1 drug, most frequently with ciprofloxacin (13/32, 41%). Among the case-patients treated with >1 antimicrobial drug, ciprofloxacin and metronidazole was the most common combination (6/10, 60%). A 4-year-old case-patient was treated with trimethoprim/sulfamethoxazole, to which the isolate was resistant. This patient's treatment was started 5 days after diarrhea onset, and no severe outcomes were observed. The median number of days from diarrhea onset to start of antimicrobial treatment was 3 days (interquartile range 1–5 days), and 23 (66%) of 35 case-patients began antimicrobial treatment within 4 days of diarrhea onset. No differences were identified between case-patients <5 versus ≥5 years of age with respect to timing of antimicrobial drug treatment initiation or proportion treated with antimicrobial drugs.

Epidemiologic Summary

Cluster 1

In this cluster, 24 of the 25 case-patients were residents of San Diego (n = 20), Orange (n = 2), and Riverside (n = 2)

Table 1. Clinical characteristics of patients with Shiga toxin 1–producing *Shigella sonnei* infections, California, June 2014–April 2015*

Characteristic	Value
Symptoms	
Diarrhea	56/56 (100.0)
Abdominal cramps	45/52 (86.5)
Fever†	36/50 (72.0)
Bloody diarrhea	36/51 (70.6)
Chills	26/41 (63.4)
Nausea	22/39 (56.4)
Vomiting	21/48 (43.8)
Headache	10/33 (30.3)
Outcomes	
Emergency department visit	27/51 (52.9)
Hospitalization	12/56 (21.4)
Hemolytic uremic syndrome	0/43
Death	0/56
Median duration of symptoms, d (IQR)‡	5 (3–7)
Median length of hospitalization, d (IQR)§	3 (1.5–4)
Antimicrobial resistance	
Trimethoprim/sulfamethoxazole	27/29 (93.1)
Ampicillin	6/25 (24.0)
Ciprofloxacin	0/22
Ceftriaxone	0/13
Treatment	
Received any treatment	48/54 (88.9)
Treated with antimicrobials	43/53 (81.1)
Antimicrobial treatment¶	
Ciprofloxacin	19 (45.2)
Metronidazole	12 (28.6)
Ceftriaxone	5 (11.9)
Levofloxacin	5 (11.9)
Trimethoprim/sulfamethoxazole	4 (9.5)
Azithromycin	4 (9.5)
Ampicillin	3 (7.1)
Amoxicillin	2 (4.8)
Other or unknown	3 (7.1)
Median interval from diarrhea onset to start of antimicrobial treatment, d (IQR)#	3 (1–5)
Treated with intravenous fluids	23/46 (50.0)
Received other treatment**	17/54 (31.5)

*Values are no. patients/no. with information available (%) except as indicated. IQR, interquartile range.
†Based on self-reported data; 19 (52.8%) of 36 patients had documented temperature of ≥38°C.
‡n = 39; includes 6 patients in cluster 2 still symptomatic at time of interview. Illness duration range 5–12 d.
§n = 11.
¶n = 42. Because 10 case-patients were treated with >1 antimicrobial drug, total is >100%.
#n = 35.
**Antiemetics, pain medication, nonprescription antidiarrheal medication.

counties in southern California. One case-patient from San Joaquin County had illness onset in June 2014 after household contact with a visitor from southern California who had an undiagnosed diarrheal illness. We included this case-patient in cluster 1 on the basis of the illness onset date, contact with the ill southern California visitor, and positive test results for an isolate with the PFGE *Xba*I pattern predominant in cluster 1.

The median age of case-patients in this cluster was 10 years (range 2–64 years; Table 2). Case-patients were more likely to be Hispanic than the general population in those 4 counties (60.0% vs. 34.7%; p = 0.0085). Nine were considered primary case-patients and 16 were categorized as secondary; of the secondary case-patients, 7 had been exposed to another confirmed case-patient, and 9 to a contact with an undiagnosed diarrheal illness (Table 2). We identified 4 clusters of household transmission involving ≥2 confirmed cases, and a range of 2–5 cases of diarrheal illness in each household.

Five of 9 primary case-patients reported travel to Mexico during their exposure period, and 9 of 16 secondary case-patients were exposed to someone with a diarrheal illness who lived in or visited Mexico (Table 2). Overall, 14 (56%) cases were epidemiologically linked to Mexico, primarily Baja California. None of the case-patients whose illness onset dates were after mid-October 2014 (n = 8) reported epidemiologic links to Mexico. Of the 5 primary case-patients with a travel history to Mexico, none were there for all of the 7 days before illness onset. No common point sources of transmission were identified in Mexico or California.

The predominant PFGE *Xba*I pattern for isolates from patients in this cluster was J16X01.3287, identified in 12 (48%) isolates, including the San Joaquin County case-patient isolate. J16X01.1810 was the second most common pattern, identified in 4 (16%) isolates; 8 additional PFGE *Xba*I patterns were identified. We observed no association between PFGE patterns and epidemiologic links to Mexico.

Cluster 2

The 31 case-patients in this cluster were residents of San Joaquin County. Their median age was 33 years (range 1–71 years; Table 2), which was substantially older than that for cluster 1 (p = 0.0035). Eighteen cases were categorized as primary and 10 as secondary; 8 were secondary to another confirmed case. A smaller proportion of case-patients in cluster 2 were categorized as secondary than in cluster 1 (35.7% vs. 64.0%; p = 0.0398).

We identified 4 clusters of household transmission involving ≥2 confirmed cases in this cluster; a range of 2–4 cases of diarrheal illness were reported in each household. None of the case-patients reported a history of travel outside of California or close contact with anyone who lived in or visited Mexico. Case-patients in cluster 2 were significantly less likely to have an epidemiologic link to Mexico than those in cluster 1 (0% vs. 56.0%; p<0.0001). Four case-patients were homeless and 7 reported illicit drug use.

We identified no common point sources of transmission for this cluster. Aside from the identification of 1 case-patient in San Joaquin County in cluster 1, which occurred 7 months before cluster 2, no epidemiologic connection to cluster 1 was established. PFGE *Xba*I pattern J16X01.1456 was identified in 22 (71%) cluster 2 isolates, but no common source was identified among these cases. One PFGE

Table 2. Epidemiologic characteristics of Shiga toxin 1–producing *Shigella sonnei* cases by cluster, California, June 2014–April 2015

Characteristic	No. (%) patients, Cluster 1, n = 25	No. (%) patients, Cluster 2, n = 31
Male sex	12 (48.0)	18 (58.1)
Age, y		
0–4	6 (24.0)	5 (16.1)
5–9	6 (24.0)	0
10–19	5 (20.0)	6 (19.4)
20–29	1 (4.0)	3 (9.7)
30–39	4 (16.0)	3 (9.7)
40–49	0	2 (6.5)
50–59	2 (8.0)	8 (25.8)
≥60	1 (4.0)	4 (12.9)
Ethnicity		
Hispanic	15 (60.0)	14 (45.2)
Non-Hispanic	10 (40.0)	12 (38.7)
Unknown	0	5 (16.1)
Case status		
Primary case	9 (36.0)	18 (64.3)*
Secondary case	16 (64.0)	10 (35.7)*
Epidemiologic risk factors		
Epidemiologic link to Mexico	14 (56.0)	0
Homeless	0	4 (12.9)
Illicit drug use	NA†	7 (22.6)
Men who have sex with men‡	0	0

*n = 28.
†Not available (data not collected in cluster 1 investigation).
‡Denominator is men ≥18 y of age with data available: n = 2 for cluster 1 and n = 8 for cluster 2.

*Xba*I pattern, J16X01.3318, was common to both clusters, identified in 2 cases from cluster 1 and 7 (23%) cases from cluster 2; however, no epidemiologic connection was found among the case-patients. Two additional PFGE *Xba*I patterns were identified in cluster 2 isolates.

Discussion

We documented 56 cases of Stx-producing *S. sonnei* in the United States in 2 clusters, beginning in June 2014. All 56 *S. sonnei* isolates were confirmed to encode stx_1 and produce active Stx1. The clinical presentation of case-patients appeared typical for illness caused by *S. sonnei,* but more patients reported bloody diarrhea than expected. Although several initial cases detected in southern California had epidemiologic links to Mexico, most cases appeared to result from sustained domestic transmission, suggesting this pathogen may become more prevalent in California and in the United States.

Overall, the patients' diarrheal illnesses appeared typical for shigellosis caused by *S. sonnei*, and no cases of HUS were identified. The proportion of patients reporting bloody diarrhea, 71%, was higher than expected, but it is unclear whether the presence of Stx1 increased the risk for bloody diarrhea in these case-patients. A study of children with shigellosis in Bangladesh reported that those infected with *S. dysenteriae* type 1 had more grossly bloody feces than did those infected with other *Shigella* species (78% vs. 33%), likely caused by Stx (*22*). In our study, the case-patient with bacteremia had several underlying medical conditions including liver cirrhosis, a condition previously associated with shigellemia (*23*). Further studies are needed regarding the effects of Stx1 production in the pathogenesis and virulence of *S. sonnei* and the risk factors associated with developing adverse outcomes atypical for *S. sonnei* infections.

In our investigation, most case-patients received antimicrobial drug treatment, and no adverse outcomes or cases of HUS were observed. Antimicrobial drug therapy is widely used for treatment of patients with shigellosis, and early and effective treatment in patients infected with *S. dysenteriae* 1 has been shown to decrease the risk for HUS (*24*). However, delayed treatment or treatment with antimicrobial drugs to which the *S. dysenteriae* 1 isolate is resistant may be associated with an increased risk for HUS (*3*). The mechanisms by which antimicrobial treatment may precipitate *S. dysenteriae* 1–associated HUS are not well understood. Antimicrobial drug treatment for STEC infections is hypothesized to promote Stx production, and consequently increase the risk of developing HUS, so it is generally not recommended (*6*,*25*). The effects of antimicrobial drug treatment on pathogenesis or Stx production of Stx1-producing *S. sonnei* infections remain unclear. We identified multiple resistance patterns among the isolates in this study, suggesting that etiologic strains are heterogeneous. Considering the increasing prevalence of drug-resistant *Shigella* in the United States (*26*), clinicians should be aware of resistance patterns and monitor for potential adverse outcomes if deciding to treat Stx1-producing *S. sonnei* infections with antimicrobial drugs.

Our clinical data are subject to limitations. We excluded undiagnosed cases of diarrheal illness in contacts of confirmed case-patients from our investigation. Some of these persons might have had clinically mild infections with Stx1-producing *S. sonnei*. However, the possibility of missing adverse clinical outcomes in some patients is small, and because HUS is a reportable condition in California, unrecognized cases of HUS are unlikely. The lag between illness onset and case-patient re-interview in cluster 1 was unlikely to bias results because most pertinent data were collected during the initial standard shigellosis or STEC case investigation. The lag in the second interviews confirmed no adverse outcomes in case-patients.

Our investigation of 2 epidemiologically distinct clusters of Stx-producing *S. sonnei* infections suggests that emerging strains of *S. sonnei* may have initially been introduced into southern California by travelers returning from Mexico but are now circulating domestically. Stx1-producing *S. sonnei* strains have not been reported in Mexico, but our investigation suggests that they may be circulating in the Baja California region.

No clear connections between the clusters in southern and northern California were established. The introductory sources for cluster 2 remain unknown, but possibilities include unrecognized spread from southern California, new unrecognized introductions by travelers returning from abroad, or conversion of a naive strain of *S. sonnei* by an Stx1-converting bacteriophage and subsequent spread. The lack of an epidemiologic link to international travel among cases in cluster 2 and the substantial number of secondary cases caused by person-to-person transmission in both clusters demonstrate the potential for continued domestic transmission and spread in the United States.

The number of cases reported in this study is likely an underestimate of the true number of infections caused by Stx1-producing *S. sonnei* in California. Additional cases have likely gone unrecognized because persons with mild infections did not seek medical care or because laboratories and healthcare providers did not identify or report specimens positive for both Stx and *S. sonnei*. Routine Stx testing and fecal culture for patients with a clinically compatible illness may increase detection of infections with this pathogen in the future. Prompt identification is reliant upon timely reporting of results of Stx tests and fecal culture. The increasing use of multipanel culture-independent diagnostic tests may aid detection of Stx-producing *Shigella* infections. However, results must be interpreted with caution, and confirmatory testing by culture will be needed to distinguish between infection with Stx-producing *Shigella* and co-infections with *Shigella* and STEC. Enhanced surveillance by public health officials is advised, particularly for the possibility of emergence and spread of Stx2-producing *Shigella* infections, which may be more clinically severe. Transmission in high-risk settings such as childcare is of particular concern because of the potential for severe clinical outcomes in the affected populations. Although severe outcomes were not observed in the patient infected with stx_{2a}-encoding *S. sonnei* reported by Nyholm et al. (*12*), the potential for increased virulence of Stx-producing *S. sonnei* cannot be ruled out at this time.

Challenges associated with surveillance, reporting, clinical management, and public health follow-up of both shigellosis and STEC in the United States may become more prevalent as the incidence of Stx-producing *Shigella* infections increases. Because Stx-producing *Shigella* infections have been identified in Europe and the Americas, clinicians and public health officials worldwide should remain vigilant for cases of HUS or other sequelae in infected persons. Since our investigation concluded, cases of Stx1-producing *S. sonnei* have continued to be identified and reported from San Joaquin County. Surveillance for additional cases is ongoing in California to better characterize the clinical and epidemiologic features of infections with Stx-producing *S. sonnei*.

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Adenovirus Type 7 Pneumonia in Children Who Died from Measles-Associated Pneumonia, Hanoi, Vietnam, 2014

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During a 2014 measles outbreak in Vietnam, postmortem pathologic examination of hospitalized children who died showed that adenovirus type 7 pneumonia was a contributory cause of death in children with measles-associated immune suppression. Adenovirus type 7 pneumonia should be recognized as a major cause of secondary infection after measles.

Measles remains a fatal infectious disease, particularly among unvaccinated children and malnourished and immunocompromised patients. Measles virus causes systemic infection, and measles-related complications have been observed in every organ system (*1*). Pneumonia is one of the most common fatal complications and is caused by measles virus alone or by secondary viral and bacterial infections (*2*). Human adenovirus (AdV), human herpes virus, *Klebsiella* spp., *Pseudomonas* spp., and *Staphylococcus aureus* are commonly identified in the autopsied lung tissues of patients who died from measles-associated pneumonia (*3*,*4*).

In 2014, a measles outbreak occurred among mostly unvaccinated children in Vietnam; ≈15,000 confirmed cases and 146 deaths were reported (*5*). During January–October, a total of 2,462 patients with laboratory-confirmed measles infection were admitted to the National Hospital of Pediatrics (NHP) in Hanoi, Vietnam; 124 patients died (case-fatality rate 5%). Measles was diagnosed based on the presence of measles-specific IgM or the detection of measles RNA by reverse transcription PCR (RT-PCR). All 124 patients died from severe pneumonia. One patient's illness was complicated with measles encephalitis; however, the main cause of death was severe pneumonia. To elucidate the underlying causes of death in children with measles who were admitted to NHP during the outbreak, we examined formalin-fixed and paraffin-embedded (FFPE) postmortem lung tissue samples by pathologic and molecular methods.

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The Study

During February–June 2014, postmortem lung biopsies were performed on 16 children (9 boys and 7 girls) who died in the NHP pediatric intensive care unit (PICU) from measles-associated pneumonia, defined as pneumonia within 30 days of rash onset. Although we attempted to obtain written, informed consent for the postmortem biopsies from the parents or legal guardians of all 124 patients, the parents/guardians of only 16 children consented. The study was approved by the Biomedical Research Ethics Committee of NHP (approval no. 14-012) and the Research and Ethics Committee of the National Institute of Infectious Diseases, Japan (approval no. 528).

Clinical and laboratory information on the patients was reviewed retrospectively (Table 1). Median age of patients was 8 months (range 4–16 months); all 16 patients were previously healthy and unvaccinated against measles. Median duration from disease onset to PICU admission was 9.5 days and from disease onset to death was 17.5 days. Co-infections with various bacteria, *Candida* spp., AdV, and cytomegalovirus (CMV) were detected in some patients (Table 1). Histopathologic studies (Table 2) revealed diffuse alveolar damage (Figure, panel A), necrotizing pneumonia (Figure, panel B), and organizing pneumonia (data not shown) in 12, 11, and 3 patients, respectively. Bacterial pneumonia was found in 1 patient (patient 16) (Tables 1, 2). Syncytial giant cells with intracytoplasmic and intranuclear eosinophilic inclusions, which are typical findings in measles giant-cell pneumonia, were found only in patient 2 (Figure, panel C). Intranuclear eosinophilic inclusions with halo or basophilic amorphous inclusion bodies with smudgy outlines, suggesting AdV infection, were found in 10 patients (patients 3–12) (Figure, panel D, inset).

We examined the virus genomes in the FFPE lung tissues by using molecular methods (*6*). First, RNA and DNA were extracted from the FFPE lung tissues of 10 patients. A multivirus real-time PCR (rPCR) system, which was used to screen for 163 types of virus genomes, detected measles virus RNA, AdV-subgroup B DNA, and CMV DNA.

Table 1. Clinical and laboratory characteristics of 16 children who died from measles-associated pneumonia in a pediatric intensive care unit, National Hospital of Pediatrics, Hanoi, Vietnam, February–June 2014*

		Days from onset to				Laboratory test results (type of test)			
Pt. no.	Age, mo/sex	PICU admission	Death	CD4/µL (CD4 %)	MEAS IgM†	NPA/TLA (culture)	Blood (rPCR)	AdV TLA (PCR)	CMV/mL blood (rPCR)
1	6/M	5	6	530 (28)	+	–	–	ND	ND
2	8/M	4	6	538 (19)	+	–	–	–	1.90×10^5
3	14/F	9	12	754 (15)	+	–	–	ND	ND
4	12/M	6	14	714 (31)	+	–	–	+	ND
5	8/F	15	17	341 (37)	+	–	ND	+	–
6	16/F	16	18	120 (34)	–	–	–	+	–
7	10/M	22	27	793 (47)	+	*Acinetobacter* spp.	*Acinetobacter* spp.	+	6.72×10^4
8	8/M	28	30	1,084 (26)	+	–	–	ND	ND
9	15/M	10	14	2,578 (52)	+	–	*Candida tropicalis, C. parapsilosis*	+	1.10×10^4
10	4/M	10	15	947 (46)	+	–	*Klebsiella pneumoniae, K. oxytoca, Enterobacter cloacae, E. aerogenes*	+	–
11	9/F	16	20	372 (29)	+	–	*C. albicans*	+	1.10×10^4
12	7/F	11	27	472 (21)	–	–	*Pseudomonas aeruginosa, Enterococcus faecalis, Stenotrophomonas maltophilia*	–	–
13	5/F	5	21	202 (22)	+	–	–	–	2.03×10^4
14	4/M	5	24	326 (12)	+	–	–	ND	4.79×10^5
15	7/M	8	25	1,732 (49)	+	*K. pneumoniae*	*Staphylococcus haemolyticus*	+	3.20×10^3
16	15/F	5	10	ND	+	*Staphylococcus aureus*	–	ND	ND

*+, positive; –, negative; AdV, adenovirus; CMV, cytomegalovirus; MEAS, measles virus, ND, not done; NPA, nasopharyngeal aspirate; Pt, patient; PICU, pediatric intensive care unit; rPCR, real-time PCR; TLA, trachea lavage aspirate.
†Specimens from patients 6 and 12 were positive for measles virus RNA.

The copy numbers for each virus genome, along with internal reference genes, were quantified by using real-time RT-PCR (rRT-PCR) or rPCR (Table 2). To determine the serotype of the AdV-subgroup B, which was detected in 6 patients (patients 3–8), we sequenced the hypervariable regions of the hexon gene (784 nt) (*7*). Specimens from 5 of these 6 patients exhibited sequences that were consistent with AdV type 7 (AdV7; GenBank accession no. AC_000018), and the specimen from 1 patient exhibited a sequence with only 1 silent mutation.

To examine the distribution of virus antigens, we immunostained the lung sections with antibodies against each

Table 2. Histologic findings and detection of virus genomes and antigens in postmortem lung tissues of 16 children who died from measles-associated pneumonia in a pediatric intensive care unit, National Hospital of Pediatrics, Hanoi, Vietnam, February–June 2014*

	Patient no.															
Analysis	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Histology																
Diffuse alveolar damage	No	Yes	Yes	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	No
Necrotizing pneumonia	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes
Interstitial pneumonia	Yes	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No
Organizing pneumonia	No	No	No	No	No	No	No	No	No	No	No	No	Yes	Yes	Yes	No
Bacterial pneumonia	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	Yes
Viral Inclusion body	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No
Virus genome in FFPE lung†																
Measles virus RNA	3+	NA	UD	UD	UD	UD	UD	UD	NA	NA	NA	NA	UD	UD	NA	UD
AdV7 DNA	UD	NA	5+	7+	6+	3+	5+	7+	NA	NA	NA	NA	UD	UD	NA	UD
Cytomegalovirus DNA	UD	NA	UD	UD	UD	UD	2+	UD	NA	NA	NA	NA	3+	4+	NA	UD
Immunohistochemistry																
Measles virus	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AdV	–	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–
Cytomegalovirus	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–

*+, positive; –, negative; AdV; adenovirus; AdV7, adenovirus type 7; FFPE, formalin-fixed and paraffin-embedded; NA, not available because the specimen sizes were too small for analysis; RT-PCR, reverse transcription PCR; UD, under detection limit.
†Virus genome copy numbers were quantified by using real-time RT-PCR or real-time PCR: 2+, 10^2–10^3 copies/µL; 3+, 10^3–10^4 copies/µL; 4+, 10^4–10^5 copies/µL; 5+, 10^5–10^6 copies/µL; 6+, 10^6–10^7 copies/µL; 7+, 10^7–10^8 copies/µL.

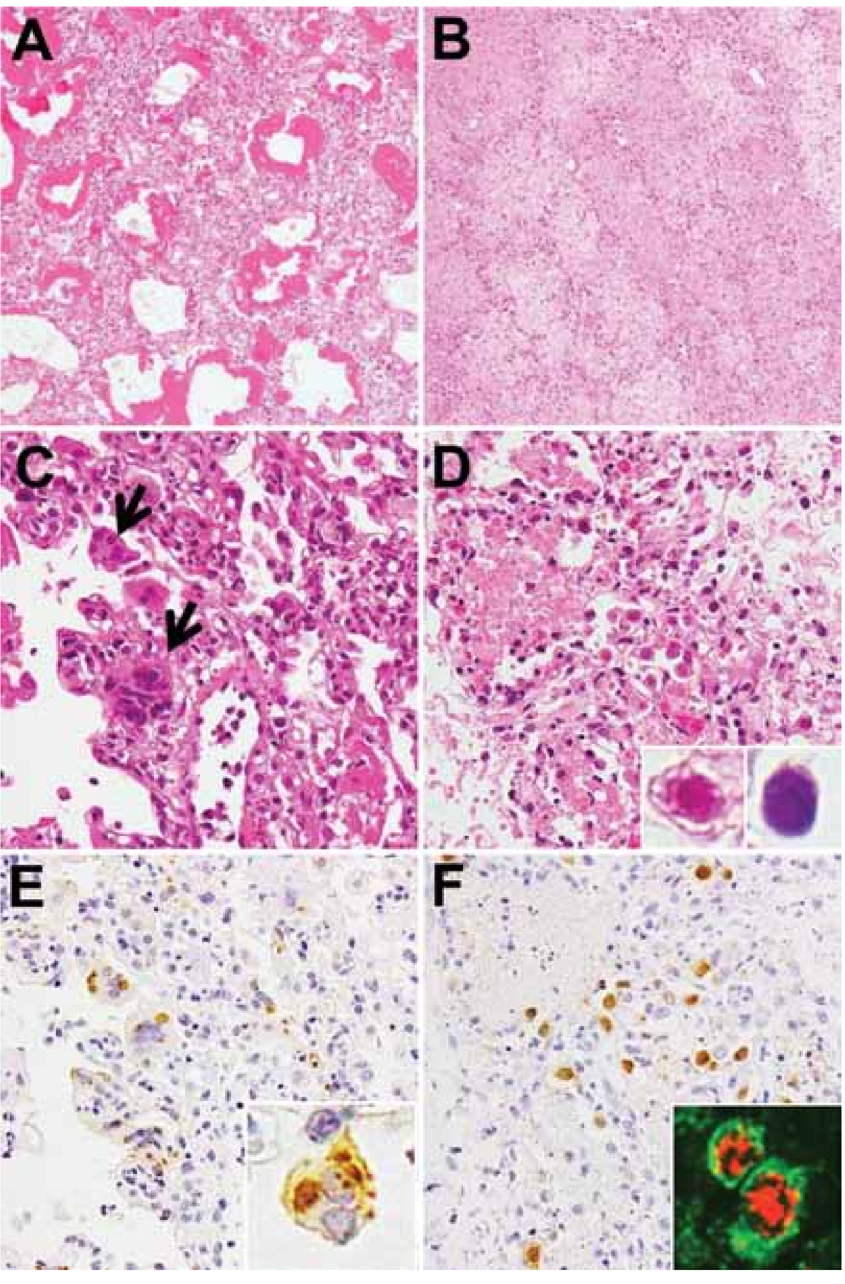

Figure. Histologic findings from postmortem lung tissues of children who died from measles-associated pneumonia in a pediatric intensive care unit, National Hospital of Pediatrics, Hanoi, Vietnam, January–October 2014. A) Diffuse alveolar damage with hyaline membrane formation (hematoxylin and eosin [H&E] stain, original magnification ×100). B) Necrotizing pneumonia with coagulation necrosis (H&E stain, original magnification ×100). C) Measles giant cell pneumonia. Arrows indicate syncytial cells with intracytoplasmic and intranuclear eosinophilic inclusions that were observed in the thickened alveolar walls (H&E stain, original magnification ×400). D) Adenovirus (AdV) pneumonia with necrotic epithelial cells and intranuclear inclusion bodies. Inset shows eosinophilic inclusion with halo and basophilic inclusion without halo (H&E stain, original magnification ×400). E) Measles nucleoprotein (brown) detected by immunohistochemisical. Inset shows inclusions in syncytial cells with measles nucleoprotein (original magnification ×400). F) AdV antigen (brown) detected by immunohistochemical analysis (H&E stain, original magnification ×400). Inset: AdV antigens (red) were detected in the epithelial membrane antigen (green)–positive pneumocytes (double immunofluorescence stain, original magnification ×400).

of the selected virus antigens. Measles virus nucleoprotein antigens were detected in the epithelial cells of patients 1 and 2 (Figure, panel E). AdV antigens were detected in all lung sections from 10 patients. Lung sections with higher copy numbers of AdV7 DNA had greater numbers of AdV antigen–positive cells (Figure, panel F). The infected cells appeared to be alveolar epithelial cells when observed after double immunofluorescence stain with epithelial membrane antigen (Figure, panel F, inset). Neither CMV inclusion bodies nor CMV antigens were observed in the CMV DNA–positive lung sections. Pathologic evidence of CMV disease was not obtained.

Conclusions

The estimated national mortality rate for the 2014 measles outbreak in Vietnam was 1% (*5*). The case-fatality rate at NPH (5%) was disproportionately higher. There are at least 3 possible reasons for the high case-fatality rate in our hospital. First, we received referred patients with severe and complicated measles from provincial hospitals in northern Vietnam. Second, the number of patients exceeded the capacity of our hospital and made it impossible to properly isolate patients with measles. Third, the patients with measles at NPH easily acquired secondary bacterial or viral infections because of the 2 aforementioned conditions.

Although autopsies are not commonly performed in Vietnam, our use of postmortem biopsies was critical for understanding the underlying contributors to measles-associated deaths, at least among the deceased children who were evaluated. Our findings showed that 10 of the 16 patients demonstrated characteristic findings of severe AdV7 pneumonia: distinctive necrotizing lesions, numerous inclusion bodies, and AdV antigens.

AdV infection commonly occurs in children. It is generally mild or subclinical and thus is not problematic. However, AdV7 can cause serious and fatal disease, particularly in closed populations such as hospitalized children and military recruits (*8*,*9*). In addition, immunocompromised children may have a much higher risk for infection with AdV disease compared with their healthy counterparts because they lack an effective cell-mediated immune response (*10*). Measles causes immune suppression that can last from several weeks to several months (*11*). In approximately half the children in this study, the percentage of CD4-positive T cells decreased to <30% of the total number of lymphocytes at PICU admission, which suggests that measles-associated immune suppression may have resulted in increased susceptibility to AdV7 infection (*12*) (Table 1). The AdV7 hypervariable region sequences that were detected in our study were the same as those found in a similar study conducted in Singapore (*13*). AdV7 should be recognized as a potentially coinfectious pathogen in patients with measles. To ensure a rapid diagnosis, AdV virus load determination and virus typing should be performed for patients who are AdV DNA–positive by PCR.

The results of our study are subject to at least 3 limitations. First, our sample size was small and included only 16 of 124 measles-associated deaths that occurred at NPH in 2014; thus, we cannot be certain that our results are representative of all of the measles-associated deaths that occurred at the hospital. Second, because a limited portion of the lung was examined, other pathologic findings may have been missed. Third, we do not have epidemiologic data to rule out a concurrent outbreak of AdV7 infection in NHP or in Hanoi.

The extent to which the complication of AdV7 infection was responsible for the increase in the case-fatality rate is unknown. However, the postmortem pathologic examination of patients in our study showed that AdV7 coinfection was one of the co-morbid causes of death.

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Elevated *Toxoplasma gondii* Infection Rates for Retinas from Eye Banks, Southern Brazil

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We found significantly higher incidence of *Toxoplasma gondii* DNA in eye bank specimens from Joinville in southern Brazil (13/15, 87%) than in São Paulo (3/42, 7%; p = 2.1 × 10E–8). PCR DNA sequence analysis was more sensitive at locus *NTS2* than at locus *B1*; a high frequency of mixed co-infections was detected.

Toxoplasma gondii is a protozoan parasite that infects nearly one third of the world's human population. Infection is typically asymptomatic, but life-threatening disease may develop in immunocompromised patients and when the infection is acquired congenitally (*1*). When parasites migrate to the eye, ocular toxoplasmosis (OT) can occur; this potentially blinding disease causes a high incidence of uveitis worldwide (*1*). In the United States, ≈2% of *Toxoplasma* infections progress to OT, whereas in southern Brazil, the proportion can be >18% (*1*). Factors leading to development of OT are poorly understood. In most OT studies, the study population is symptomatic persons. Little is known about OT infection status among a random population. We investigated the incidence of OT in a random population in Brazil and determined the sensitivity of 3 PCR diagnostic markers used for detecting parasite DNA and genotype.

The Study

In southern Brazil, incidence of OT ranges from 9.8% to 22% (*2–4*). In 1985, as part of a study to determine prevalence of toxoplasmic retinochoroidal lesions among a random population, 270 archived eyes submitted to the São Paulo Eye Bank consecutively were examined for macroscopic chorioretinal lesions or histologic evidence of retinochoroiditis (R. Belfort, Jr., unpub. data). Each was from a different person (no bilateral specimens were received). Eighteen (6.7%) eyes had chorioretinitis and 9 (3.3%) possessed cicatrial lesions; total prevalence was 10% (27/270). Of these 27 eyes, 8 (3.0%) had mononuclear cells, and 1 (0.4%) had acute, inflammatory infiltrate consistent with OT (Figure 1). These eyes were no longer available for molecular testing.

To determine updated infection prevalence rates, we obtained 114 eyes collected during 2009 from eye banks in 2 disease-endemic regions of Brazil with different OT incidence rates, the south (Joinville, n = 15) and southeast (São Paulo, n = 42) (Figure 2). The eyes were collected consecutively from deceased persons who donated to eye banks, 15 in Joinville and 42 in São Paulo. All eyes were from patients who died of natural causes or in an accident and who had no history or symptoms of ophthalmologic disease. The average age of donors was 54 years (range 20–76 years); exclusion criteria included a history of lymphoma, leukemia, leptospirosis, hepatitis B or C, HIV, rabies, or bacteria associated with endocarditis. Matched serum samples for each eye were not available.

We extracted DNA from eyes to ascertain *T. gondii* prevalence and *Toxoplasma* genotype. Eyes were not examined macroscopically. Retinal DNA was extracted by using a QIAamp DNA Blood Midi Kit (QIAGEN, Valencia, CA, USA). Multilocus PCR DNA sequencing was performed by using B1, NTS2, and GRA7 loci, as described (*5*,*6*). Positive (type I RH DNA) and negative (water only) controls were run alongside each PCR. PCR products were treated with ExoSAP-IT (USB Corp., Cleveland, OH, USA) before DNA sequencing (RML Genomics, Hamilton, MT, USA).

Specimens from 3 (7.1%) of 42 donors from São Paulo tested positive at NTS2 (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/22/4/15-1192-Techapp1.pdf), similar to the incidence rate determined among the eyes examined macroscopically in 1985 and consistent with previously published work (*7*). Our results show that no significant change in relative frequency of *Toxoplasma* infection occurred in eyes donated in São Paulo over a 25-year period. DNA sequence analysis of the 3 positive samples identified an archetypal allele shared by types II and III strains at NTS2. Only 1 NTS2-positive eye (no. 37) was positive at B1 (sensitivity 33% relative to NTS2; B1 is a standard diagnostic marker [*5*]). It possessed a unique allele (online Technical Appendix Table). No samples were PCR positive at the GRA7 marker.

Of the 15 donors from Joinville, 13 (87%) tested positive for *Toxoplasma* DNA at NTS2. Six samples possessed

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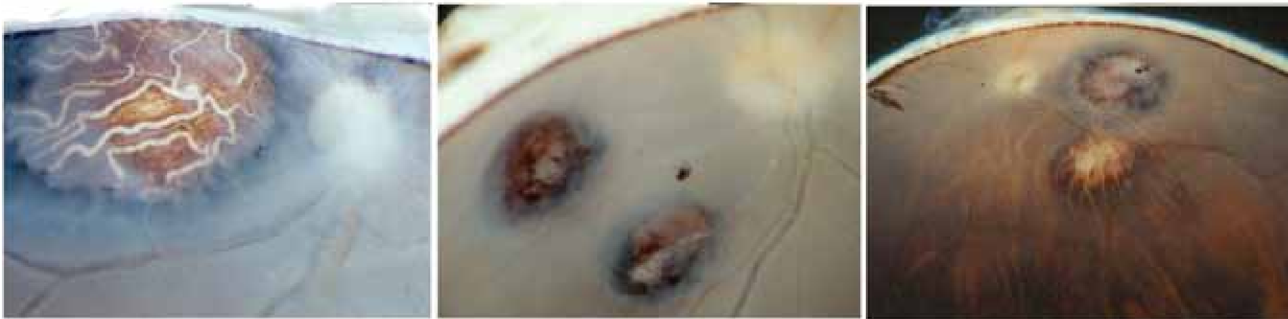

Figure 1. Representative retinochoroidal lesions depicted for 3 of 18 archived eyes examined by optical microscopy for evidence of *Toxoplasma gondii* infection; 270 single eyes were examined, each from a different donor, obtained during 1985 and 2009 in São Paulo, São Paulo State, Brazil. Original magnification (left to right): ×20, ×20, ×12.

type I alleles, 1 had a unique allele sharing phylogenetic ancestry with type I, 2 had drifted type II or type III alleles, and 4 possessed a mixture of 2 allelic types, indicative of mixed strain co-infection. The detection of 5, 3, and 3 distinct and mostly novel alleles at NTS2, B1, and GRA7, respectively, argue against the possibility that the PCR results were due to a false-negative amplification. A high incidence of mixed infection has been observed previously in Australian marsupials (*8*), but it is considered rare in human infection (*9*). In contrast, only 6 samples were positive at B1 (sensitivity 46% relative to NTS2); 1 sample had a type I allele, 4 had a unique allele, and 1 (no. 5) had a mixture of 2 different alleles. Three samples were positive at GRA7 (sensitivity 23% relative to NTS2); a type I allele, and 2 alleles sharing phylogenetic ancestry with type I and type III, respectively (online Technical Appendix Table).

Figure 2. Cities where retinochoroidal scars consistent with *Toxoplasma gondii* infection were found on eyes donated to eye banks in Brazil during 1985 and 2009: São Paulo, São Paulo (SP) State, and its relative position in southern Brazil to Joinville, Santa Catarina (SC) State; 522 km separates the 2 cities.

Southern Brazil experiences different incidence rates and severity of OT, although overall toxoplasmosis prevalence rates are similar. Studies in metropolitan São Paulo identified *Toxoplasma* antibodies in samples from pregnant patients that ranged from 59% to 65% across 2 decades, but no data on frequency of OT was reported (*10,11*). One 2004 study showed that ≈10% of the metropolitan Sao Paulo population has OT (*7*). Seroprevalence in São Jose do Rio Preto, another city in São Paulo State, is 74.5%, and the incidence of OT in 2009–2010 was 20.3% (*2*). Seropositivity in Erechim, a southern city in Rio Grande do Sul, is ≈88%; OT occurs frequently (18%) and is severe (*12*).

Conclusions

Our results reveal differences in the ability of parasites to infect eyes collected from the general population in different geographic regions of Brazil. The difference in eye infection prevalence rates suggests that, although most of the São Paulo population is seropositive, only 7% of the population harbor *Toxoplasma* parasites capable of migrating to the eye. However, 87% of eyes from Joinville tested positive for parasite DNA (a 1:1 correlation with seroprevalence), which may indicate that no apparent barrier to parasite entry into the eye exists in this geographic region. Although not all patients bearing parasite infections in their eyes develop OT, similar to the finding that only 30%–50% of AIDS patients experience recrudescent disease (*13*), our results may explain the increased incidence of OT in southern Brazil and help clarify possible factors controlling the disease.

Our findings also show that the 110 gene-copy NTS2 marker proved more sensitive than B1, a marker traditionally used to detect infection (*5*). In addition, NTS2 polymorphisms enabled mixed strain co-infections to be detected in the eyes of donor no. 5. Multilocus typing established that atypical strains common to Brazil caused most eye infections and identified different *Toxoplasma* genotypes circulating between the 2 locations; e.g., strains bearing alleles that share phylogenetic ancestry with type I

were only identified in Joinville. Previous studies suggested a link between parasite genotype and development of OT (*14*). Our work using serologic typing has established that the parasite genotype is associated with more serious congenital and ocular disease among patients in the United States and Europe (*6*,*15*). Ultimately, to determine whether increased eye infection rates in Joinville is dependent on parasite genotype, or an associated host genetic risk factor, prospective sampling should be done on eyes donated to eye banks. The serostatus of the donor, whether retinal scars are present, and the geographic origin of the donor can be recorded to determine factors associated with parasite infection and whether such donated eyes should be precluded from transplantation.

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Arenavirus Diversity and Phylogeography of *Mastomys natalensis* Rodents, Nigeria

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Mastomys natalensis rodents are natural hosts for Lassa virus (LASV). Detection of LASV in 2 mitochondrial phylogroups of the rodent near the Niger and Benue Rivers in Nigeria underlines the potential for LASV emergence in fresh phylogroups of this rodent. A Mobala-like sequence was also detected in eastern Nigeria.

Lassa fever, a viral hemorrhagic disease, is estimated to infect 150,000–300,000 persons every year, killing ≈5,000 (*1*). Within West Africa, Lassa fever is endemic to 2 regions: 1) Guinea, Sierra Leone, and Liberia; and 2) Nigeria. Even within most of these countries, Lassa fever is endemic to certain areas but rare or completely absent in others (*2*). Zoonotic disease nidality describes the phenomenon in which geographic occurrence of a zoonotic disease is markedly focused or fragmented, as opposed to occurring continuously or spreading in a consistent pattern (*3*). Zoonotic disease nidality might result when only select phyletic groups in a host species are capable of serving as reservoirs for the pathogen (*4*).

The natural host for Lassa virus (LASV), the arenavirus that causes Lassa fever, is the multimammate rat *Mastomys natalensis* (*5*). This rodent, which is distributed all over sub-Saharan African, is also host to other arenaviruses such as the Mopeia virus in southeastern Africa (*6*), Morogoro and Gairo viruses in Tanzania (*7*,*8*), and Luna virus in Zambia (*9*).

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In a genetic study of *M. natalensis* rodents across Africa (*10*), which analyzed cytochrome *b* sequences, researchers found that populations of these rodents in western Africa belong to the same monophylogenetic phylogroup, A-I. However, those authors detected phylogroup A-I of *M. natalensis* rodents in countries west of Nigeria and phylogroup A-II in countries east of Nigeria, but they did not sample Nigeria, the contact zone for rodents of these phylogroups. As a country in which Lassa fever is endemic in the western and eastern areas (*2*), Nigeria presents an excellent opportunity for investigation of patterns of LASV and arenavirus occurrence in 2 phylogroups of *M. natalensis* rodents. Our objectives in this study were to 1) determine which *M. natalensis* rodent cytochrome *b* phylogroups (A-I and A-II) are infected with LASV and other arenaviruses, and 2) identify the limits of distribution of these phylogroups within Nigeria.

The Study

From January 2011 through March 2013, small mammals were captured in H.B. Sherman live animal traps (https://www.shermantraps.com/) at 8 sites across Lassa fever–endemic and –nonendemic areas in Nigeria (Figure 1). We classified Lassa fever–nonendemic areas as areas where no cases of Lassa fever have been documented (*2*). Permission to trap rodents in various localities was granted by the Ministry of Environment, Osun State; Gwer West Local Government Council, Benue State; and the Ministry of Health, Taraba State.

Among 782 small mammals, 274 *M. natalensis* rodents were trapped. Identification of the animals in the field was based on external morphology and later confirmed genetically by cytochrome *b* gene sequencing. The rodents were euthanized, and biopsy samples (blood, liver, kidneys, spleen) were collected for laboratory analyses. Precautions for working with animals potentially infected with dangerous pathogens were strictly followed (*11*).

Using a QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA), we extracted total RNA from 20 mL of whole blood frozen at –80°C. Extracted RNA was tested with a panarenavirus protocol designed to amplify the L (polymerase) gene (340 nt) (*12*) and with another reverse transcription PCR specific for LASV, selective for the glycoprotein precursor (GPC) gene (303 nt) (*13*). We conducted further PCR amplification of the GPC fragment (using primers in online Technical Appendix 1 Table, http://wwwnc.cdc.gov/EID/article/22/4/15-0155-Techapp1.pdf)

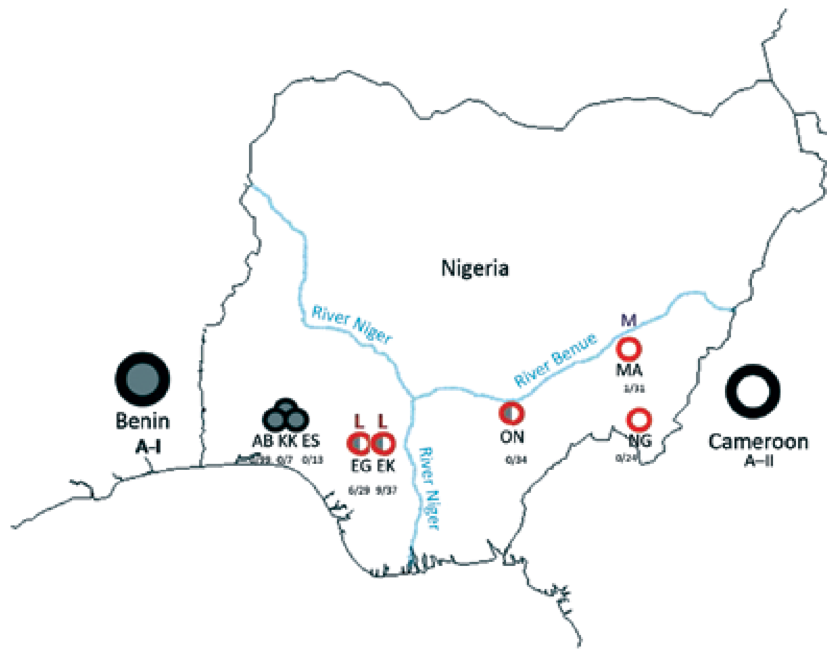

Figure 1. Sites at which *Mastomys natalensis* rodents were captured in Nigeria during January 2011–March 2013. Red circles represent sites within the Lassa fever–endemic zone; black circles represent sites outside the Lassa fever–endemic zone. Within the circles, gray indicates *M. natalensis* phylogroup A-I rodents; white indicates *M. natalensis* phylogroup A-II rodents; both colors within 1 circle indicate that rodents of both phylogroups were present at that site. Numbers under each site indicate number of arenavirus-positive *M. natalensis* rodents/number of *M. natalensis* captured. L indicates sites with Lassa virus–positive *M. natalensis* rodents; M indicates sites with Mobala-like virus–positive *M. natalensis* rodents. AB, Abagboro 07°32′38.0″N, 04°30′47.2″E; KK, Kako 07°41′26.3″N, 04°37′09.8″E; ES, Esira 07°42′04.7″N, 04°39′19.4″E; EG, Eguare-Egoro 06°46′22.7″N, 06°05′32.5″E; EK, Ekpoma 06°44′29.1″N, 06°06′17.6″E; ON, Onmba-Abena 07°38′27.5″N, 08°24′23.6″E; MA, Mayo-Ranewo 08°49′27.2″N, 10°55′15.2″E; NG, Ngel-Nyaki 07°05′30.8″N, 11°05′27.9″E.

for specimens positive on initial screening. Phylogenies were iferred by use of the Bayesian Markov Chain MonteCarlo method implemented in BEAST version 1.6.2 (http://beast.bio.ed.ac.uk/).

Of the 274 *M. natalensis* rodents from the 8 sampled sites, 16 were positive by PCR for arenavirus (Figure 1; Table). Phylogenetic analyses of the GPC and L gene sequences showed that 15 of the viruses were Lassa and 1 was a Mobala-like virus (Figure 2). The LASV sequences from Ekpoma and Eguare-Egoro belonged to lineage II and clustered with strains Nig08-A4, A37, A41, and A47 from patients in Edo State (*14*). Nucleotide identities between

Table. Source and testing results for arenavirus-positive *Mastomys natalensis* rodents, Nigeria, January 2011–March 2013*

Specimen no.	Date of capture	Village	Habitat	GPC gene (*13*)	GenBank accession no.	L gene (*12*)	GenBank accession no.
88	2011 Mar 22	Eguare-Egoro	Indoors	+	KP640562	+	KP688321
97	2011 Mar 22	Eguare-Egoro	Indoors	+	KP640563	+	KP688322
106	2011 Mar 23	Eguare-Egoro	Indoors	+	KP640564	+	KP688323
109	2011 Mar 23	Eguare-Egoro	Indoors	+	KP640565	+	KP688324
240	2011 Oct 17	Eguare-Egoro	Indoors	+	KP640566	+	KP688325
271	2011 Oct 19	Eguare-Egoro	Indoors	+	KP640567	+	KP688326
447	2012 Mar 26	Ekpoma	Peridomestic outdoor vegetation	+	KP640568	+	KP688327
508	2012 Oct 18	Ekpoma	Indoors	+	KP640569	+	KP688328
512	2012 Oct 18	Ekpoma	Peridomestic outdoor vegetation	+	KP640570	+	KP688329
518	2012 Oct 18	Ekpoma	Indoors	+	KP640571	+	KP688330
521	2012 Oct 18	Ekpoma	Peridomestic outdoor vegetation	+	KP640572	+	KP688331
539	2012 Oct 18	Ekpoma	Peridomestic outdoor vegetation	+	KP640573	+	KP688332
549	2012 Oct 18	Ekpoma	Peridomestic outdoor vegetation	+	KP640574	+	KP688333
555	2012 Oct 18	Ekpoma	Indoors	+	KP640575	+	KP688334
601	2012 Oct 21	Ekpoma	Peridomestic outdoor vegetation	+	KP640576	+	KP688335
748	2013 Mar 04	Mayo-Ranewo	Indoors	–	KP640577†	+	KP688336

*The cytochrome *b* sequences from these 16 arenavirus-positive *M. natalensis* rodents were assigned GenBank accession nos. KP688337–KP688352. +, positive; –, negative.
†Sequence KP640577 was eventually obtained by using primers OWS-1 and OWS-1000 (online Technical Appendix 1 Table, http://wwwnc.cdc.gov/EID/article/22/4/15-0155-Techapp1.pdf).

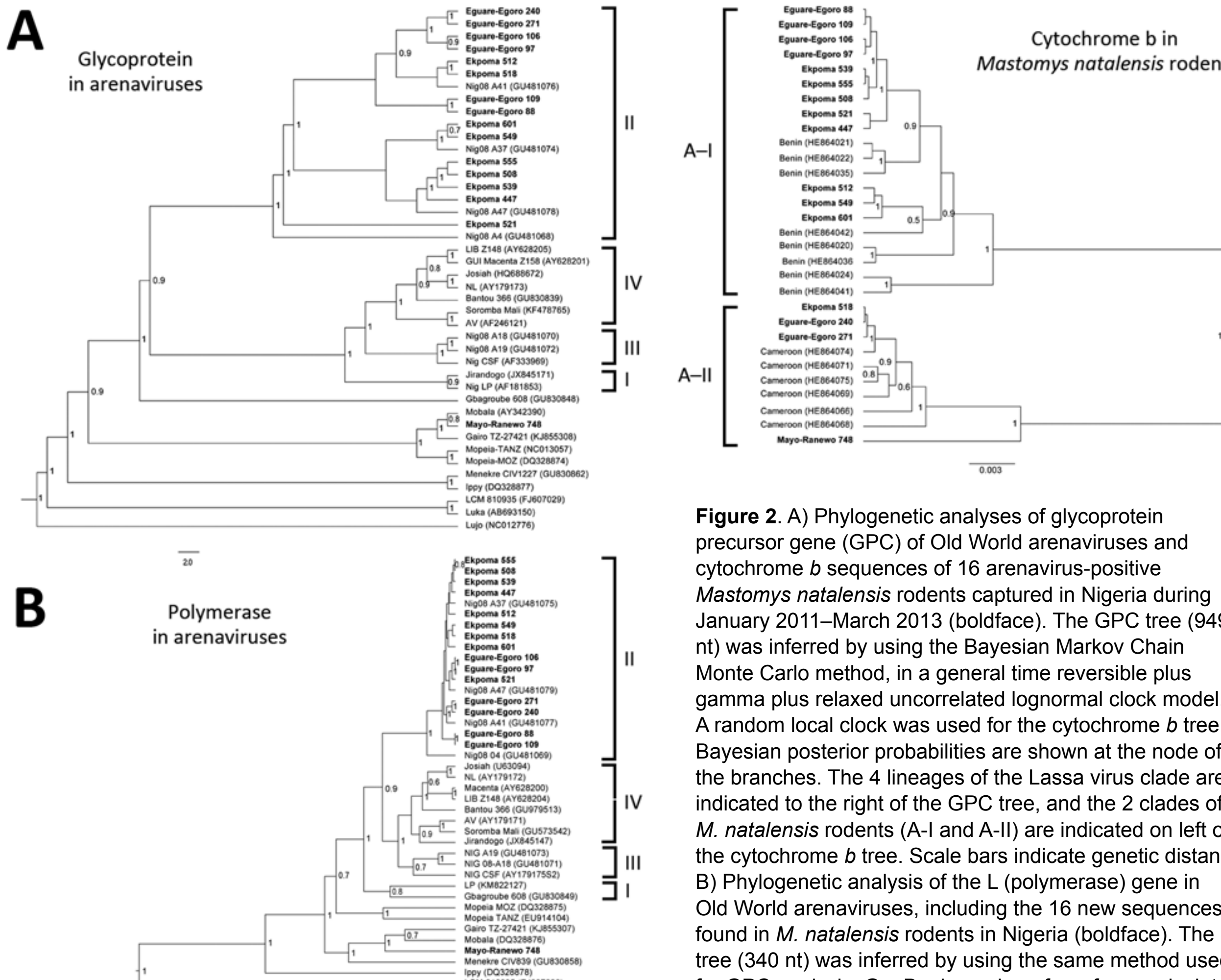

Figure 2. A) Phylogenetic analyses of glycoprotein precursor gene (GPC) of Old World arenaviruses and cytochrome *b* sequences of 16 arenavirus-positive *Mastomys natalensis* rodents captured in Nigeria during January 2011–March 2013 (boldface). The GPC tree (949 nt) was inferred by using the Bayesian Markov Chain Monte Carlo method, in a general time reversible plus gamma plus relaxed uncorrelated lognormal clock model. A random local clock was used for the cytochrome *b* tree. Bayesian posterior probabilities are shown at the node of the branches. The 4 lineages of the Lassa virus clade are indicated to the right of the GPC tree, and the 2 clades of *M. natalensis* rodents (A-I and A-II) are indicated on left of the cytochrome *b* tree. Scale bars indicate genetic distance. B) Phylogenetic analysis of the L (polymerase) gene in Old World arenaviruses, including the 16 new sequences found in *M. natalensis* rodents in Nigeria (boldface). The L tree (340 nt) was inferred by using the same method used for GPC analysis. GenBank numbers for reference isolate sequences are shown in parentheses.

the sequences of LASV from the rodents and those from the human patients were 82%–96% (GPC) and 85%–97% (L), and amino acid identities were 95%–99% (GPC) and 93%–100% (L), respectively (online Technical Appendix 2, http://wwwnc.cdc.gov/EID/article/22/4/15-0155-Techapp2.pdf). The sequence from Mayo Ranewo was conversely more distant from those from Mobala and Gairo; nucleotides identities were 69%–73% (GPC) and 77%–80% (L), and amino acid identities were 75%–77% (GPC) and 90%–95% (L), respectively (online Technical Appendix 2).

Sequence analysis of the region coding cytochrome *b* indicated that *M. natalensis* rodents from Nigeria cluster in 2 clades. The first clade corresponds to phylogroup A-I, which clusters with sequences from rodents from Benin, which is west of Nigeria (Figures 1, 2; online Technical Appendix 2). Phylogroup A-I, including sequences from Abagboro, Esira, Kako, Eguare-Egoro, Ekpoma from western Nigeria, extends across the Niger and Benue Rivers into Onmba-Abena in eastern Nigeria.

The second clade corresponds to phylogroup A-II, which clustered with sequences from Cameroon, which is east of Nigeria (Figures 1, 2; online Technical Appendix 1 Figure). Phylogroup A-II within Nigeria is represented by *M. natalensis* rodents from Ngel-Nyaki, Mayo-Ranewo, and Onmba-Abena in eastern Nigeria, but this phylogroup also overlaps the Niger and Benue Rivers westward into Eguare-Egoro and Ekpoma. The contact zone between rodents of phylogroups A-I and A-II in Nigeria was detected at sites relatively close to the Niger and Benue Rivers (Eguare-Egoro, Ekpoma, Onmba-Abena) (Figure 1). The Niger River has been demonstrated to be a natural barrier for some rodents (*15*) but seems to delimit these 2 phylogroups only to an extent. Human-assisted long-distance migration of commensal rodents could influence their genetic structure, which may be what happened for rodents

of the same *M. natalensis* phylogroup that were detected on opposite banks of the Niger River.

Conclusions

M. natalensis phylogroup A-I rodents were infected with LASV in Eguare-Egoro and Ekpoma but not in Abagboro, Kako, and Esira. Because all rodents from these sites belong to the same phylogroup, some factor other than cytochrome *b* genetic structure might be responsible for the focal prevalence of LASV. It could be, however, that our study was limited by use of the cytochrome *b* mitochondrial marker only, which is maternally inherited. Therefore, other biparentally inherited genetic markers, such as microsatellites, should be investigated. Environmental variables such as humidity and temperature could also be considered (*2*).

M. natalensis phylogroup A-II rodents were infected by LASV and a Mobala-like virus. We did not detect any LASV-positive, phylogroup A-II rodents east of the Niger River, although all the sites sampled in this area lie within the Lassa fever–endemic zone and regularly experience epidemics (*2*). It is worth exploring the possibility that other small mammals might also host LASV. LASV-positive members of phylogroup A-II, however, were found on the west bank of the Niger River in Eguare-Egoro and Ekpoma (along with LASV-positive members of phylogroup A-I). A crucial implication of these findings is the potential that new, previously naive populations and phylogroups of *M. natalensis* rodents could become infected with LASV and the disease could emerge in new regions in western Africa.

Detection of the Mobala-like virus in *M. natalensis* rodents within Mayo-Ranewo in eastern Nigeria deserves further study. We included Mayo-Ranewo among our survey sites because an epidemic of hemorrhagic fever, considered but not confirmed to be Lassa fever, occurred there in 2012. Whether the Mobala-like arenavirus detected in this village has pathogenic properties remains to be determined.

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Neisseria meningitidis Serogroup X in Sub-Saharan Africa

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The epidemiology of meningococcal disease varies by geography and time. Whole-genome sequencing of *Neisseria meningitidis* serogroup X isolates from sub-Saharan Africa and Europe showed that serogroup X emergence in sub-Saharan Africa resulted from expansion of particular variants within clonal complex 181. Virulence of these isolates in experimental mouse models was high.

The epidemiology of meningococcal disease, caused by infection with the bacterium *Neisseria meningitidis*, varies substantially by geography and time. The disease can occur as sporadic cases, outbreaks, and large epidemics. Most cases occur in what has been termed the "meningitis belt" in sub-Saharan Africa, where the World Health Organization estimated that 12,464 suspected cases occurred during the 2013 meningitis season and that 1,131 were fatal (case-fatality ratio 9%) (*1*).

The number of cases was lower during the 2013 season than during previous seasons and can be explained by the introduction of mass vaccination with the meningococcal A conjugate vaccine MenAfriVac (Serum Institute of India, Pune, India) (*1*). Cyclic epidemics spanned the meningitis belt and were caused mainly by isolates of *N. meningitidis* serogroup A that belonged to several genetic lineages called clonal complexes (CCs). Molecular typing of meningococci has used multilocus sequence typing (MLST) to determine CCs on the basis of the polymorphism of 7 housekeeping genes (*2*). Since the 1990s, isolates of other *N. meningitidis* serogroups, such as serogroup X, have been reported in several countries within the meningitis belt (*3*). In 2006, high incidence of serogroup X was first reported in Niamey, Niger (*4*), and thereafter in other countries, reinforcing the need for an effective vaccine against serogroup X (*5*). Serogroup X has rarely been detected in Europe (*6*).

MLST may not be able to resolve the variations associated with the emergence of meningococcal isolates. Whole-genome sequencing can provide data that enable allelic comparisons between meningococcal isolates (*7*,*8*) and tracing of the emergence and spread of meningococcal isolates.

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The Study

As part of our mission as a World Health Organization collaborating center, we performed MLST on *N. meningitidis* serogroup X isolates sent to the Institut Pasteur during 1998–2005 (Table 1). We further performed whole-genome sequencing on 8 invasive serogroup X isolates obtained during 1998–2005 from several countries within the meningitis belt and 3 invasive isolates from France (Table 1).

Genomic DNA was extracted by using a Genomic-tip 20/G kit (QIAGEN, Valencia CA, USA) from culture grown overnight on gonococcal medium base agar plates with Kellogg supplements (*9*). Whole-genome sequencing was performed by using an Illumina HiSeq 2000 sequencer (Illumina, San Diego, CA, USA) and assembled as described (*10*). Sequences are available through the PubMLST database (http://pubmlst.org/neisseria/), which runs on the Bacterial Isolate Genome Sequence Database (BIGSdb) platform (*11*); identification numbers are 34731–34732, 34734–34741, and 34745. We analyzed the 11 isolates from sub-Saharan Africa and France and all other serogroup X genomes available in BIGSdb by using the BIGSdb genome comparator tool.

The isolates from Africa were genetically related, belonged to CC181, and were separated from all the other isolates that belonged to other CCs (Table 1; Figure 1, panel A). The CC181 isolates formed a single main lineage comprising 2 sublineages, 181.1 and 182.2. Sublineage 181.1 was detected only in isolates obtained in 2006, but sublineage 181.2 was detected in isolates from both periods (1990s and since 2006). Genomes from the 3 isolates from France and those available in the PubMLST database were highly diverse with no close clustering (Figure 1, panel A).

Because we assembled the sequences by using the same method, we further focused on the 11 genomes according to particular groups of genes involved in meningococcal virulence (biosynthesis of the capsule, serogroup B vaccine antigens, lipooligosaccharide, pilin, and iron acquisition). Among genes involved in iron acquisition, the isolates from Africa, unlike those from France, lacked the *hpuA* and *hpuB* genes that mediate heme-iron acquisition from hemoglobin and hemoglobin–haptoglobin complexes. However, the isolates from Africa harbored the hemoglobin receptor that was missing in the isolates from France. The hemoglobin receptor is detected more frequently among isolates involved with disease than among those involved with carriage (*12*). The analysis of the polymorphism of these

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Table 1. Characteristics of *Neisseria meningitidis* serogroup X isolates, sub-Saharan Africa and France, 1995–2008*

Isolate	Year collected	Country	Clonal complex	Allele *lpt3*	Reactivity of monoclonal antibody immunotype L3,7, 9
LNP13407	1995	Chad	ST-181	45	–
LNP14354	1996	Niger	ST-181	136	+
LNP14355	1996	Niger	ST-181	136	+
LNP15075	1997	Burkina Faso	ST-181	136	+
LNP14964	1997	Niger	ST-181	136	+
LNP19504	2002	France	ST-254	1	+
LNP23557	2006	Niger	ST-181	45	–
LNP2006100	2006	Niger	ST-181	45	–
LNP23552	2006	Niger	ST-181	45	–
2005166	2006	Niger	ST-181	45	–
LNP23558	2006	Niger	ST-181	45	–
LNP24287	2007	France	ST-750	–	+
LNP24196	2007	France	Unassigned	–	+
2008223B	2008	Burkina Faso	ST-181	45	–
2008112	2008	Benin	ST-181	45	–

*ST, sequence type; –, absent; +, present.

iron-acquisition genes showed that the isolates from Africa clustered together, separate from the isolates from France (Figure 1, panel B).

We tested the virulence of these isolates in a relevant animal model, transgenic mice expressing the human transferrin (*13*). The animal experiments were conducted in accordance with the European Union Directive 2010/63/EU (and its revision 86/609/EEC) for the protection of animals used for scientific purposes and were approved by the Institut Pasteur Review Board, which is part of the Regional Committee of Ethics of Animal Experiments of the Paris region (permit 99–174). We constructed bioluminescent variants of isolates LNP19504 and LNP14354 by transformation with the previously published recombinant plasmid pDG34 harboring the *luxCDABE* operon expressed by the PporB meningococcal promoter (*14*). The structure was

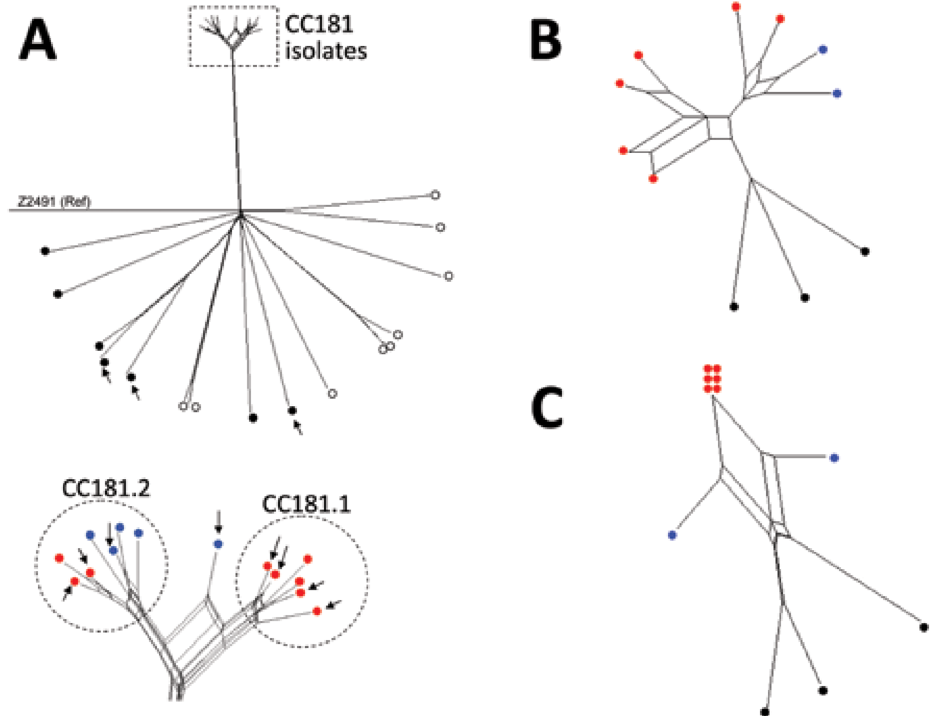

Figure 1. Neighbor-Net SplitsTree graphs generated using SplitsTree4 version 4.13.1 (http://www.splitstree.org) to visualize trees of *Neisseria meningitidis* serogroup X isolates. A) All 30 *Neisseria meningitidis* serogroup X isolates available on BIGSdb (*11*) were analyzed, including the 11 isolates from this study (8 from sub-Saharan Africa and 3 from France), 9 carriage isolates, 3 invasive isolates from Europe, 1 isolate from the United States, and 6 isolates from sub-Saharan African countries. Open (white) circles indicate carriage isolates; black circles indicate invasive isolates obtained outside of Africa; red circles indicate isolates obtained in sub-Saharan Africa since 2006; blue circles indicate isolates obtained from sub-Saharan Africa during the 1990s. Arrows indicate the 11 isolates obtained during this study. All isolates were compared with reference (Ref) meningococcal strain Z2491. The dashed rectangle indicates the cluster of all the clonal complex (CC) 181 isolates from sub-Saharan Africa (enlarged view at bottom of panel). B) The 11 isolates obtained during this study were compared for iron-acquisition genes (*tpbA*, *tbpB*, *hpuA*, *hpuB*, *lbpA*, *lbpB*, and *hmbR*). C) Genes of the 11 isolates obtained during this study compared with the 41 genes that differed between all the isolates obtained in Africa since 2006 and the isolate LNP13407 or the isolate LNP14354 (obtained during the 1990s).

recombined in *N. meningitidis* downstream of the recombinogenic *pilE* gene to obtain the strains LNP19504lux and LNP14354lux. The insertion of the *lux* operon did not modify bacterial invasion in this animal model (*13*). These 2 bioluminescent strains were then used to infect transgenic mice by intraperitoneal injection of 5×10^6 CFU in 500 mL of the corresponding bacterial suspension. Dynamic bioluminescence imaging was used to follow the infection. After 30 minutes of intraperitoneal challenge, bacteria were found mainly in the peritoneal cavity and the image signals did not differ substantially. Bacterial infection then started to spread, enabling detection of bioluminescent signals at distal anatomic sites; signals in the skull and all over the infected mice suggested systemic infection and septicemia (*13*). However, after 6 hours of infection, the signal decreased more rapidly in transgenic mice infected by isolate 19504lux from France than in the mice infected by isolate LNP14354lux from Africa, suggesting significantly higher virulence for the serogroup X isolate from Africa (Figure 2).

We next focused on differences between CC181 isolates obtained since 2006 and those obtained in the 1990s. We detected 18 genes for which alleles were identical in all isolates obtained since 2006 and in LNP14354 (obtained in the 1990s) but differed in LNP13407 (obtained in the 1990s), and we detected 23 genes for which alleles were identical in all isolates obtained since 2006 and in LNP13407 but differed in LNP14354 (Table 2).

Most of these 41 genes encode hypothetical proteins; several encode proteins that are involved in meningococcal survival in the blood (Table 2; Figure 1, panel C). In particular, genes of interest were *fHbp* (encodes factor H binding protein), *lpt3* (encodes the phosphoethanolamine transferase), and *lot3* (encodes the lipooligosaccharides, O-acetyltransferase). All serogroup X isolates obtained since the outbreak of 2006 (including those from the BIGSdb platform) harbored the *lpt3* allele 45, which differs from allele 136 by the deletion of 2 codons (encoding Glu-211 and Ser-222 residues). These isolates do not react with the monoclonal antibody that recognizes immunotype L3,7,9 on the lipooligosaccharide (*15*). Replacing allele 45 with allele 136 by transformation enabled re-recognition of the epitope L3,7,9 (data not shown). The epitope L3,7,9 occurs frequently among invasive isolates, and antibodies against this epitope seem to be of importance for protection. Adding phosphoethanolamine on lipooligosaccharide may hide the epitope. This lipooligosaccharide modification may have enabled serogroup X to escape population immunity against lipooligosaccharides and contributed to its reemergence (*15*).

Conclusions

The extensive characterization of *N. meningitidis* serogroup X isolates in the meningitis belt of Africa supports

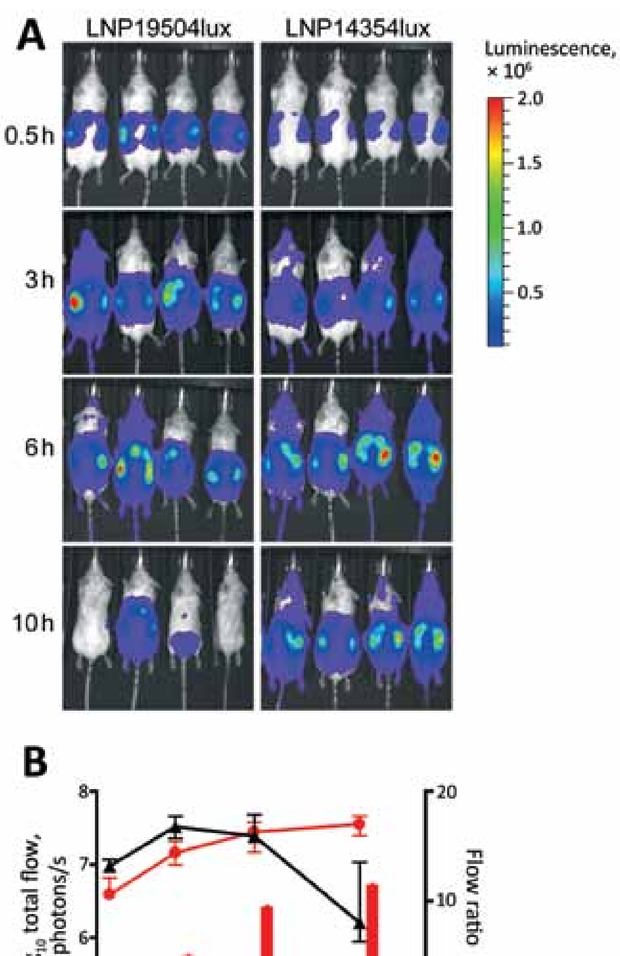

Figure 2. Dynamic imaging showing the multiplication and spread of *Neisseria meningitidis* in BALB/c transgenic mice expressing the human transferrin. A) Dorsal views of 8 mice (4/group) analyzed for bioluminescence at intervals after infection, as shown on left. Mice were infected by intraperitoneal injection of 5×10^6 CFU of *N. meningitidis* strain LNP19504lux (derived from an isolate from France) or LNP14354lux (derived from an isolate from Africa). Both strains expressed the luciferase (*lux*) operon. Photographs are overlaid with color representations of luminescence intensity, measured in total photons per second and indicated on the scales: red, most intense (2.00×10^6 p/s/cm^2/steradian); blue, least intense (1.00×10^4 p/s/cm^2/steradian). B) Luminescence for LNP19504lux (black) and LNP14354lux (red) was quantified and expressed by using GraphPad PRISM version 4.00 (http://www.graphpad.com/). Means ± 95% CIs of total photons per second (lines, left y-axis) were calculated by defining the specific representative region of interest encompassing the entire animal. Signals differed significantly between the 2 groups after 10 h of infection (p = 0.01 by *t*-test). After 24 h, all mice had survived and signals declined for both isolates but remained detectable for LNP14354lux (not shown). The data are also expressed as flow ratio (total photons per second for each point/total photons at the first point [after 0.5 h of infection]) (bars, right y-axis).

Table 2. Identical loci in all *Neisseria meningitidis* isolates obtained since 2006 and in 1 isolate obtained in the 1990s (LNP14354 or LNP13407), sub-Saharan Africa and France, 1995–2008

All isolates obtained since 2006 and in LNP14354, n = 18 (gene)	All isolates obtained since 2006 and in LNP13407, n = 23 (gene)
16S_ribosomal DNA	NEIS0047 (*rfbB*)
NEIS0056 (*ctrB*)	NEIS0048 (*galE*)
NEIS0057 (*ctrC*)	NEIS0066 (*ctrE*)
NEIS0187	NEIS0092
NEIS0235	NEIS0095
NEIS0291 (*lot*)	NEIS0107
NEIS0303	NEIS0108
NEIS0342	NEIS0224
NEIS0620 (*maeA*)	NEIS0352
NEIS0665	NEIS0378
NEIS0695	NEIS0554
NEIS1034	NEIS0689
NEIS1558	NEIS0691
NEIS1651	NEIS0795
NEIS2083 (*mafA_MGI-3*)	NEIS1300
NEIS2474	NEIS1357
NEIS2484	NEIS1594
fHbp_PEPTIDEfrag_Pasteur	NEIS1706
	NEIS1777
	NEIS1978
	NEIS1986 (*lpt3*)
	NEIS2149
	NEIS2486

the emergence of virulent serogroup X isolates and the belief that CC181 may be considered a hyperinvasive genetic lineage of meningococci. The occurrence of the periodic epidemic waves may be caused by the emergence and spread of successive new clones of meningococci with enhanced virulence and an ability to escape population immunity. Our data underline the need for whole-genome sequencing for reliable tracking of meningococcal isolates and emphasizes the need for an effective vaccine against serogroup X (*5*).

This work was supported by the Institut Pasteur and by funding from the Fondation Total including a fellowship to A.A. This study made use of the Neisseria Multilocus Sequence Typing website (http://pubmlst.org/neisseria/), developed by Keith Jolley and sited at the University of Oxford, and the dynamic imaging facilities of Imagopole at the Institut Pasteur, Paris. The development of this site has been funded by the Wellcome Trust and European Union. This study also made use of the Meningitis Research Foundation Meningococcus Genome Library (http://www.meningitis.org/research/genome) developed by Public Health England, the Wellcome Trust Sanger Institute, and the University of Oxford.

Dr. Agnememel is a microbiologist at the Institut Pasteur, Paris, and works on development of bacterial meningitis rapid diagnostic tools. His research interests include development of a universal vaccine against meningococcal meningitis.

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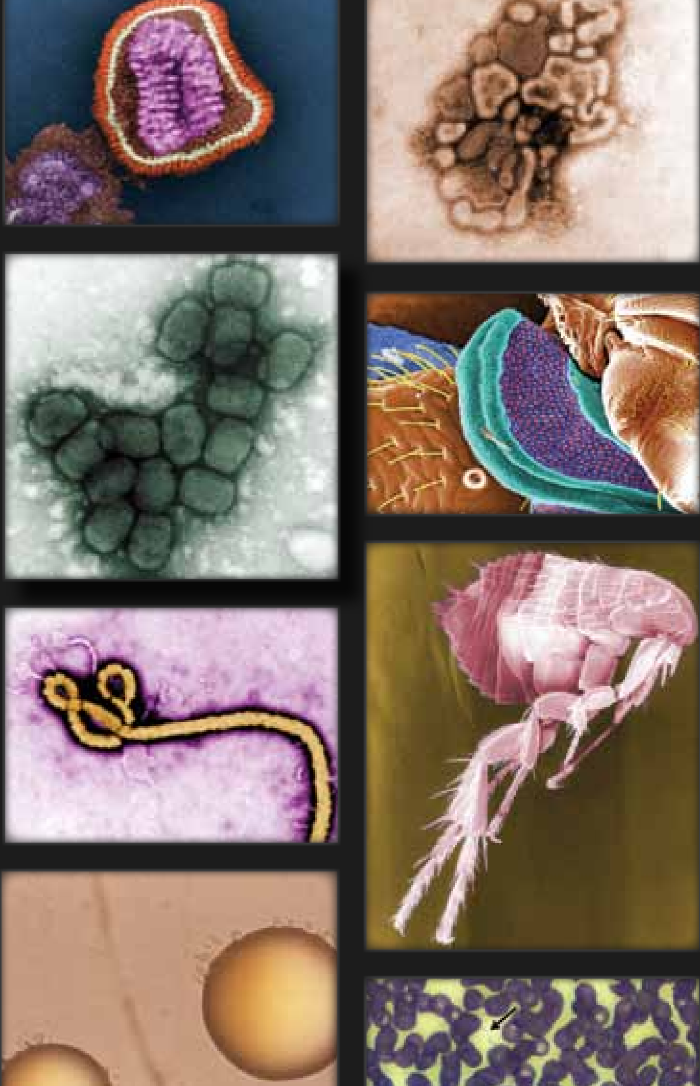

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Cross-Neutralization between Human and African Bat Mumps Viruses

Hiroshi Katoh, Toru Kubota, Toshiaki Ihara, Ken Maeda, Makoto Takeda, Minoru Kidokoro

Recently, a new paramyxovirus closely related to human mumps virus (MuV) was detected in bats. We generated recombinant MuVs carrying either or both of the fusion and hemagglutinin-neuraminidase bat virus glycoproteins. These viruses showed replication kinetics similar to human MuV in cultured cells and were neutralized efficiently by serum from healthy humans.

Many batborne paramyxoviruses closely related to mammalian paramyxoviruses recently have been identified, suggesting a possible risk for transmission of batborne paramyxoviruses to humans (*1*). Although no infectious virus has been isolated, the genome of a new paramyxovirus detected in an epauletted fruit bat (*Epomophorus* sp.) in the Democratic Republic of the Congo was closely related to the mumps virus (MuV, genus *Rubulavirus*) (*2*).

Mumps is typically characterized by inflammation of the parotid glands but also can be accompanied by orchitis, aseptic meningitis, pancreatitis, and deafness (*3*). Mumps vaccines have been used worldwide for >20 years. MuV is serologically monotypic (*4*). The fusion (F) and hemagglutinin-neuraminidase (HN) proteins, but not the small hydrophobic (SH) membrane protein, are the major targets of neutralizing (NT) antibodies (*5*,*6*).

By using expression plasmids, in 2015, Kruger et al. determined that the envelope proteins of the new paramyxovirus African bat MuV (ABMuV) were serologically and functionally related to those of MuV (*7*). We generated infectious recombinant MuVs (rMuVs) carrying either or both of the F and HN glycoproteins of ABMuV to analyze their functions and serologic cross-reactivities in the context of virus infection.

The Study

A full-length genomic cDNA of the MuV Odate strain (pMuV-Odate) (*8*) was constructed, and the open reading frames of the F and HN genes were exchanged individually or together with those of ABMuV, which were obtained by an artificial composition (GenBank accession no. HQ660095) (pMuV-Odate/ABMuV-F, /ABMuV-HN, and /ABMuV-FHN) (Figure 1, panel A). All infectious viruses were rescued by transfecting the plasmids pMuV-Odate, /ABMuV-F, /ABMuV-HN, and /ABMuV-FHN, along with helper plasmids, into BHK/T7–9 cells. Rescued viruses were propagated in Vero cells.

First, we examined syncytium formation in the Vero cells infected with all of the rMuVs (Figure 1, panel B). Although rOdate/ABMuV-F produced a cytopathic effect similar to that of rOdate, larger syncytia and severe cell detachment were observed in the cells infected with rOdate/ABMuV-HN and -FHN. To understand the basis for the enhanced syncytium formation by rMuVs carrying the ABMuV HN protein, we expressed the F and HN proteins of the Odate and ABMuV strains using expression plasmids and examined for syncytium formation. Expression of the ABMuV HN protein did not enhance syncytium formation when used in expression plasmids (Figure 1, panel C). These data were consistent with a previous study (*7*). The matrix protein was expressed together with the HN and F proteins because it can modulate syncytium formation. However, it did not affect the fusion activity. Therefore, the findings with infectious viruses appeared to differ from those obtained with expression plasmids (*7*).

All rMuVs were propagated efficiently in Vero cells (Figure 1, panel D). Although at 24 and 48 h postinfection the titers of rOdate/ABMuV-F were lower than those of the other 3 viruses, they later increased to ≈10^8 PFU/mL, which was comparable to the titers of rOdate and rOdate/ABMuV-FHN. On the other hand, the peak titer of rOdate/ABMuV-HN was as low as 5×10^6 PFU/mL. To determine whether the envelope proteins affect the cell tropisms of MuV in vitro, we evaluated the viral growth in human- and bat-derived cell lines. Human lung epithelial A549 and human monocytic THP-1 cells were used because epithelial cells and monocytes are the primary targets of MuV in vivo (*9*). In A549 cells, rOdate/ABMuV-HN showed the highest titer by up to >10^6 PFU/mL at 96 h postinfection (Figure 1, panel E). The other 3 rMuVs also replicated well in A549 cells up to ≈10^5 PFU/mL, with rOdate/ABMuV-FHN showing much faster kinetics than the others. All 4 viruses grew to similar titers of up to 10^7 PFU/mL in THP-1 cells (Figure 1, panel F). Growth was also efficient in the fruit bat–derived FBKT1 cells, although the peak titers of rOdate were lowest (Figure 1, panel G). Collectively, these

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findings using culture cells suggested that the envelope proteins are not a critical determinant of host specificity between ABMuV and MuV.

We conducted NT assays and ELISA using human serum obtained from 12 healthy adults (18–58 years of age) under approval by the Ethical Committees of National

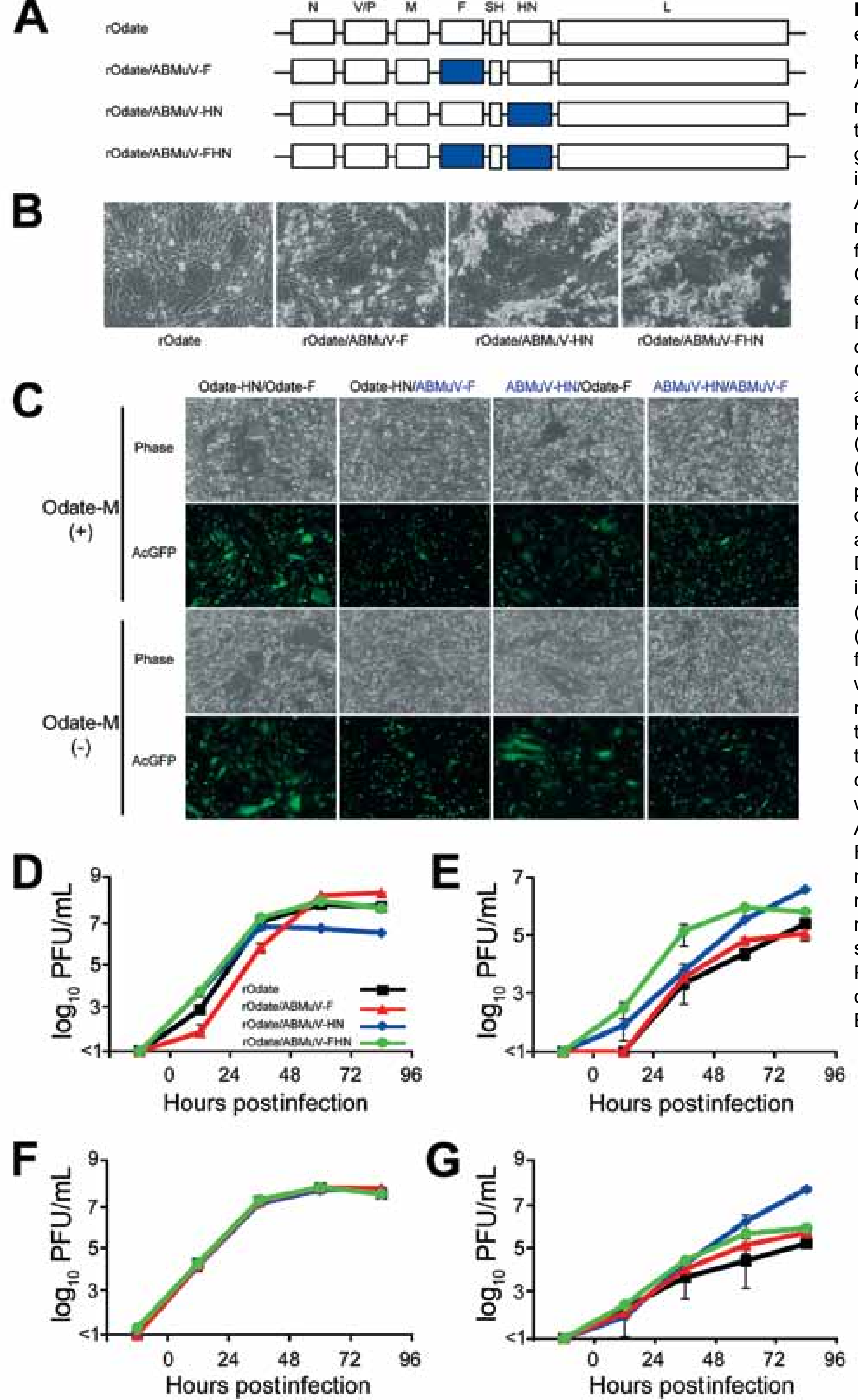

Figure 1. Construction of rMuVs expressing the ABMuV envelope proteins for study of their functions. A) Genome structures of the rMuVs. The 7 boxes indicate the N, V/P, M, F, SH, HN, and L genes of MuV. The blue boxes indicate the genes derived from ABMuV. B) Cytopathic effect of rMuV infection of the Vero cells followed by incubation for 48 hr. C) BHK cells were transfected with expression plasmids of the HN and F proteins (pCAGGS-Odate-HN or -ABMuV-HN and pCAGGS-Odate-F or -ABMuV-F). They were also cotransfected with expression plasmids of the M protein (pCAGGS-Odate-M) and AcGFP (pAcGFP-C1, Clontech). At 48 h posttransfection, the cells were observed under a phase-contrast and a fluorescence microscope. D–G) Growth kinetics of the rMuVs in Vero (African green monkey) (D), A549 (human) (E), THP-1 (human) (F) and FBKT1 (Ryukyu fruit bat) (G) cells. Each cell line was infected with the rMuVs at a multiplicity of infection of 0.01. At the indicated times postinfection, the culture supernatants were collected, and the infectious titers were determined by plaque assay. ABMuV, African bat mumps virus; F, fusion; HN, hemagglutinin-neuraminidase; L, large; M, matrix; N, nucleocapsid; rMuV, recombinant mumps virus; SH, small hydrophobic; P, phosphoprotein; V, historically considered the fifth viral protein. Error bars indicate SD.

Table. Mumps virus neutralization test for serum of healthy human adults, Japan*

Serum sample no.	Patient age, y	Mumps history: Natural Infection	Mumps history: Vaccinated	EIA titer‡	NT titer†: rOdate	NT titer†: rOdate/ABMuV-F	NT titer†: rOdate/ABMuV-HN	NT titer†: rOdate/ABMuV-FHN
1	32	Unknown	+	$2^{2.35}$	121	36	133	122
2	40	Unknown	–	$2^{1.36}$	21	38	78	109
3	49	Unknown	–	$2^{2.09}$	26	7	31	12
4	48	+	–	$2^{2.94}$	176	122	183	149
5	58	+	–	$2^{2.80}$	41	31	41	33
6	56	+	–	$2^{2.55}$	31	8	85	27
7	40	Unknown	–	$2^{1.84}$	88	105	58	33
8	35	+	–	$2^{3.32}$	186	60	131	118
9	33	Unknown	–	$2^{2.48}$	83	30	81	37
10	31	+	–	$2^{2.78}$	53	5	122	26
11	18	Unknown	–	$2^{0.74}$	<4	<4	<4	<4
12	18	Unknown	–	$2^{0.64}$	<4	<4	<4	<4

*ABMuV, African bat mumps virus; EIA, enzyme immunoassay; F, fusion; HN, hemagglutinin-neuraminidase; NT, neutralizing; r, recombinant; +, positive; –, negative.
†NT titer was determined as the dilution of serum that gave 50% plaque reduction compared with the average number of plaques formed in the absence of serum using the method of Reed and Muench.
‡Determined by using a commercially available indirect IgG eEIA kit (Mumps IgG-EIA kit; Denka Seiken Co., Niigata, Japan) according to the manufacturer's instruction. Titers $<2^1$ are seronegative, 2^1–2^2 are indeterminate, and $>2^2$ are seropositive.

Institute of Infectious Diseases. Ten of 12 serum specimens (nos. 1–10) were seropositive or indeterminate (titer $>2^1$) and neutralized rOdate (NT titer ≥4-fold) (Table). The MuV-NT serum samples showed cross-neutralization between rOdate and 3 chimeric MuVs (Table). Correlations of the NT titers were significant among rOdate and rOdate/ABMuV-F, -HN and –FHN of 0.67 ($p<0.05$), 0.77 ($p<0.01$), and 0.71 ($p<0.05$), respectively, by Pearson product-moment correlation (Figure 2). In addition, serum from a rabbit vaccinated with a genotype B mumps vaccine strain also neutralized the rMuVs carrying the ABMuV envelope proteins (data not shown). All data demonstrated that MuV and ABMuV were serologically cross-reactive.

Conclusions

To our knowledge, no infectious ABMuV has been isolated, although the entire genome sequence was detected in bats. To study the context of virus infection, we generated rMuVs carrying the ABMuV envelope proteins by reverse genetics. By using expression plasmids, Kruger et al. reported that the functions, such as fusion, hemadsorption, and neuraminidase activities, of the envelope proteins were conserved and compatible between MuV and ABMuV (*7*). These findings agreed with our data using the recombinant viruses, but notable differences existed. For example, Kruger et al. reported that the ABMuV envelope proteins induce smaller syncytia than the MuV proteins, whereas we observed enhanced syncytium formation by rMuV carrying the ABMuV HN protein. However, the enhancement was not due simply to the functional difference between MuV and ABMuV HN proteins because the HN proteins showed similar fusion-supporting capacities when expressed using expression plasmids. Further investigation of the involvement of other viral proteins modulating the HN protein function could lead to elucidation of the mechanism underlying this difference. Moreover, although Kruger et al. mentioned that the fusion activity might restrict the viral species specificity, our data indicated that the envelope proteins of MuV are not critical determinants of the host specificity in cultured cells. Our study also demonstrated that a synthetic genome strategy, which has also been used for the study of a bat influenza virus (*10*,*11*), is useful for

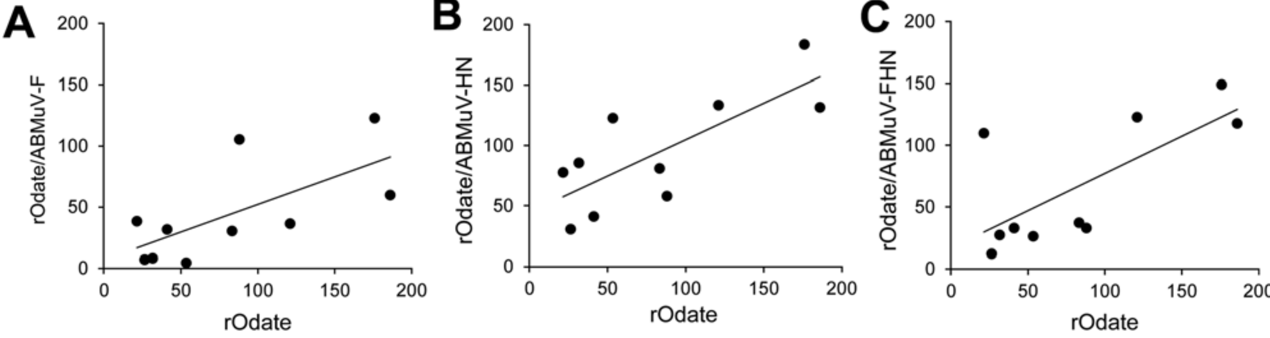

Figure 2. Comparison of the NT titer of rOdate versus rOdate/ABMuV-F (A), -HN (B), and -FHN (C) in a study of serologic cross-reactivities. r and p values, calculated by using the Pearson product-moment correlation, are as follows: (A) r = 0.67, $p<0.05$; (B) r = 0.77, $p<0.01$; (C) r = 0.71, $p<0.05$. ABMuV, African bat mumps virus; F, fusion; HN, hemagglutinin-neuraminidase; NT, neutralizing.

the characterization and risk assessment of emerging viruses, even when the authentic viruses have not been isolated.

Our data showed extensive cross-neutralization between MuV and ABMuV. Although NT antibodies might play an essential role for MuV protection, a definitive NT titer for MuV protection is still under debate (*12*). Cortese et al. suggested that case-patients generally had lower pre-outbreak mumps antibody levels than non–case-patients; however, no cutoff NT titer was defined in their study (*12*). Our findings suggest that antibodies induced by either mumps vaccines or infection with wild-type MuV generally neutralize ABMuV efficiently. Because cell-mediated immunity might also contribute to MuV protection (*13*), further investigations are needed to clarify the definitive parameters of MuV and ABMuV protection. Nonetheless, our data demonstrate that the current MuV vaccination program reduces the risk for an emerging infection of ABMuV in humans.

Acknowledgments

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Definitive Hosts of *Versteria* Tapeworms (Cestoda: Taeniidae) Causing Fatal Infection in North America

Laura M. Lee, Roberta S. Wallace, Victoria L. Clyde, Annette Gendron-Fitzpatrick, Samuel D. Sibley, Margot Stuchin, Michael Lauck, David H. O'Connor, Minoru Nakao, Antti Lavikainen, Eric P. Hoberg, Tony L. Goldberg

We previously reported fatal infection of a captive Bornean orangutan with metacestodes of a novel taeniid tapeworm, *Versteria* sp. New data implicate mustelids as definitive hosts of these tapeworms in North America. At least 2 parasite genetic lineages circulate in North America, representing separate introductions from Eurasia.

Taeniid tapeworms (Cestoda: Taeniidae) comprise 4 proposed genera: *Taenia*, *Echinococcus*, *Hydatigera*, and *Versteria* (*1*). Until recently, genetic data were absent for *Versteria* sp. in North America. However, in 2014, we reported an unusual case of fatal metacestode (larval stage of tapeworm) infection in a captive Bornean orangutan (*Pongo pygmaeus*); the causative agent was identified as a novel *Versteria* genotype (*2*).

As previously described (*2*), the orangutan was born at a zoo in Colorado, USA, and was rejected by his birth mother. Approximately 10 months later, he was transported to the Milwaukee County Zoo in Milwaukee, Wisconsin, USA, for adoption by a surrogate mother. At ≈5 years of age, he died unexpectedly from acute respiratory distress due to disseminated infection with an unknown agent. A combination of metagenomics and gene-specific DNA sequencing revealed the etiologic agent to be a previously unknown *Versteria* lineage in its larval form.

We obtained wild mustelids (carnivores of the family Mustelidae) from Colorado, near where the animal was born, and Wisconsin, near where the animal died. We targeted mustelids because they are definitive hosts of *V. mustelae* tapeworms in Europe (*3*) and *V. brachyacantha* tapeworms in Africa (*4*). Colorado samples were submissions to the Denver Museum of Nature and Science, and Wisconsin samples were obtained from a local fur trapper. We also examined mustelids from Oregon, USA, as part of an ongoing investigation of *Versteria* spp. tapeworms in the Nearctic region and Eurasia.

From 4 mustelids from Colorado (1 otter [*Lontra canadensis*], 2 ermine [*Mustela erminea*], and 1 mink [*Neovison vison*]), we recovered 1 adult tapeworm from a female ermine collected 178 km from where the orangutan was born. From 17 mustelids from Wisconsin (1 mink, 5 long-tailed weasels [*M. frenata*], and 11 ermine), we recovered 1 adult tapeworm from a male ermine collected 56 km from where the orangutan died. From 17 mustelids from Oregon (1 mink, 1 ermine, and 15 long-tailed weasels), we recovered 1 adult tapeworm from an adult mink of unknown sex. All tapeworm specimens were fragmented, lacking intact scolices, or both, which prevented complete morphologic description; however, microscope examination of mature segments from the Wisconsin and Oregon specimens revealed structures consistent with those of parasites in the genus *Versteria* (Figure 1).

We sequenced 396 bp of the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene from the 3 new adult tapeworm specimens according to previously published methods (*2*). The sequences of the tapeworms from the Colorado ermine and the Oregon mink were 99.5% and 99.2% similar, respectively, to the sequence from the orangutan, placing these new specimens confidently within the same *Versteria* lineage (Figure 2). By contrast, the sequence from the tapeworm from the Wisconsin ermine was only 90.7% similar to the sequence from the orangutan, making it a heretofore unrecognized lineage that clusters more closely with parasites from Eurasia (Figure 2).

To investigate the hosts from which adult tapeworms were recovered, we amplified and sequenced 751 bp of the mustelid cytochrome b (*cytb*) gene, using DNA extracts from adult tapeworm material. To do this, we used previously published methods (*7*,*8*) with modified PCR primers MVZ45_must_F (5′-CAGTNATAGCAACAGCATTCATAGG-3′) and MVZ14_must_R (5′-GCTCTCCATTTTTGGTTTACAAGAC-3′). This effort was successful, demonstrating that adult tapeworm material

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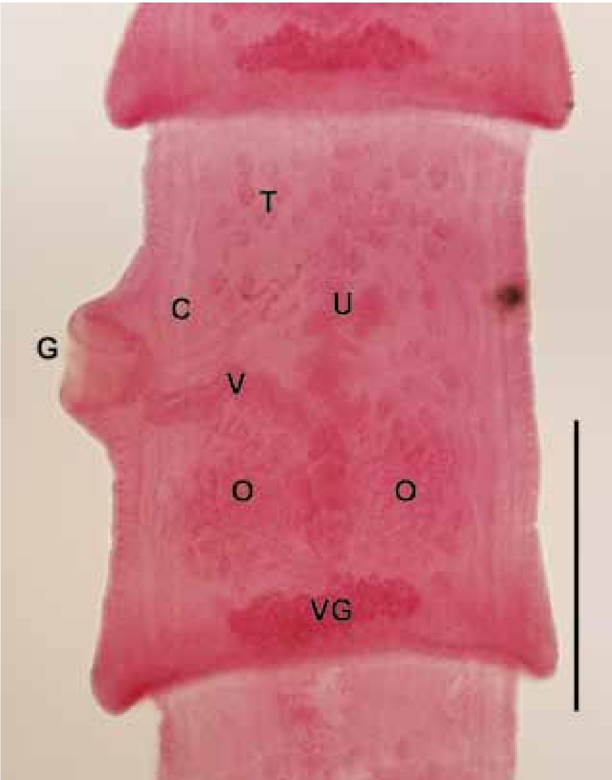

Figure 1. Microscope image of a mature segment of an adult *Versteria* sp. tapeworm recovered from an ermine in Wisconsin, USA (original magnification ×10). Characteristic reproductive structures are visible, including genital pore (G), cirrus sac (C), vagina (V), ovary (O), testes (T), uterine stem (U), and vitelline gland (VG). Tapeworm specimens were preserved in 70% ethanol for concurrent morphologic and molecular analyses. A series of proglottids was subsampled from each worm as a basis for sequencing; remaining strobila was stained, cleared and mounted in Canada balsam as permanent vouchers based on standard methods (*5*). Specimens are deposited in the Museum of Southwestern Biology, Parasitology Division, University of New Mexico, Albuquerque, New Mexico, USA (accession no. MSB 23169), and in the collections of the Denver Museum of Nature and Science, Denver, Colorado, USA (accession no. DZTM.3170). Scale bar indicates 500 μm.

contains sufficient host-associated DNA for such analyses. The resulting sequences indicated that the ermine from Colorado and Wisconsin (GenBank accession nos. KT223030 and KT223032, respectively) belonged to the continental clade, which is broadly distributed across the Nearctic region (*9*). The sequence for the Oregon mink (GenBank accession no. KT223031) closely matched published sequences for other mink from North America but was otherwise phylogeographically uninformative.

We next assessed whether parasites of the genus *Versteria* were present in wild carnivores on and near the grounds of the Milwaukee County Zoo. We conducted systematic searches of the zoo grounds (i.e., outside of enclosures) for carnivore feces. We also trapped carnivores, using live traps baited with fish, and then immediately released them, collecting any feces deposited in the trap, and we collected feces from mink from a local wildlife rehabilitation center. These efforts yielded 51 samples (3 from skunks [*Mephitis mephitis*], 3 from coyotes [*Canis latrans*], 6 from long-tailed weasels, 8 from mink, and 31 from raccoons [*Procyon lotor*]), which we examined by microscope, using standard flotation and sedimentation methods to identify parasite eggs (Table 1). We then used the ZR Fecal DNA MiniPrep kit (Zymo Research, Irvine, CA, USA) to extract DNA directly from feces samples and amplified and sequenced the *cox1* gene (*2*). Resulting sequences were unambiguous and revealed a diversity of parasites (not only taeniids, and in some cases representing putative novel lineages), but none matched *Versteria* spp. (Table 2).

Our results demonstrate that the genus *Versteria* is a species complex in North America. The new lineage identified in Wisconsin clusters with parasites formerly known only from Eurasia (Figure 2). Preliminary assessments suggest that this same lineage infects ermine as far away as the Northwest Territories (NWT) of Canada (E.P. Hoberg et al., unpub. data) and that the lineage responsible for fatal infection of an orangutan (*2*) also infects muskrats (*Ondatra zibethicus*) in Idaho, USA, and the NWT (A. Lavikainen et al., unpub. data). To the extent that current sampling reflects the distribution of *Versteria* lineages in North America, there appears to be a western lineage (represented by Colorado, Oregon, and Idaho, and the NWT) and a northern continental lineage (represented by Wisconsin and the NWT), with sympatry in the NWT. We note that this pattern parallels the phylogeography of ermine in North America, perhaps reflecting postglacial expansion of host and parasite after the Pleistocene (*9*). More *Versteria* lineages will likely be found, as evidenced by a novel lineage in zokor (*Eospalax baileyi*, a fossorial rodent) recently described from Tibet (*10*) (Figure 2). *Versteria* lineages have clearly entered the Nearctic region from the Palearctic region at least twice, probably reflecting Beringian biogeographic processes of faunal expansion that are important drivers of the evolution of mammals and the organisms parasitizing them (*11*).

Our findings shed light on the origins of the infection that proved fatal to the orangutan. We found an adult *Versteria* sp. tapeworm with a nearly identical DNA sequence in Colorado, where the orangutan was born; however, a *Versteria* sp. tapeworm from Wisconsin, where the orangutan died, was genetically divergent. Moreover, we found no evidence of *Versteria* tapeworms on the grounds of the Milwaukee County Zoo or nearby. Taeniid metacestodes encyst and can remain dormant for years before asexual multiplication (*12*). We therefore suspect that the orangutan became infected where it was born (Colorado) and

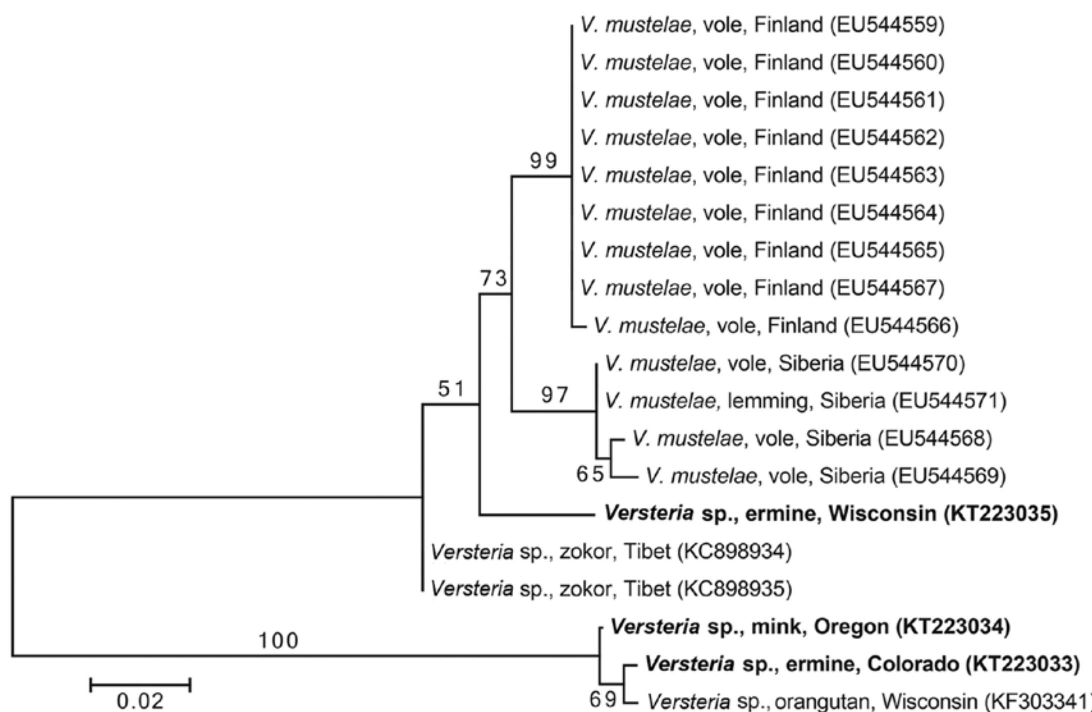

Figure 2. Phylogenetic tree of members of the genus *Versteria* (Cestoda: Taeniidae). The tree was constructed from a DNA sequence alignment of cytochrome c oxidase subunit 1 genes. The maximum-likelihood method was used, with the likeliest model of molecular evolution (Hasegawa-Kishino-Yano model with invariable positions), which was chosen by using MEGA6 (*6*). Numbers next to branches indicate bootstrap values (%), estimated from 1,000 resamplings of the data, and the tree is rooted at the midpoint of the longest branch. Taxon labels indicate parasite species, intermediate or definitive host, and geographic origin (GenBank accession nos. in parentheses). Bold indicates sequences from this study (from adult parasites and definitive hosts). Scale bar indicates nucleotide substitutions per site

carried the latent infection to where it died ≈4 years later (Wisconsin). This animal's sudden progression to disease remains a mystery, perhaps indicating immune deficiency or another precipitating factor, consistent with reports of disseminated taeniid infection in other hosts (*13*).

In general, our findings underscore that exotic animals in zoo settings are susceptible to infections harbored by local wildlife and that transport of such animals can complicate inferences about the origins of these locally acquired infections. We reiterate that taeniid tapeworms of the genus *Versteria* should be considered a threat to captive apes (*2*), and we recommend that wild mustelids, such as ermine and mink, be excluded or removed from the grounds of zoos where apes have access to outdoor environments. Given the close relationship between apes and humans, we also suggest increased vigilance for zoonotic infections.

Acknowledgments

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Table 1. Parasites in wild carnivore feces samples collected on the grounds of and near the Milwaukee County Zoo, Milwaukee, Wisconsin, USA, 2014*

	Host, % positive				
Parasite	Coyote, n = 3	Long-tailed weasel, n = 6	Mink, n = 8	Raccoon, n = 31	Skunk, n = 3
Ascarid	–	–	–	12.9	–
Baylisascaris procyonis	–	–	–	35.5	–
Cestode	33.3	50.0	16.7	25.8	66.7
Coccidia	66.7	33.3	–	22.6	33.3
Cystoisospora spp.	–	–	–	3.2	–
Giardia spp.	–	–	16.7	25.8	–
Hookworm	–	–	–	12.9	33.3
Strongylid	–	16.7	33.3	12.9	33.3
Trichuris spp.	–	–	33.3	22.6	–
Other†	66.7	33.3	50.0	3.2	–

*–, not present.
†Other parasites were unidentified metastrongyles, nematodes, and protozoans.

Table 2. Parasites identified by DNA sequencing of the *cox1* gene in samples of wild carnivore feces collected on the grounds of and near the Milwaukee County Zoo, Milwaukee, Wisconsin, USA, 2014*

Host	GenBank accession no.†	Most similar to	% Similarity‡
Mink	KT223036	*Alaria alata*	91.2 (HM022221)
Raccoon	KT223037	*Baylisascaris procyonis*	100.0 (KC172104)
Long-tailed weasel	KT223038	*Parafilaroides normani*	89.6 (KJ801815)
Skunk	KT223039	*Taenia crassiceps*	86.9 (EU544549)
Mink	KT223040	*Toxascaris leonina*	93.1 (JF780951)

*Only samples with parasite eggs resembling those of cestodes were tested; mink samples were from a local wildlife rehabilitation center. Sequences matching noncestode parasites indicate nonspecificity of PCR primers; results do not exclude the possibility of mixed infections. *cox1*, cytochrome c oxidase subunit 1.
†For parasite sequences newly generated during this study.
‡Nucleotide similarity to the most similar sequence in GenBank (accession no. in parentheses) as of June 26, 2015.

and the Denver Museum of Nature and Science for access to mustelid specimens from Colorado, Katie Moriarty and Nicole Edmison for access to mustelid specimens from Oregon, Slade Noreen and Jorden Manasse for assistance with mustelid specimens from Wisconsin, and Daron Graves and Patricia Khan for assistance with obtaining samples from the grounds of the Milwaukee County Zoo.

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Ms. Lee is a veterinary student who participated in this research as part of the University of Wisconsin-Madison School of Veterinary Medicine's Summer Scholars Program. Her interests include wildlife medicine, small animal medicine, and infectious disease.

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Effectiveness of a Mobile Short-Message-Service–Based Disease Outbreak Alert System in Kenya

Mitsuru Toda, Ian Njeru, Dejan Zurovac, Shikanga O-Tipo, David Kareko, Matilu Mwau, Kouichi Morita

We conducted a randomized, controlled trial to test the effectiveness of a text-messaging system used for notification of disease outbreaks in Kenya. Health facilities that used the system had more timely notifications than those that did not (19.2% vs. 2.6%), indicating that technology can enhance disease surveillance in resource-limited settings.

Outbreaks of epidemic diseases pose serious public health risks (*1*). Kenya, like other Africa countries, lacks the means to deliver adequate healthcare services. This weakness compromises the success of the World Health Organization's Integrated Disease Surveillance and Response (IDSR) and International Health Regulations (IHR) strategies and often results in incomplete, delayed, and poor-quality (i.e., not following standard case definitions in the IDSR guidelines) paper-based reporting from health facilities in remote areas. Furthermore, inadequate reporting limits health managers' ability to take appropriate and timely action in response to health events (*2,3*).

Widespread expansion of mobile phone coverage in Africa (*4*) offers opportunities to overcome weaknesses in health systems and to improve medical and public health practice through mobile health (mHealth) (*5*). Despite many mHealth projects undertaken in Africa, their effectiveness has rarely been rigorously evaluated, limiting evidence-based policy adoptions or project expansion in scope or geography (*6–9*). In particular, evidence of effectiveness of mHealth interventions for enhancing disease surveillance is scarce (*10*). We undertook a clustered, randomized, controlled trial with 135 health facilities in Busia and Kajiado Counties in Kenya during November 2013–April 2014 to test the effectiveness of a mobile short-message-service (SMS)–based disease outbreak alert system (mSOS) for reporting immediately notifiable diseases.

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The Study

mSOS is a formatted text-messaging system that enables communications between healthcare facility workers and Ministry of Health managers and uses a Web-based portal to monitor disease notifications and response actions taken by health managers (Figure 1; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/4/15-1459-Techapp1.pdf). In our trial, health workers used mSOS for 6 months to send information about suspected cases or health events that required notification within 24 hours. Twelve diseases and conditions were selected for the study (online Technical Appendix Table 1). Before mSOS was implemented, we conducted a 1-day refresher training course on IDSR for in-charges (i.e., medical officers in charge) of 135 participating health facilities; the training focused on case definitions of notifiable diseases and on paper-based reporting. During the training, facilities were randomized into intervention and control groups; the intervention group received an additional day of training on mSOS. Paper-based reporting continued throughout the study period for both groups, so the intervention group would report cases 2 ways.

Our primary outcome was determining how many of the cases that required immediate notification were reported within the time specified. Our secondary outcome was determining, from among the cases for which notifications were sent, the proportion for which response actions were taken. For evaluation purposes, data from health facilities were collected for 6-month periods before and after the intervention launch (i.e., IDSR and mSOS training and use of mSOS for 6 months). Cases detected, notifications submitted, and responses undertaken were extracted from facility records in both study groups. Notifications sent by SMS were retrieved from the mSOS system. Our primary analysis was intention-to-treat (i.e., analysis of cases from all health facilities as they were randomized, regardless of intervention exposure). Our secondary analysis was per-protocol (i.e., our trial protocol) and was restricted to cases reported by facilities whose in-charges had received training (i.e., IDSR training for control group; IDSR and mSOS training for intervention group; Figure 2).

Characteristics of health facilities and in-charges were similar; data from preintervention and postintervention

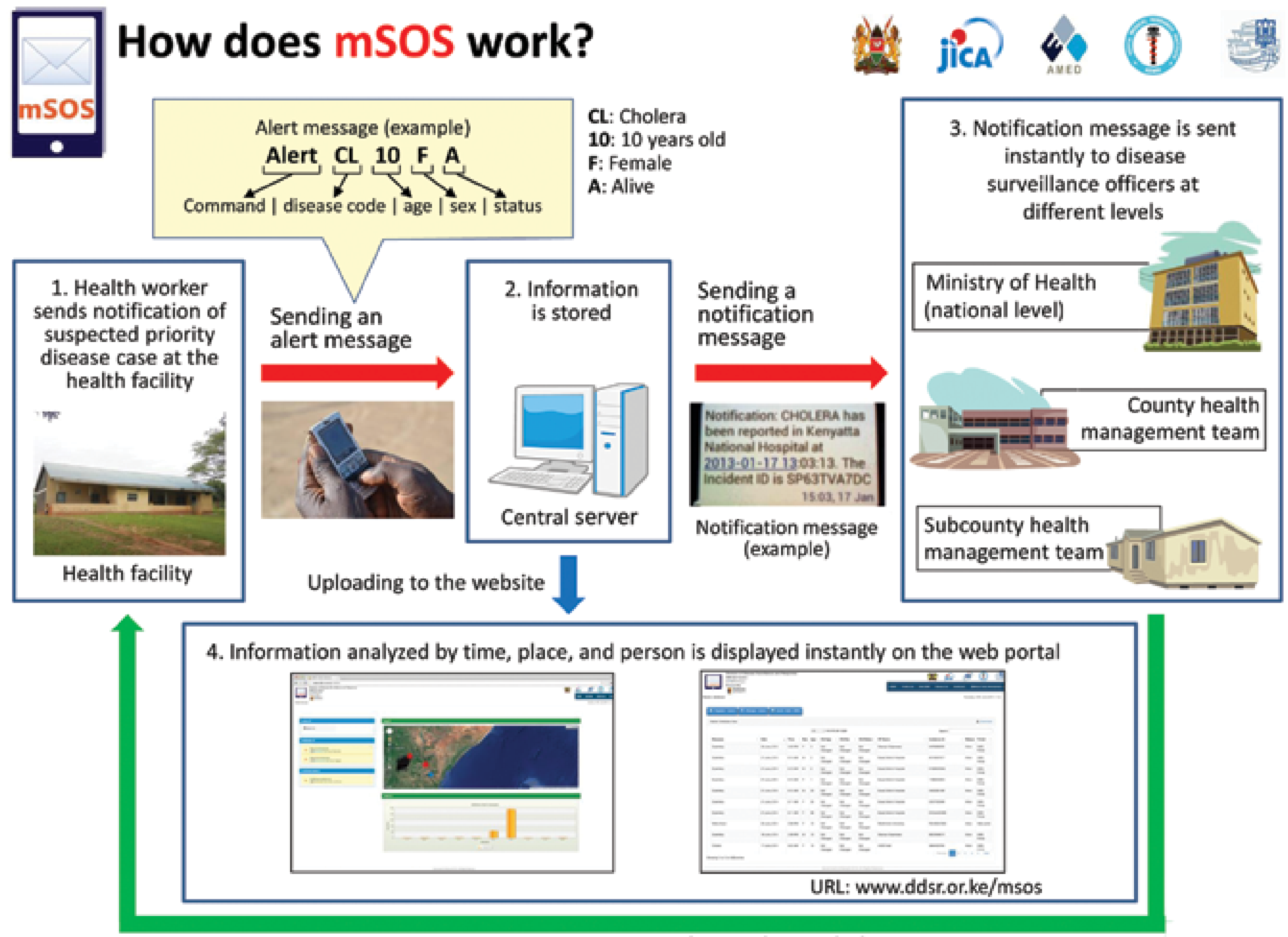

Figure 1. Structure and communication flow of a mobile short-message-service–based disease outbreak alert system (mSOS) in Kenya. Source: mSOS Technical Working Group, Ministry of Health Kenya.

surveys showed no significant differences between control and intervention groups (Table 1). Follow-up surveys conducted 6 months after the intervention showed that 34 (51.6%) of 66 intervention group in-charges received mSOS and IDSR training and 32 (49.2%) of 65 control group in-charges received IDSR training (Figure 2; online Technical Appendix).

A retrospective review of the baseline (preintervention) surveys showed that 36 cases (19 for intervention group, 17 for control group), all measles, required immediate notification. Of these 36 cases, only 1 immediately notifiable case was reported (from a control facility using paper forms). During the 6-month period after the intervention, 169 immediately notifiable cases (130 for the intervention group, 39 for the control group) were detected: 160 measles, 6 anthrax, 2 Q fever, and 1 guinea worm. Of the 39 cases detected in the control group, notification of only 1 case (2.6%), which was measles, was sent. Of the 130 immediately notifiable cases detected in the intervention group, 25 (19.2%) were reported to disease surveillance coordinators at the subcounty, county, and national levels. This proportion of cases reported was significantly higher than that reported by the control group (% difference 16.7, 95% CI 2.71–25.07; Table 2).

All 25 cases for which notifications were sent from the intervention group were measles cases reported through mSOS; 2 cases were also reported with paper forms. For these 25 mSOS notifications, the threshold for a measles outbreak response (5 suspected cases) was met once, and disease surveillance coordinators at the subcounty level responded to this event. Furthermore, 24 (96%) of the 25 suspected measles cases were reported within 24 hours.

In the per-protocol analysis, the percentage of cases for which notification was sent was greater in the intervention group than in the control group (27.3% vs. 4.8%), but the difference was of borderline statistical significance (% difference 22.5, 95% CI –0.32 to 34.13 by Wilson procedure with continuity correction [*11*]). Similar differences were found when the analysis was restricted to health

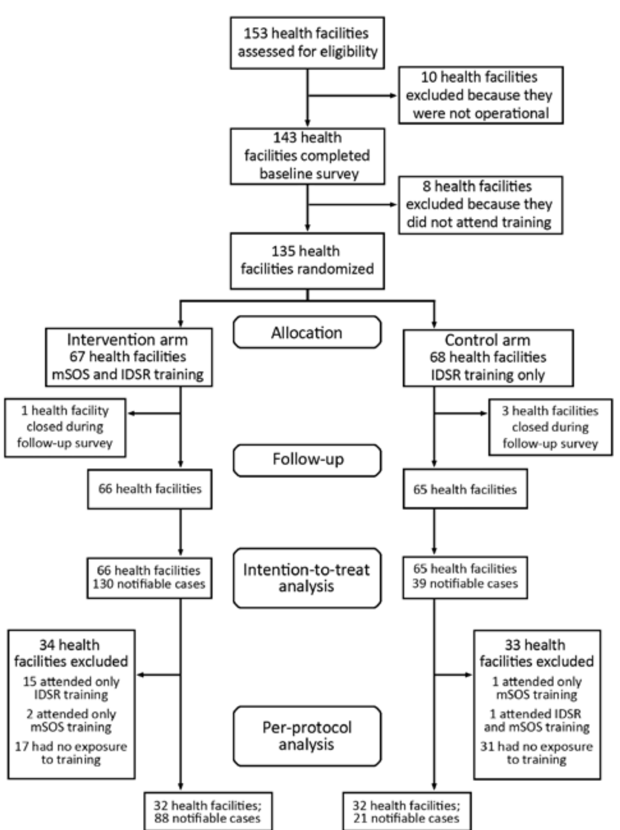

Figure 2. Profile of control and intervention health facilities and exclusions during the course of a study of a mobile short-message-service–based disease outbreak alert system (mSOS) in Kenya. IDSR, Integrated Disease Surveillance and Response.

facilities that stocked paper-based tools (i.e., control group, 1/18 [5.6%] vs. intervention group, 22/78 [22.6%]; % difference 17.0, 95% CI -2.93 to 35.30).

Conclusions

This study showed that SMS intervention significantly increased timely notifications; however, despite a relatively large improvement, response remained suboptimal, with timely notifications of only one fifth of detected cases. These findings mirror results of a study in Tanzania, which showed that SMS considerably increased vital registration coverage but fell far short of reporting actual birth and death events in the community (*12*).

Our study has implications for health managers who implement interventions to improve disease surveillance in resource-limited settings. First, the number of detected cases requiring immediate notification increased postintervention. This effect was observed in both intervention and control groups but was higher in the group using SMS; this group had a 7-fold increase in detected cases compared with baseline findings. IDSR refresher training may have contributed to increased case detection, and the combined interventions, including the technology component, resulted in a greater detection effect. Second, expecting health workers to complete paper-based forms and deliver them without incentive within 24 hours is ineffective for ensuring notification of cases, with or without exposure to the refresher training. Third, we observed a large drop-out rate (47.4%) for health facility in-charges participating in the study. The study took place during a period of health management decentralization in Kenya, resulting in 47 new counties and in health worker transfers. Lack of on-the-job training for staff who did not attend the training and lack of support through posttraining follow-up and supportive supervision were weaknesses in the intervention. These systemic challenges, reported in other IDSR (*13*) and mHealth surveillance (*14*) projects, must be addressed to avoid compromising the sustainability of such interventions. Finally, attrition of health workers exposed to the intervention and lack of paper-based tools explain only part of our results. The short duration of the training deployed (*15*) and the possibly suboptimal quality of the training delivered (*3*) may have contributed to the unrealized full potential of the intervention.

Despite its limitations (online Technical Appendix), this study shows how technology in the form of mSOS can increase the rate of notifications of suspected disease outbreaks and enhance IHR compliance in resource-limited settings. Further investigation into ways to optimize the quality of delivery of mSOS interventions in countries with weak healthcare systems is justified.

Acknowledgments

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Table 1. Characteristics of health facilities and their in-charges for intervention and control groups and study periods, Kajiado County, Kenya*

Characteristic	Preintervention, no. (%)		Postintervention, no. (%)		
	Control, N = 65	Intervention, n = 66	Control, n = 65	Intervention, n = 66	p value†
Health facilities, Kajiado County	42 (64.6)	41 (62.1)	42 (64.6)	41 (62.1)	0.767
Ownership					
Public	39 (60.0)	45 (68.2)	39 (60.0)	45 (68.2)	0.329
Private	15 (23.1)	13 (19.7)	15 (23.1)	13 (19.7)	0.637
FBO/NGO	11 (16.9)	8 (12.1)	11 (16.9)	8 (12.1)	0.435
Level of care					
Hospital/health center	20 (30.8)	19 (28.8)	20 (30.8)	19 (28.8)	0.804
Dispensary	40 (61.54)	43 (65.15)	40 (61.5)	43 (65.2)	0.668
Other facility	5 (7.7)	4 (6.1)	5 (7.7)	4 (6.1)	0.712
Resource availability					
Mobile phone	65 (100)	66 (100)	65 (100)	66 (100)	–
Electricity	45 (69.2)	47 (71.2)	54 (83.1)	49 (74.2)	0.217
Water	54 (83.1)	47 (71.2)	51 (78.5)	50 (75.8)	0.713
Surveillance focal person	48 (73.9)	44 (67.7)	44 (67.7)	47 (71.2)	0.662
IDSR reporting tool‡	22 (33.9)	23 (34.9)	34 (52.3)	32 (48.5)	0.662
IDSR job aid	44 (67.7)	44 (66.7)	49 (75.4)	55 (83.3)	0.261
Characteristic of in-charge					
Female sex	32 (49.2)	39 (59.1)	32 (49.2)	39 (59.1)	0.257
Median age, y (IQR)§	34 (29–48)	35 (30–42)	36 (30–49.5)	37 (30–44)	0.677
Doctor/clinical officer	12 (18.5)	15 (22.7)	16 (24.6)	13 (19.7)	0.498
Nurse	46 (70.8)	48 (72.7)	44 (67.7)	48 (72.7)	0.529
Other healthcare worker	7 (10.8)	3 (4.6)	5 (7.7)	5 (7.6)	0.980

*The table does not show data for Busia County because values will be inverse of data for Kajiado County (i.e., N minus n). N = total facilities in both counties. The intervention group is the group of facility in-charges who were exposed to IDSR and mSOS training and to the mSOS intervention; the control group is the group of in-charges who were exposed to IDSR training only. FBO, faith-based organization; IDSR, Integrated Disease Surveillance and Response; in-charge, medical officer in charge of facility; IQR, interquartile range; NGO, nongovernment organization.
†χ^2 test was used to compare the proportions between control and intervention groups. Wilcoxon Mann Whitney test was used to compare medians between control and intervention groups (i.e., age of in-charges). Analyses were conducted by using an α level of 0.05. The p value is shown for the postintervention period only.
‡Standardized IDSR paper-based reporting form for immediately notifiable diseases.
§Data are median and Interquartile range rather than numbers and percentages. Denominator excludes 3 facilities with missing values in the preintervention control group and 1 facility with missing values for each of the remaining 3 study groups.

Table 2. Postintervention reporting of immediately notifiable cases by study group under the intention-to-treat and per-protocol analysis*

Type of analysis	Control		Intervention		% Difference (95% CI)
	Total	Cases notified, no. (%)	Total	Cases notified, no. (%)	
Intention to treat	39	1 (2.6)	130	25 (19.2)	+16.7 (2.71–25.07)
Per protocol	21	1 (4.8)	88	24 (27.3)	+22.5 (–0.32 to 34.13)

*Intention-to-treat analysis indicates analysis of treatment groups as they were randomized, regardless of the intervention exposure; per-protocol analysis indicates restricted analysis of groups that completed the entire study according to the trial protocol.

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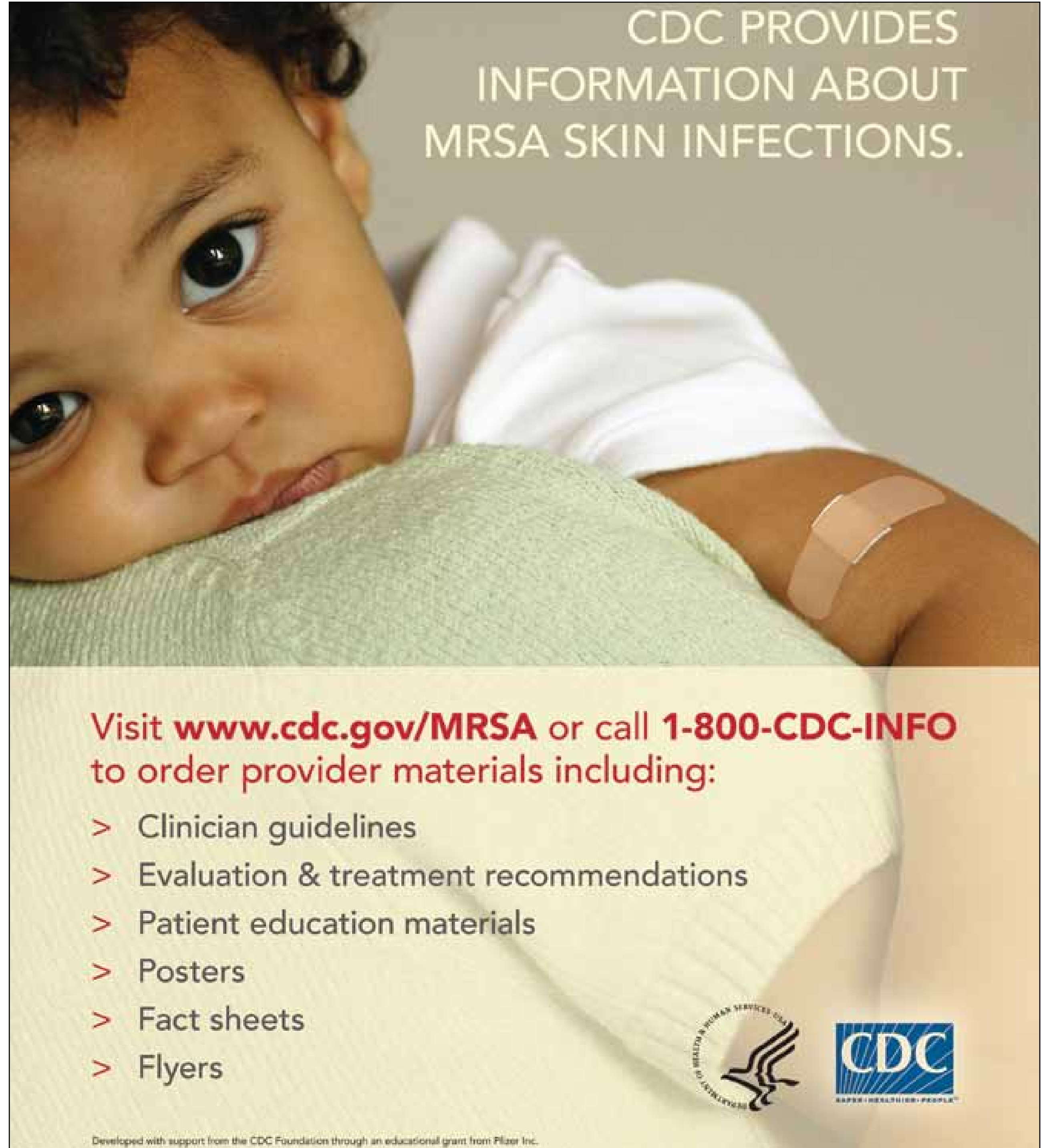

Deletion Variants of Middle East Respiratory Syndrome Coronavirus from Humans, Jordan, 2015

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We characterized Middle East respiratory syndrome coronaviruses from a hospital outbreak in Jordan in 2015. The viruses from Jordan were highly similar to isolates from Riyadh, Saudi Arabia, except for deletions in open reading frames 4a and 3. Transmissibility and pathogenicity of this strain remains to be determined.

Middle East respiratory syndrome coronavirus (MERS-CoV) was first recognized in 2012 as the cause of severe lower respiratory tract infection in humans (*1*). As of November 13, 2015, a total of 1,618 cases of laboratory-confirmed MERS-CoV infection and 579 associated deaths had been reported to the World Health Organization (*2*). Human-to-human transmission of MERS-CoV can occur in households and hospitals, but thus far, major genomic changes associated with host switching, as have occurred with severe acute respiratory syndrome (SARS) CoV, have not been noted for MERS-CoV (*3*–*9*). We characterized MERS-CoVs from a recent hospital outbreak in Jordan.

The Study

During August and September 2015, an outbreak of MERS-CoV infection occurred in hospitals in Jordan (*10*). MERS-CoV–positive throat swab or bronchiolar lavage samples were obtained from each patient; of these, 13 samples were labeled Jordan-1-2015 through Jordan-13-2015 and shipped on dry ice to Erasmus University Medical Center (Rotterdam, the Netherlands) for testing. Total RNA was isolated from 140 µL of each sample by use of a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) and quantified by TaqMan assay targeting the envelope gene, as described previously (*11*). Full MERS-CoV genome sequencing was performed on the sample with the highest viral load, obtained on August 24, 2015, from a 60-year-old man who died of the disease 3 days later. At the end of July 2015, this man had traveled from Jeddah, Saudi Arabia, to Jordan for his annual vacation. From the throat swab sample from this man, 76,082 sequence reads—of which 851 were specific for MERS-CoV—were obtained by using 454 deep-sequencing (*12*), revealing ≈85% of the MERS-CoV genome with coverage of 1–178 reads at single-nucleotide positions. Missing sequences and low-coverage regions were obtained by conventional Sanger sequencing, except for 6 nt at the 5′ end.

Phylogenetic analysis of the complete viral genome of this sample revealed that this virus, tentatively called Jordan-1-2015, clusters with viruses detected in humans in Riyadh in 2015 (Figure 1). Alignment of the full genomic sequences of this virus with the Riyadh 2015 isolates showed that they were >99.7% similar. Amino acids in the spike protein were the same in Jordan-1-2015 and the consensus sequence of the Riyadh 2015 viruses. Of note, Jordan-1-2015 contained a 48-nt (16 aa) in-frame deletion in open reading frame (ORF) 4a that had not been detected in any other MERS-CoV (Figure 2, panel A). This ORF is present only in lineage 2c betacoronaviruses and encodes protein 4a, which has recently been shown to inhibit type I interferon production, presumably by binding and masking double-stranded RNA from detection by RNA sensors such as RIG-1 (retinoic acid–inducible gene 1)–like helicases and the double-stranded RNA-binding protein PACT (protein activator of the interferon-induced protein kinase) (*13*,*14*). Subsequent sequence analysis showed that all 13 viruses from patients sampled during this outbreak contained the deletion and that the flanking regions were identical, indicating that this deletion mutant was transmitted (Figure 2, panel B).

To further characterize the viruses in the clinical samples, we subjected all samples to titration on Vero cells (American Type Culture Collection no. CCL-81). We observed cytopathic changes in cells inoculated with samples Jordan-1-2015 and Jordan-10-2015 (obtained on September 17 from a 29-year-old man, who survived the illness); however, no cytopathic changes were observed in the cells inoculated with the other samples.

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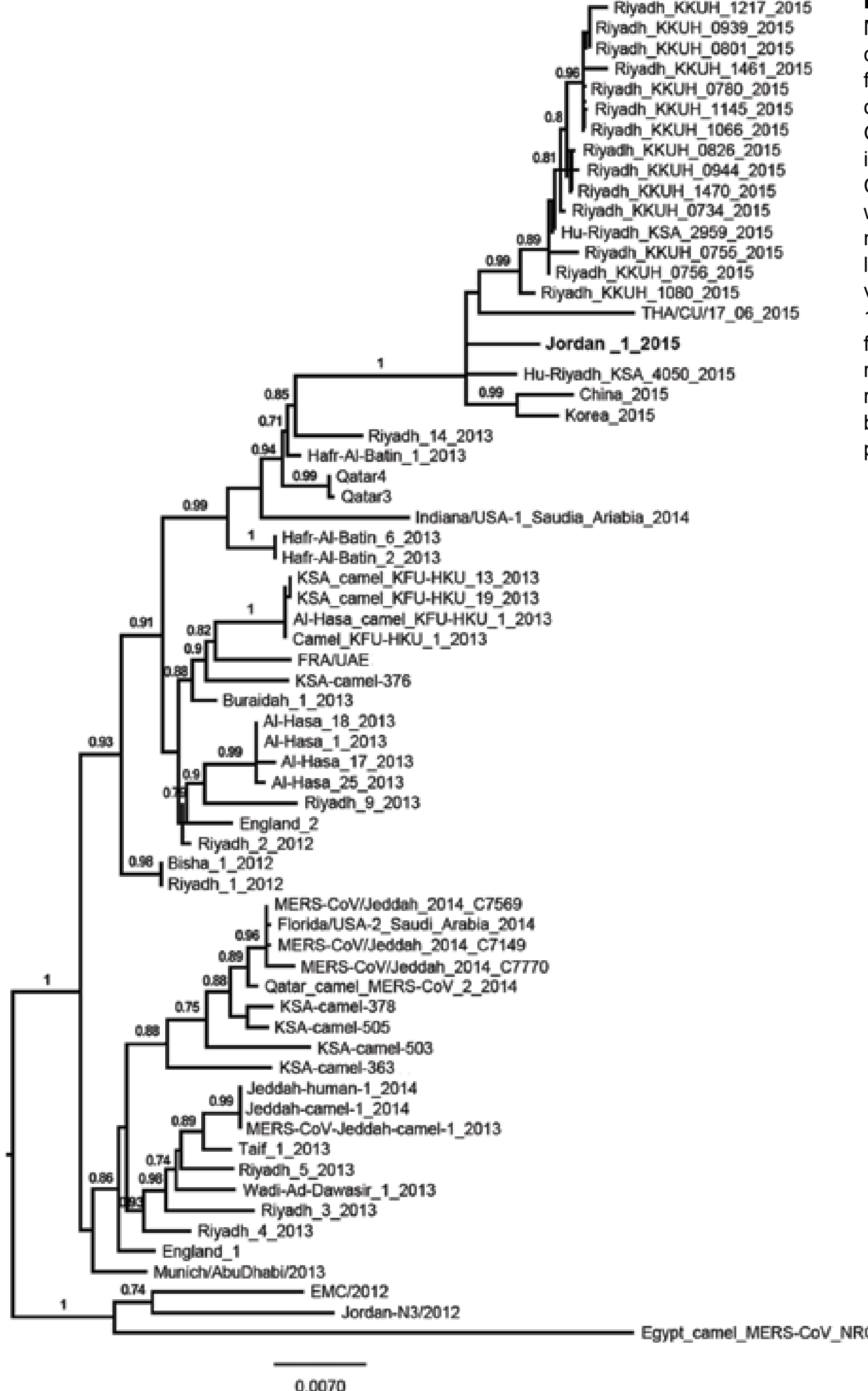

Figure 1. Phylogenetic analysis of Middle East respiratory syndrome coronavirus (MERS-CoV) isolated from Jordan (Jordan-1-2015; boldface) compared with reference strains. Genome sequences of representative isolates were aligned by using ClustalW, and a phylogenetic tree was constructed by using the PhyML method in Seaview 4 (http://pbil.univ-lyon1.fr/software/seaview); the tree was visualized by using FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree). Values at branches show the result of the approximate likelihood ratio; values <0.70 are not shown. Scale bar indicates nucleotide substitutions per site.

Subsequently, we obtained complete genomes by deep sequencing of the second-passage viruses, with high coverage of reads at single-nucleotide positions. No cell culture adaptive mutations in the genome sequence were observed for the virus isolated from clinical sample Jordan-1-2015. Pairwise comparison of Jordan-1-2015 with Jordan-10-2015 revealed that both viruses were highly similar (99.99% nt identity) and confirmed that both carried the 48-nt deletion in ORF4a. However, in addition to this deletion, a 9-nt (3-aa) in-frame deletion was observed in the genome of Jordan-10-2015. This deletion was located in ORF3, which encodes a protein with an unknown function. The presence of this deletion in the virus from clinical sample Jordan-10-2015 was

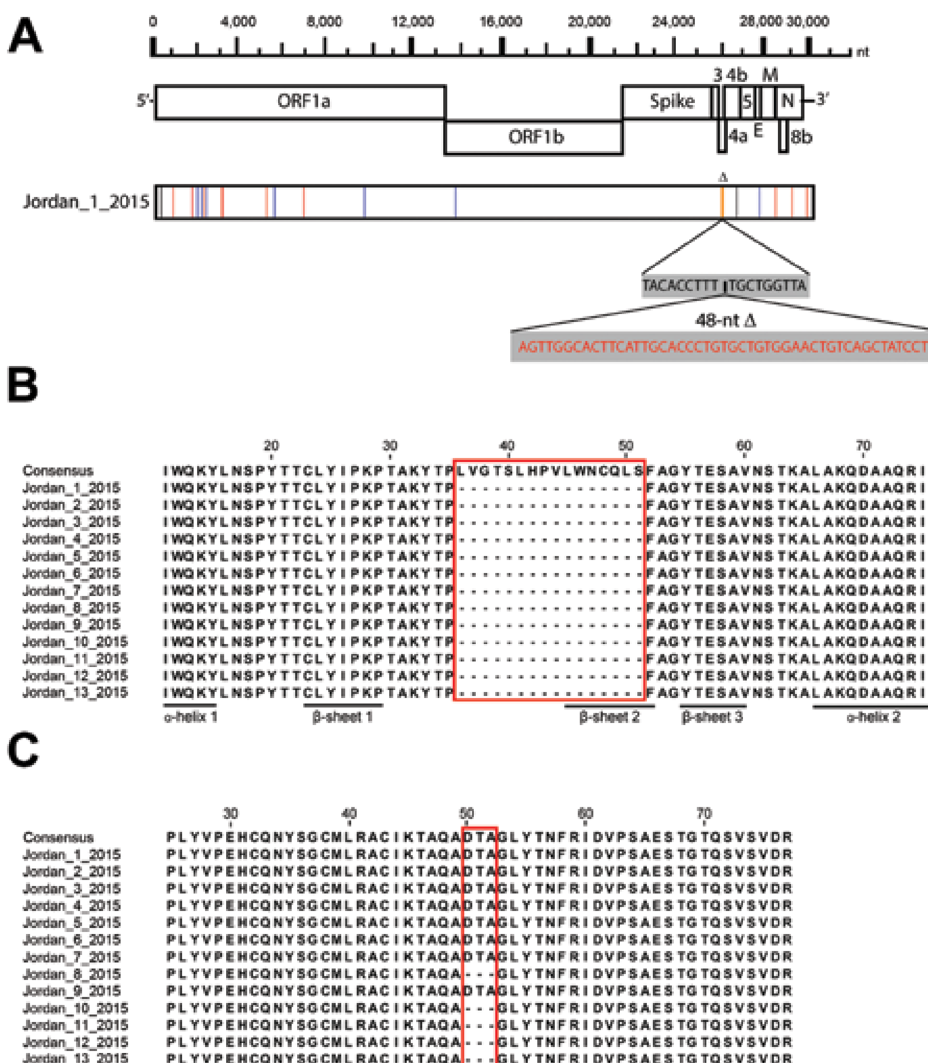

Figure 2. Genomic characterization of Middle East respiratory syndrome coronaviruses (MERS-CoVs) from Jordan. A) Nucleotide differences between MERS-CoV strain Jordan-1-2015 and the consensus sequence of the Riyadh 2015 cluster. Nucleotide changes are indicated by vertical lines in bar. B) Protein sequence alignment of open reading frame (ORF) 4a (residues 10–76) of all 13 MERS-CoV strains from Jordan in 2015 compared with the consensus sequence of the Riyadh 2015 cluster. No nucleotide substitutions were observed in this region between MERS-CoVs from Jordan. Predicted secondary structures are indicated (13). Classical double-stranded RNA binding proteins have αβββα architecture. C) Protein sequence alignment of ORF3 (residues 25–77) of all 13 MERS-CoV strains from Jordan in 2015 compared with the consensus sequence of the Riyadh 2015 cluster. No nucleotide substitutions were observed in this region between MERS-CoVs from Jordan. Alignments were generated with the ClustalW program (http://pbil.univ-lyon1.fr/software/seaview) and visualized by using Jalview 2.9 (http://www.jalview.org/). Boxes in panels B and C indicate regions where amino acids have been deleted in the viruses from Jordan.

confirmed by Sanger sequencing, excluding the possibility that it was an adaptation to cell culture. Next, sequencing of ORF3 from all other clinical samples revealed that the ORF3 deletion was present in a subgroup of clinical samples (Figure 2, panel C). These samples had been collected toward the end of the outbreak, suggesting that the ORF3 deletion may be an adaptation resulting from sustained transmission between humans. All sequences obtained in this study were deposited in GenBank (accession nos. KU233362–KU233377). Identical deletions in ORF3 and ORF4a in the same sample set from Jordan were independently confirmed by Erasmus University Medical Center and the Centers for Disease Control and Prevention (Atlanta, GA, USA).

Conclusions

Despite the human-to-human transmission capacity of MERS-CoV and substantial sequencing efforts, major genomic changes associated with human adaptation have not been described (*3–8*). We observed a large-scale deletion in ORF4a and a small deletion in ORF3 in MERS-CoVs that caused the outbreak in Jordan.

One of the most striking genomic changes observed in SARS-CoV isolated from humans soon after its transmission from animals was the acquisition of a 29-nt deletion in ORF8, an accessory protein with an unresolved function (*9*). The SARS-CoV ORF8 deletion disrupted the reading frame of the early-stage human isolates and created 2 new ORFs, designated ORF8a and ORF8b. Although no clear

roles have been designated for both ORFs, viruses containing this deletion predominated later during the epidemic, showing that these viruses were able to spread efficiently from human to human.

The deletion we describe in ORF4a of MERS-CoV does not result in the removal of an entire gene, because the reading frame is not disrupted, suggesting that this mutation is not a loss-of-function mutation. However, whether this ORF4a deletion variant can still bind double-stranded RNA needs to be assessed because the deleted region contains a predicted β-sheet belonging to the classical double-stranded RNA binding αβββα fold of this protein (*13;* Figure 2, panel B). The data from this study indicate that adaptive pressures possibly exerted by the human host may operate on the ORF4a and ORF3 regions. Alternatively, proteins encoded by these ORFs could have functions specific for the camel host, causing them to be redundant in humans and enabling accumulation of mutations that do not affect viral fitness in humans. Future studies of the roles of these proteins are needed.

The deletion located in the type I interferon antagonist protein 4a was detected in all samples collected during the outbreak. Another small-scale deletion was detected in ORF3 in a subset of samples collected later in the outbreak. Overall, the data suggest that MERS-CoV ORF4a and ORF3 can acquire deletions by selection or by chance. Whether these deletions affect the transmissibility or pathogenicity of this particular MERS-CoV strain needs to be addressed. The finding that all viruses analyzed contained the ORF4a deletion suggests that all patients were infected with the same virus. Although unlikely, some MERS patients may have indirectly become infected through independent transmission of viruses in dromedaries carrying highly related viruses, some of which acquired deletions in their genome. Therefore, a more detailed epidemiologic investigation of this outbreak is needed. These data underscore the need for close monitoring of the molecular evolution of MERS-CoV.

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Low-Cost National Media-Based Surveillance System for Public Health Events, Bangladesh

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We assessed a media-based public health surveillance system in Bangladesh during 2010–2011. The system is a highly effective, low-cost, locally appropriate, and sustainable outbreak detection tool that could be used in other low-income, resource-poor settings to meet the capacity for surveillance outlined in the International Health Regulations 2005.

The International Health Regulations (IHR) 2005 state that an effective public health system should conduct surveillance activities to enhance detection, reporting, notification, verification, response, and collaboration in the event of an outbreak (*1*).Traditional approaches to disease surveillance heavily rely on laboratories, points of care, or population-based surveillance for disease reporting. Although these approaches are generally effective, traditional surveillance systems are limited by reporting delays, high cost, and reliance on an overburdened healthcare infrastructure for information collection.

In 2009 the Institute of Epidemiology, Disease Control and Research (IEDCR) of Bangladesh, the national agency responsible for disease surveillance and outbreak investigation, established a surveillance system to monitor print and television media to quickly detect outbreaks. We describe this media-based surveillance system and report results from a formal evaluation of its characteristics.

The Study

IEDCR contracted for a media scanning company to identify relevant news stories for this surveillance system (Figure). Each day, the company collected the major daily newspapers available in the capital city of Dhaka shortly after morning distribution. Trained staff members at the company read through each paper to identify any health-related article and scan them into PDF file format. Other staff members scanned television news reports and recorded relevant video clips.

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DOI: http://dx.doi.org/10.3201/eid2204.150330

The national rapid response team, consisting of key staff members from IEDCR, received a daily email containing all identified health-related newspaper articles and video clips. The team examined each news item and decided whether it warranted an outbreak response on the basis of expert clinical and epidemiologic knowledge; public health importance (e.g., number of cases and deaths reported, severity of symptoms); and verification by local health officials. For the purposes of this analysis, IEDCR retrospectively created a database of reported events sent by the media scanning company, which included the number of reported events, outbreak etiology, news source, and the outcome of each investigation. The outbreaks reported were classified by media type, etiology, and season.

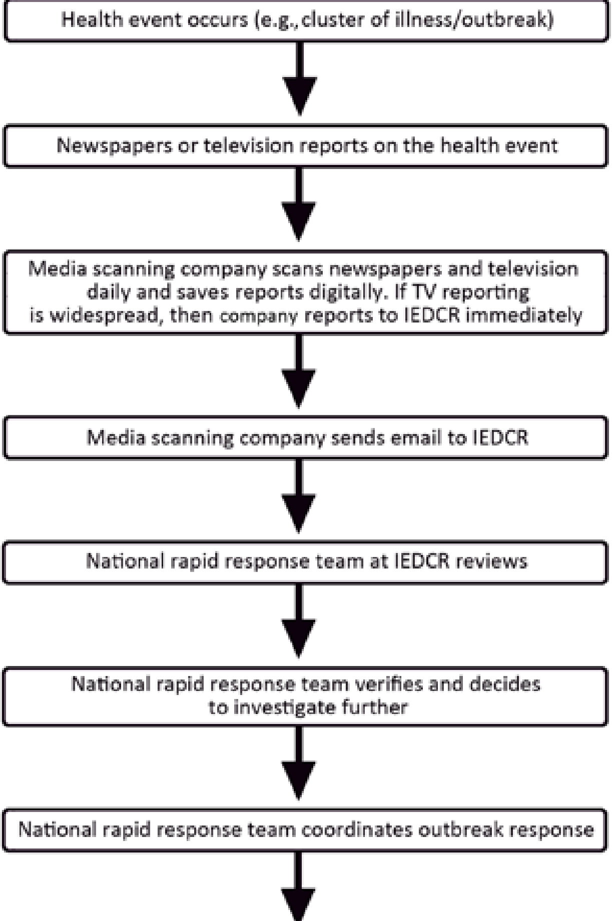

Figure. Information flow for national media-based public health surveillance system, Bangladesh. IEDCR, Institute of Epidemiology, Disease Control and Research.

Table 1. Outbreaks first identified by event-based surveillance and investigated by IEDCR, Bangladesh, May 2010–September 2011*

Outbreak no.	Date	Etiology reported by media	Confirmed etiology
1	2010 May	Anthrax	Cutaneous anthrax
2	2010 Jun	Anthrax	Cutaneous anthrax
3	2010 Jul	Unknown poisoning	Unintentional pesticide poisoning
4	2010 Jul	Mass psychogenic illness	Mass psychogenic illness
5	2010 Jul	Suspected pneumonia	Bronchiolitis
6	2010 Jul	Unknown animal scratch	Rabies
7	2010 Jul	Food poisoning	Food poisoning
8	2010 Jul	Diarrhea	Diarrhea
9	2010 Aug	Cutaneous anthrax	Cutaneous anthrax
10	2010 Nov	Diarrhea	Cholera
11	2010 Nov	Suspected high-energy biscuit poisoning	Mass psychogenic illness after biscuit consumption
12	2010 Dec	Suspected pneumonia	Bronchiolitis
13	2010 Dec	Suspected rabies	Rabies
14	2011 Apr	Diarrhea	Cholera
15	2011 May	Cutaneous anthrax	Cutaneous anthrax (5 outbreaks)
16	2011 Jun	Cutaneous anthrax	Cutaneous anthrax (2 outbreaks)
17†	2011 Jun	Unusual duck and geese mortality†	Avian influenza, subtype H5N1, in geese, but no human cases detected
18	2011 Jul	Cutaneous anthrax	Cutaneous anthrax
19	2011 Jul	Cutaneous anthrax	Cutaneous anthrax
20	2011 Aug	Unknown disease	Influenza B virus infection
21	2011 Aug	Cutaneous anthrax	Cutaneous anthrax

*IEDCR, Institute of Epidemiology, Disease Control and Research, Bangladesh.
†Non–human-related outbreak.

To assess the surveillance system's performance, we followed the guidelines for evaluating public health surveillance set by the US Centers for Disease Control and Prevention (CDC) (*2*).We aimed to assess all 9 recommended system qualities: simplicity, flexibility, data quality, acceptability, sensitivity, positive predictive value (PPV), representativeness, timeliness, and stability (*3*). However, because we did not have external data to serve as a reference standard or comparator, we were unable to assess the sensitivity and PPV of the system. We calculated the proportion of all outbreak investigations conducted by IEDCR that were first detected through the media surveillance. We interviewed key stakeholders, including the manager and staff of the contracted media scanning company, the director of IEDCR, and national rapid response team members. We also conducted a group discussion with 4 journalists representing large national newspapers to explore the processes of obtaining information for health events and timeline of reporting.

A total of 36 news sources were scanned regularly in this media surveillance system: 23 (64%) were Bengali language newspapers, 6 (17%) were English language newspapers, and 7 (19%) were Bengali language television channels. From May 2010 through September 2011, the media scanning company captured and delivered 2,821 news stories to IEDCR. Of those, 2,501 (89%) were health related, 810 items included the term "outbreak" (29% of total), and 196 were the first reports of a possible outbreak (7%); 90% of all outbreak reports came from Bengali language media sources.

During the same period, the national rapid response team investigated 30 outbreaks, 21 (70%) of which were first detected through this surveillance system. At the rate of US$125/year to hire the scanning company, the total cost for the 16-month period was US$167. The cost of contracting with the scanning company for each outbreak detected with this system was therefore approximately US$8 (US$167 divided by 21 outbreaks). Outbreaks of diarrhea, measles, and anthrax were reported through this system (Table 1). Outbreaks were reported year-round and from 51 of the 64 administrative districts in Bangladesh (Table 2).

Key informant interviews consistently indicated that the system was simple, flexible, timely, and acceptable because it used existing media infrastructure and required only minimal costs to contract with a company to compile daily reports of news items. Changes to the system could

Table 2. Case numbers for media-reported outbreaks, by cause and season, Bangladesh, May 2010–September 2011

	No. (%) cases				
Reported etiology	Pre-monsoon, Mar–May	Monsoon, Jun–Sep	Post-monsoon, Oct–Nov	Winter, Dec–Feb	Total
Diarrhea	24 (50)	7 (15)	10 (21)	7 (15)	48 (100)
Anthrax	3 (14)	19 (86)	0	0	22 (100)
Mass psychogenic illness	9 (45)	10 (50)	0	1 (5)	20 (100)
Upper respiratory infection	0	2 (33)	3 (50)	1 (17)	6 (100)
Measles	3 (60)	0	0	2 (40)	5 (100)
Other/unknown	31 (33)	49 (52)	5 (5)	10 (11)	95 (100)
Total	70 (36)	87 (44)	18 (9)	21 (11)	196 (100)

be implemented effectively through frequent communications between the media scanning company and IEDCR. The system was widely acceptable by all stakeholders and was considered a valuable component of disease surveillance in Bangladesh.

We were unable to quantitatively assess the coverage of remote areas by national newspapers, especially those lacking easy access to telecommunication infrastructure. The system moderately captured a representative sample of possible sources of information in the country. Both Bengali- and English-language news items were collected, although only newspapers available in Dhaka were included, so some newspapers with only local circulation were not available for review.

Timeliness and stability of the system were both high. Although time from outbreak onset to reporting in the media source might vary, once media sources learned of an outbreak, publication occurred within 24 hours. Because it was low-cost, low-tech, and highly acceptable to all stakeholders, the system was highly stable.

This media surveillance identified outbreaks of emerging infections that might not have been otherwise investigated, including several outbreaks that were potential public health events of international concern. In the context of global health security, international donors should support media-based surveillance to further strengthen existing traditional indicator-based approaches.

The media-based surveillance system in Bangladesh fills a gap that is not covered by other global event-based surveillance systems, which collect publically available information about potential health threats mostly from Internet sources (*4*), such as ProMED (*5*), BioCaster, and HealthMap (*6*). Although these systems collect and analyze enormous amounts of information from the Internet regarding potential health threats, they are limited by the inability to process information in local, non-English languages or to capture information not on the Internet. In 2013, ≈341 million persons in the world (5%) spoke English as a first language (http://www.nationsonline.org/oneworld/most_spoken_languages.htm); clearly, most news sources in the world are written in languages other than English and, therefore, are beyond the reach of these English language event-based surveillance systems.

Although all stakeholders were knowledgeable about their duties and responsibilities and the procedures which needed to be followed, no written standard operating procedures were in place by which we could evaluate process performance. Written procedures, for both the media scanning company and IEDCR staff, could enhance system performance by fostering sustainability and ensuring standardization.

Our evaluation of this surveillance system was limited in 2 key ways. Because the database for the system was created retrospectively over a short period, some reports may have been missing from the database. In addition, the absence of other data sources meant that we were unable to determine the sensitivity and PPV of the system. However, neither of these limitations influences the high proportion of outbreaks detected through this system nor the low cost per outbreak detected, the most critical findings from our evaluation.

Conclusions

IEDCR in Bangladesh has created an innovative, low-cost, locally appropriate solution for event-based surveillance that helps to meet the purposes of the country's surveillance goals and IHR requirements. This surveillance system could serve as a model for outbreak detection in other resource-poor countries.

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Exportations of Symptomatic Cases of MERS-CoV Infection to Countries outside the Middle East

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In 2012, an outbreak of infection with Middle East respiratory syndrome coronavirus (MERS-CoV), was detected in the Arabian Peninsula. Modeling can produce estimates of the expected annual number of symptomatic cases of MERS-CoV infection exported and the likelihood of exportation from source countries in the Middle East to countries outside the region.

In September 2012, the first confirmed case of Middle East respiratory syndrome coronavirus (MERS-CoV) infection was reported in the Kingdom of Saudi Arabia (KSA) (*1*). Approximately 900 laboratory-confirmed cases had been identified as of January 16, 2015; more than 300 (37%) have resulted in death. Although KSA has reported >80% of cases, infections have been confirmed in other Middle Eastern, European, North American, and, more recently, Asian countries (*2*). Multiple introductions from an animal reservoir have occurred (*3*), but human-to-human transmission of MERS-CoV has also been documented among family members of case-patients and healthcare personnel who cared for case-patients (*4–6*). More than a dozen cases have been identified among travelers returning from the Middle East to their home countries; onward transmission in destination countries also occurred (*7*,*8*).

The Study

We calculated the expected annual number of exportations of symptomatic cases of infection with MERS-CoV and the likelihood of exportation from the Middle Eastern countries where additional cases have been detected (KSA, Jordan, Qatar, and the United Arab Emirates [UAE], which we refer to as source countries) to countries outside the Middle East. We defined exportation as the arrival of a person infected with MERS-CoV in a country other than KSA, Jordan, Qatar, and UAE as a result of MERS-CoV infection in those source countries and subsequent outbound travel (not including medical evacuation). Because it is unclear whether MERS-CoV cases in Middle Eastern countries other than the source countries resulted from importation or a local outbreak, those countries were excluded from the analysis. Exportations can occur by visitors returning to their home countries or source country residents traveling abroad. We produced example calculations for destination countries outside the Middle East where exportation of MERS-CoV infection cases has been confirmed (Algeria, Austria, France, Greece, Italy, Malaysia, Netherlands, Tunisia, United Kingdom, and the United States) as of January 16, 2015 (Table 1, http://wwwnc.cdc.gov/EID/article/22/4/15-0976-T1.htm).

To calculate exportations, we used a simple multiplier model whereby MERS-CoV incidence rates among source country residents were extrapolated to rates among visitors. In particular, for each source country, the expected number of cases among travelers was calculated by multiplying the infection rate among residents in the affected countries (total number of cases divided by number of residents) by the number of travelers and the days travelers spend, on average, in the affected countries (*9*). To calculate incidence rates in destination countries, the number of symptomatic cases was considered to be 10 times greater than the reported number of cases (*8*). Calculations were made by using Excel (Microsoft Corporation, Redmond, Washington, USA). The probability of exportation was calculated while assuming the number of exportations was Poisson distributed, with the mean equal to the expected exportations (online Technical Appendix 1, http://wwwnc.cdc.gov/eid/article/22/4/15-0976-Techapp1.pdf, and online Technical Appendix 2, http://wwwnc.cdc.gov/eid/article/22/4/15-0976-Techapp2.xlsx).

Expected annual exportations of symptomatic MERS-CoV infection cases among visitors to the Middle East source countries were highest among visitors to KSA (Table 2). For visitors returning from KSA, expected exportations were higher among visitors returning to Algeria, where the number of expected exportations was 1 (95% CI 0–5) and the likelihood of ≥1 exportation was 58%; and Malaysia, where the number of expected exportations was 1 (95% CI 0–5) and the likelihood of ≥1 exportation was 47%.

Expected exportations among residents from the Middle East source countries traveling abroad also were estimated to be higher for visitors from KSA (Table 3). Expected exportations among residents in KSA visiting abroad were highest for the United States, where the

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Table 2. Estimated annual number of symptomatic cases of MERS-CoV infection exported and likelihood of ≥1 exportation for countries outside the Middle East among visitors to the source countries*

Country of destination	Observed no. symptomatic cases exported	No. cases exported from source countries (95% CI); % likelihood for ≥1 exportation†		
		Jordan	KSA	UAE
Algeria	2 from KSA	0 (0–4); 0	1 (0–5); 58	NA
Austria	0	0 (0–4); 0	0 (0–4); 1	NA
France	1 from UAE	0 (0–4); 0	0 (0–4); 11	0 (0–4); 9
Italy	1 from Jordan	0 (0–4); 0	0 (0–4); 3	0 (0–4); 9
Greece	0	0 (0–4); 0	0 (0–4); 1	NA
Netherlands	2 from KSA	0 (0–4); 0	0 (0–4); 5	0 (0–4); 4
Malaysia	1 from KSA	0 (0–4); 0	1 (0–5); 47	NA
Tunisia	1 from Qatar	0 (0–4); 0	0 (0–4); 19	NA
United Kingdom	1 from KSA	0 (0–4); 0	0 (0–4); 24	0 (0–4); 35
United States	0	0 (0–4); 1	0 (0–4); 27	0 (0–4); 31

*The incubation period considered was 5.5 days (95% CI 3.6–10.2 days) (*8*). MERS-CoV, Middle Eastern respiratory syndrome coronavirus; KSA, Kingdom of Saudi Arabia; UAE, United Arab Emirates; NA, not available.
†Information on tourists to Qatar was not available.

number of expected exportations was 1 (95% CI 0–5) and the likelihood of ≥1 exportation was 51%.

Conclusions

More complex models have been developed to characterize exportations of infectious disease cases via infectious travelers (*10*,*11*). The main advantage of our approach is its simplicity and reproducibility within short timeframes. This model also complements previous work on the risk for MERS-CoV exportation by global air travel, which attempted to quantify travel volume to the affected areas and calculate exportations among Hajj pilgrims in KSA (*12*,*13*). In our calculations, however, the visitor data included persons traveling by air, land, and sea and distinguished between visitors to the Middle East and persons from the Middle East traveling abroad over the period of 1 year. Unlike Khan et al. (*12*), we accounted for the rate of infection in the source countries by calculating source country–specific MERS-CoV incidence rates. Furthermore, by accounting for the different travel volume of visitors to the Middle East compared with Middle East residents traveling to non–Middle Eastern countries, we suggested the mode of exportation (visitor to the Middle East vs. Middle East resident visiting abroad). For Malaysia, estimated expected exportations were higher among visitors to source countries than among source country residents visiting abroad, consistent with the number of exportations detected by surveillance. However, the opposite was true for the United States, where 2 source country residents were found to have MERS-CoV infection during their US visit (*7*).

Our findings have limitations. The estimates are based on historical incidence data. Thus, they are especially applicable for periods in which the incidence of the disease remains stable. For outbreaks characterized by a highly transmissible pathogen, the model might severely underestimate exportations if incidence rates are not projected to the period for which exportations are calculated. In addition, our small multiplier only took into account exportations of symptomatic cases. Given that most of the detected exportations resulted in hospitalization of the infected case-patient, the number of exportations calculated here likely refers to exportations of severe symptomatic cases. Recent results from a serologic survey performed during December 2012–December 2013 indicate that there might be as many as 44,951 (95% CI 26,971–71,922) persons infected with MERS-CoV in KSA (*14*). During that period, there were 125 reported confirmed cases; thus, the ratio of asymptomatic to reported cases might be much higher, possibly

Table 3. Estimated annual number of symptomatic cases of MERS-CoV infection exported and likelihood of ≥1 exportation for countries outside the Middle East among source country residents traveling outside the Middle East*

Country of destination	Observed no. symptomatic cases exported	No. cases exported from source countries (95% CI); % likelihood for ≥1 exportation†			
		Jordan	KSA	Qatar	UAE
Italy‡	0	0 (0–4); 1	0 (0–4); 18	NA	0 (0–4); 8
Malaysia§	0	0 (0–4); 0	0 (0–4); 33	0 (0–4); 1	0 (0–4); 2
Tunisia‡	0	0 (0–4); 0	0 (0–4); 2	0 (0–4); 0	0 (0–4); 0
United Kingdom§	0	0 (0–4); 0	0 (0–4); 35	0 (0–4); 3	0 (0–4); 25
United States§	2 from KSA	0 (0–4); 0	1 (0–5); 51	0 (0–4); 2	0 (0–4); 8

*The incubation period considered was 5.5 days (95% CI 3.6–10.2 days) (*8*). *MERS-CoV, Middle Eastern respiratory syndrome coronavirus; KSA, Kingdom of Saudi Arabia; UAE, United Arab Emirates; NA, not available.
†Information on Middle Eastern visitors to France, Netherlands, and Greece was not available. Number of Middle Eastern residents visiting Algeria and Austria was not available by country of residence.
‡Number of Middle Eastern visitors traveling to Italy and Tunisia corresponds to persons whose nationality is that of Middle Eastern source countries.
§Number of Middle Eastern visitors traveling to Malaysia, the United Kingdom, and the United States corresponds to residents in the Middle East.

in the hundreds. This finding suggests that exportations of asymptomatic cases might be several orders of magnitude higher than those of severe symptomatic cases.

The upper bound of exportations for the countries least likely to receive symptomatic case importations was 4, suggesting that small numbers of importations are largely stochastic events. Although every country is at risk, as illustrated by the recent outbreak of MERS-CoV infections in South Korea that was triggered by just 1 importation (*15*), our model can be used to assess risk level. Combined with other indicators, our model can help determine the level of additional public health measures (e.g., border screenings) required for infectious diseases threats from abroad.

No exportations of symptomatic cases were detected in some of the top travel volume countries for trips originating in KSA, Jordan, UAE, and Qatar. Travel volume to Egypt, India, and Pakistan was several times higher than that for other countries where MERS-CoV has been detected. However, only 1 case of MERS-CoV infection has been detected in Egypt (in a KSA resident) (http://www.who.int/csr/don/2014_05_01_mers), and no MERS-CoV cases have been detected in India or Pakistan. This finding suggests that MERS-CoV introductions may have already occurred in these countries but have not been detected.

In summary, by adapting a simple model of disease spread, we estimated the expected number of MERS-CoV symptomatic case exportations during a 1-year period among visitors to the Middle East and visitors from KSA, Jordan, Qatar, and UAE to non–Middle Eastern countries where case exportations have occurred. Our estimations suggest that the risk for repeated exportations of severe symptomatic MERS-CoV cases is low, although the number of asymptomatic case exportations might be higher.

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Nontyphoidal Salmonella Infection, Guangdong Province, China, 2012[1]

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We used active and passive surveillance to estimate nontyphoidal *Salmonella* (NTS) infection during 2012 in Guangdong Province, China. Under passive surveillance, for every reported NTS infection, an estimated 414.8 cases occurred annually. Under active surveillance, an estimated 35.8 cases occurred. Active surveillance provides remarkable advantages in incidence estimate.

Gastrointestinal illness or diarrhea caused by foodborne pathogens, such as nontyphoidal *Salmonella* (NTS), is a global public health concern (*1*). Many countries (e.g., the United States, England and Wales, Australia, Canada, Jordan, and Japan) have estimated the incidence of gastrointestinal illness caused by specific pathogens (*2–8*). However, in China, information is limited about the incidence of specific foodborne pathogens.

In 2003, China initiated a national, internet-based disease reporting system called the National Notifiable Disease Reporting System (NNDRS). This system legally requires routine reporting from all medical institutions and public health units of a list of infectious diseases. In this system, diarrheal pathogens other than *Vibiro cholerae* and *Shigella dysenteriae*, such as *Salmonella* spp., *Escherichia coli*, and *Listeria* spp., are reported as "other infectious diarrhea"; information about etiology is provided as an additional comment (*9*). Because NNDRS is passive, few reports include laboratory confirmation. According to previous data from passive surveillance, <1,000 NTS cases were reported in Guangdong Province annually since 2009, representing only a small proportion of actual infections.

In 2009, Guangdong Provincial Center for Disease Control and Prevention (Guangdong CDC) established laboratory-based active surveillance for NTS infection. In 2012, this system covered more than half of the Guangdong Province prefectures, capturing 61.5% of the population served by 27 sentinel hospitals (21 general hospitals and 6 specialized hospitals, including pediatric and gynecologic). In the surveillance system, patients with ≥3 loose stools in a 24-hour period plus fever, vomiting, or abdominal pain who visited the sentinel hospitals were enrolled as cases, and fecal samples were collected. The sentinel hospitals were required to forward *Salmonella* isolates to Guangdong CDC, along with epidemiologic data, for analysis. Culture-confirmed cases were then reported to NNDRS with pathogen information. Based on the pyramid model of burden of illness, we used data from active and passive surveillance to estimate NTS infection and to clarify the advantages and disadvantages of each system (*2*,*7*,*10*).

The Study

The estimation requires multiple steps. First, a person must have symptoms severe enough for medical care (multiplier 1). Second, the physician must collect patients' specimens (multiplier 2) and forward them for testing by bacterial culture (multiplier 3). Third, the sample test result must be positive (multiplier 4), and the confirmed case must be reported (multiplier 5) (*2*,*7*,*8*).

To obtain multiplier 1, we conducted a 12-month population-based household survey during March 1, 2012–February 28, 2013 (approved by the Ethics Committee of Guangdong CDC). Respondents were randomly selected from 4 districts in western, eastern, and central Guangdong Province. The case definition was the same as that for active surveillance. We used a standard questionnaire to collect information about diarrhea in the previous 4 weeks. The incidence rate of diarrhea was 0.1081 (95% CI 0.1004–0.1158) episodes/person-year; 38.6% of the household survey respondents with diarrhea sought medical care. Multipliers 2 and 3 were based on data from sentinel hospitals and comprised the overall number of diarrhea cases, samples collected, and samples submitted for culture during the year. A total of 75,583 (45.3%) samples of 166,729 registered diarrhea cases in the sentinel hospitals were collected, of which 22,577 (29.9%) were tested. Laboratories of sentinel hospitals cultured samples for *Salmonella* in accordance with standard pro-

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[2]These authors contributed equally to this article.

Table 1. Active and passive surveillance multipliers used to determine the incidence of nontyphoidal *Salmonella* infections, Guangdong Province, China, 2012*

	Active surveillance			Passive surveillance, by age group, y					
Surveillance steps	Overall	General hospitals	Specialized hospitals	Overall	0–4	5–24	25–44	45–64	≥65
Multiplier 1: Patient seeks medical care	2.59	2.59	2.59	2.59	1.25	5.0	8.0	2.0	3.0
Multiplier 2: Physician obtains samples	2.21	2.01	2.31	2.21	2.21	2.21	2.21	2.21	2.21
Multiplier 3: Samples tested for *Salmonella*	3.35	2.45	6.73	3.35	3.35	3.35	3.35	3.35	3.35
Multiplier 4: Positive laboratory test result	1.14	1.14	1.14	2.07	2.07	2.07	2.07	2.07	2.07
Multiplier 5: Confirmed cases reported	1.64	1.60	1.82	10.45	10.45	10.45	10.45	10.45	10.45
Overall	35.8	23.3	83.7	414.8	200.1	800.6	1,280.9	320.2	480.4

*Incidence is cases per 100,000 persons.

tocol provided by the national reference laboratory by using MacConkey agar as plating medium. According to a proficiency testing program, the *Salmonella* isolation sensitivity rate of these laboratories was 87.5% (multiplier 4). The numbers of *Salmonella* isolates identified and reported to NNDRS as NTS infectious diarrhea by all sentinel hospitals yielded the proportion of cases reported (648/1,061, 61.1%) (multiplier 5). We estimated the number of infections using the above 5 multipliers. Thus, active surveillance for each reported NTS infection identified 35.8 cases. We also analyzed multipliers of specialized hospitals (Table 1).

In the population survey–based passive surveillance system, multiplier 1 was the same as for active surveillance. According to a comparison with samples from the submission proportion in a survey of physician-diagnosed diarrhea in Guangdong Province during 2009 (Mann-Whitney test, p = 0.246) (*11*), and a comparison between medical institutions that charged and did not charge for testing (Kolmogorov-Smirnov test, p = 0.837), the proportion of samples submitted and tested from active surveillance were also used as estimates of passive surveillance. The average test sensitivity of sentinel laboratories before active surveillance began was used as an estimate of all medical institutions (i.e., the sensitivity of passive surveillance [48.2%]). Using numbers of *Salmonella* isolates in Guangdong Province from laboratory data, and number of reported NTS cases by all medical institutions, we determined the proportion of reported NTS was 9.6% (991/10,360). Thus, for each reported NTS case under passive surveillance, 414.8 cases actually occurred. Multipliers of 5 age groups also were presented (Table 1).

To generate a more robust estimate, we conducted uncertainty and sensitivity analyses (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/4/15-1372-Techapp1.pdf) on passive surveillance data using Monte Carlo simulation (@Risk 6.0; Palisade, Ithaca, NY, USA) (*12*). We used β distribution to describe the uncertainty of proportions and negative binominal distribution to estimate the number of cases. The sensitivity analysis helped determine factors that provide higher uncertainty in the estimate.

The uncertainty analysis model predicted a 411.9 (95% CI 308.4–592.7) overall multiplier and estimated that 408,499 (95% CI 302,899–591,901) *Salmonella* cases occurred per year when the overall multiplier was applied to the 991 reported NTS cases, resulting in 391.6 (95% CI 290.3–567.4) cases/100,000 persons in 2012. Incidence for 5 age groups was also estimated (Table 2). The rank correlation of various factors in the model showed that patients seeking medical care provided the highest uncertainty in the overall estimate (influence rate 96%) (Figure).

Table 2. Results of Monte Carlo simulation characterizing the uncertainty about the number of cases and the passive surveillance multipliers and the annual reported and estimated cases and incidence of nontyphoidal *Salmonella* infections, Guangdong Province, China, 2012

			No. cases		Annual incidence, cases/100,000	
Age group, y	Annual no. cases, median (95% CI)	Multipliers, median (95% CI)	Reported	Estimated (95% CI)	Reported	Estimated (95% CI)
0–4	87,195 (68,367–138,725)	209.6 (168.5–332.1)	415	87,195 (68,367–138,725)	6.13	1,287.9 (1,009.8–2,049.0)
5–24	222,558 (90,222–1,376,278)	605.3 (248.9–3720.3)	367	222,558 (90,222–1,376,278)	0.90	547.7 (222.0–3387.1)
25–44	119,409 (49,292–488,534)	1038.3 (436.4–4250.9)	115	119,409 (49,292–488,534)	0.32	333.4 (137.6–1,364.1)
45–64	18,577 (11,400–36,234)	320.2 (216.8–603.2)	58	18,577 (11,400–36,234)	0.40	126.6 (77.7–247.0)
≥65	16,421 (8,351–42,273)	457.1 (259.7–1156.5)	36	16,421 (8,351–42,273)	0.56	255.1 (129.7–656.8)
Overall	408,499 (302,899–591,901)	411.9 (308.4–592.7)	991	408,499 (302,899–591,901)	0.95	391.6 (290.3–567.4)

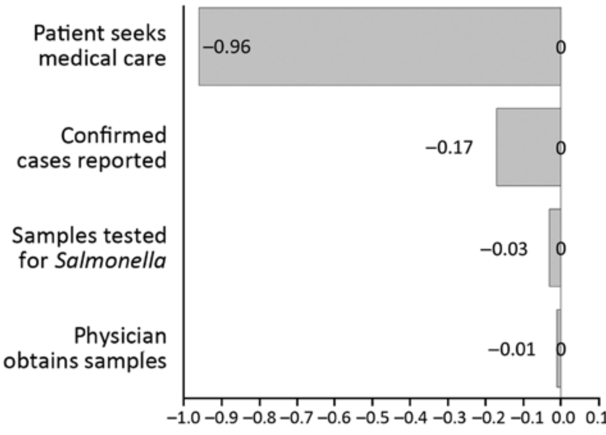

Figure. Rank correlations for the total number of nontyphoidal Salmonella cases in the population (tornado diagram), Guangdong Province, China, 2012.

Conclusions

Our estimated NTS incidence was lower than the incidence in China as determined from a literature review (626.5 cases/100,000 persons) (*13*) but close to that in the United States (352.1 cases/100,000 persons) (*3*). However, incidences for persons <5 years of age and 5–24 years of age in our study were higher than those for persons in China and the United States, highlighting that *Salmonella* represents a major health problem in Guangdong Province, especially among younger persons. Our estimated active surveillance rate (35.8) of NTS infections per reported case is similar to estimates in the United States (38.6 and 39) (*2*,*10*) but different from those for England (3.2), Jordan (278), and Japan (63) (*7*,*8*,*14*). Such differences might be due to differences in methods used and to actual differences in *Salmonella* infections.

With fewer missing cases and less underestimation, active surveillance has lower overall multipliers than passive surveillance, indicating smaller surveillance artifacts and more accurate incidence estimate and presents remarkable advantages over passive surveillance. The estimate for active surveillance also showed that if we seek to reduce uncertainty in the overall estimate, we should first focus on encouraging patients to seek medical care.

Our study provides policymakers in China with a reference for the importance of *Salmonella* incidence and calls for balanced surveillance on both foodborne infections and foods and enlarging active surveillance scales. More surveillance guidelines need to be developed to help physicians identify timing of sampling, tests, and performance. Laws requiring reporting of foodborne diseases and pathogens need to be enacted to increase quantity and quality of reporting. The result suggests that to increase care seeking and sample submission, government health insurance schemes should be further developed to cover diagnostic tests and treatments of diseases of public health significance.

Acknowledgments

We thank professionals from prefectural and county level CDCs for the household survey and physicians at sentinel hospitals for data collection. We also thank Olga Henao, Dana Cole, and Shua Chai for suggestions on the study and Jianghui Zhu and Xiaoyu Song for statistical review.

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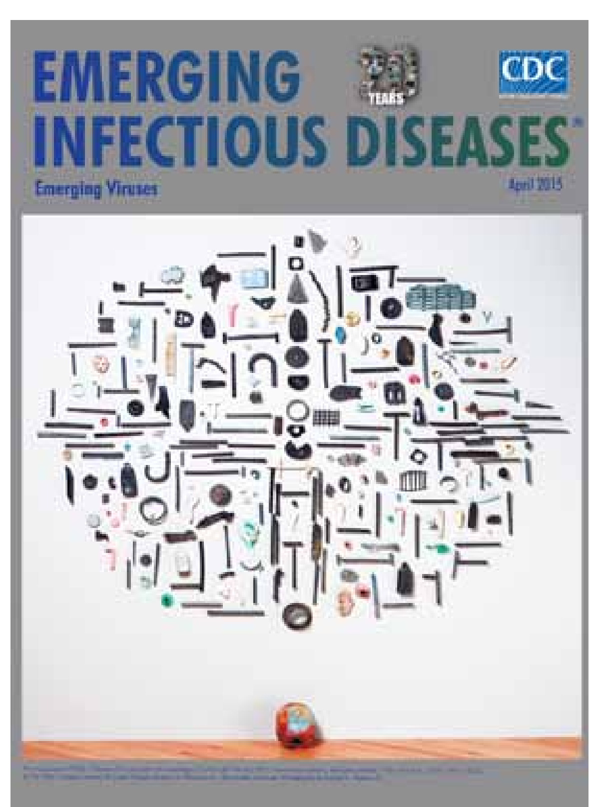
April 2015: **Emerging Viruses**

Including:

- Reappearance of Chikungunya, Formerly Called Dengue, in the Americas
- Hantavirus Pulmonary Syndrome, Southern Chile, 1995–2012
- Animal-Associated Exposure to Rabies Virus among Travelers, 1997–2012
- Evolution of Ebola Virus Disease from Exotic Infection to Global Health Priority, Liberia, Mid-2014
- Norovirus Genotype Profiles Associated with Foodborne Transmission, 1999–2012



- Deaths Associated with Respiratory Syncytial and Influenza Viruses among Persons ≥5 Years of Age in HIV-Prevalent Area, South Africa, 1998–2009
- Sequence Variability and Geographic Distribution of Lassa Virus, Sierra Leone
- Influenza A(H7N9) Virus Transmission between Finches and Poultry
- Highly Pathogenic Avian Influenza A(H5N1) Virus Infection among Workers at Live Bird Markets, Bangladesh, 2009–2010
- Increased Risk for Group B Streptococcus Sepsis in Young Infants Exposed to HIV, Soweto, South Africa, 2004–2008
- Population Structure and Antimicrobial Resistance of Invasive Serotype IV Group B Streptococcus, Toronto, Ontario, Canada

http://wwwnc.cdc.gov/eid/articles/issue/21/4/table-of-contents

Severe Infections with Human Adenovirus 7d in 2 Adults in Family, Illinois, USA, 2014

Adriana E. Kajon,[1] Michael G. Ison[1]

Human adenovirus 7d, a genomic variant with no reported circulation in the United States, was isolated from 2 adults with severe respiratory infections in Illinois. Molecular typing identified a close relationship with strains of the same genome type isolated from cases of respiratory disease in several provinces of China since 2009.

Species B human adenoviruses are frequent causative agents of acute respiratory disease requiring hospitalization mainly for children but occasionally also for adults. In particular, respiratory infections with human adenovirus 7 (HAdV7) have a well-documented association with severe clinical manifestations, nosocomial transmission, prolonged hospitalizations, pulmonary sequela, and deaths (*1–6*). Adenovirus pneumonia among immunocompetent adults is still relatively infrequent and therefore attracts considerable attention (*4*).

In the absence of active sentinel surveillance for adenovirus infections, there is no current sense of what the actual burden of adenovirus-associated respiratory disease is in the United States or which are the most prevalent pathogenic types circulating in this country. Only large outbreaks or severe cases elicit enough interest to pursue etiology investigation. We report characterization of adenovirus strains isolated from 2 adults in the same family who had severe respiratory infections in Illinois, USA, during December 2014.

The Study

This study was approved by the Northwestern University institutional review board. A waiver of consent was provided by this board.

Patient 1 was a 44-year-old man (electrician) who had no previous medical problems. He came to a hospital on December 2, 2014, with chest pain, fever (temperature up to 101°F), cough and shortness of breath, a few days after returning from a visit with family in Alabama. Two respiratory specimens were positive for adenovirus by PCR.

The patient was transferred to Northwestern Memorial Hospital (NMH) (Chicago, IL, USA) because of respiratory failure on December 13. A nasal swab specimen obtained at admission to NMH was positive for adenovirus B/E by the GenMarkDx eSensor Respiratory Virus Panel (Genmark Diagnostics, Inc., Carlsbad, CA, USA). He was given a diagnosis of adenovirus pneumonia, acute heart failure, and respiratory failure that required veno-venous extracorporeal membrane oxygenation during December 14-19. He slowly recovered but remained hospitalized until cardiac function normalized and was discharged on January 17, 2015. No other viral, bacterial or fungal pathogens were detected in any multiple blood, induced sputum, and bronchoalveolar lavage (BAL) specimen cultures performed during hospitalization. A BAL specimen obtained on December 17 was positive for adenovirus B/E by PCR. Sputum specimens were negative for *Mycoplasma* spp. and *Chlamydia*. spp.

Patient 2, the father of patient 1, was a retired 68-year-old man with a history of coronary artery disease, chronic obstructive pulmonary disease, chronic kidney disease, and diabetes. He came to a hospital on December 14, 2014, with fever, weakness, and shortness of breath. He was transferred to NMH because of respiratory failure, admitted to the intensive care unit, and required intubation and mechanical ventilation during December 16-21. A BAL specimen obtained on December 17 was positive for adenovirus B/E by the GenMarkDx eSensor Respiratory Virus Panel. No other pathogens were detected in this specimen. The patient had a nearly complete recovery and was discharged from the hospital on December 25.

No cases of respiratory illness were reported for other family members. Admission chest radiographs for both patients are shown in Figure 1, and a timeline of events are shown in Figure 2 (http://wwwnc.cdc/gov/EID/article/22/4/15-1403-F2.htm).

Adenovirus-positive clinical specimens (1 nasal swab specimen and 1 BAL specimen from patient 1 and 1 BAL specimen from patient 2) were shipped from NMH to Lovelace Respiratory Research Institute (Albuquerque, NM, USA) for molecular typing of the detected virus. HAdV was readily isolated in A549 cells from the nasal swab specimen obtained on December 13 at admission of patient 1at NMH, and from the initial culture of the BAL specimen obtained from patient 2 on December 17. These findings indicated that a high infectious virus load was present in the respiratory tract of both patients at the time of specimen collection.

Virus isolates from both patients were typed as HAdV7 by amplification and sequencing of the hexon gene

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[1]These authors contributed equally to this article.

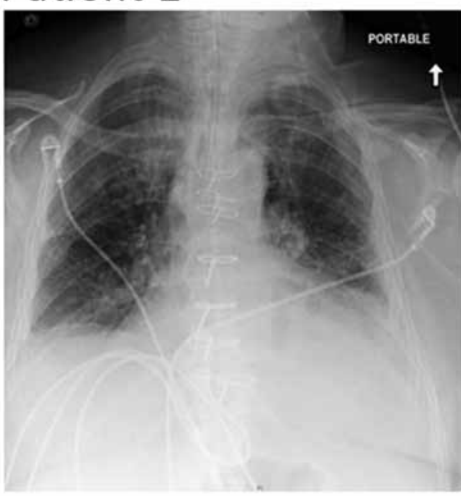

Figure 1. Admission chest radiographs of 2 adults with severe infections with adenovirus type 7 in family, Illinois, USA, 2014. Chest radiograph of patient 1 shows diffuse parenchymal consolidation involving all lobes. Chest radiograph of patient 2 shows interstitial changes in all lung fields and cardiomegaly.

hypervariable regions 1–7 and the complete fiber gene by using previously described primer pairs (*7*). Sequences of both amplicons (GenBank accession nos. KT266797–KT266800) were identical for the 2 isolates.

DNAs extracted from infected A549 cells by described procedures (*8*) were examined by restriction enzyme analysis, which is a powerful molecular approach for discrimination of intratypic genetic variability (*9*). Both isolates showed identical restriction profiles for endonucleases *Bam*HI, *Bcl*I, *Bst*EII, *Hpa*I, and *Sma*I, which confirmed the epidemiologic link and determined their genome type as 7d (*9*). A comparison between these profiles (Figure 3, panel A) and the corresponding in silico-generated profiles for HAdV7 genomic sequences available from GenBank (accession nos. KC440171 [Figure 3, panel B], JF800905, JX625134, KC857700, and KJ019880–KJ019888), and phylogenetic analysis of hexon and fiber gene sequences performed by using MEGA version 6.0 software (*10*) (Figure 3, panels C, D) showed a close relationship of the strain from Illinois with strains described in association with cases of severe pneumonia recently reported in difference provinces in China (*11*) and in Taiwan (*12*).

Conclusions

HAdV7 has been detected sporadically among cases of acute respiratory infection in the United States over the past 15 years, and genomic variants 7b, 7d2, and 7h have been identified among the isolated strains (*2*,*7*,*13,*). Our molecular typing findings suggest that genome type 7d might be circulating in the United States as a recently introduced newly emerging variant probably imported from Southeast Asia where increased incidence of severe HAdV7-associated respiratory disease has been reported (*11*,*12*,*14*). The 2 patients in this study had not traveled outside the United States and had not had contact with persons who recently traveled to Asia.

Two clusters of cases of severe respiratory disease associated with detection of HAdV7 were recently identified in the northwestern United States and are currently under investigation at the Centers for Disease Control and Prevention (Atlanta, GA) (K.S. Scott et al., pers. comm.). Comparison of complete genomic sequences of isolated strains and genome typing data will be critical for investigation of possible epidemiologic links between these cases.

Although no adequate data are available to compare clinical presentations associated with infection by different HAdV7 genome types, differences in virulence and transmissibility are likely among them. This issue merits further investigation. Severe respiratory disease has been reported in association with various genomic variants of HAdV7 (*1*,*11*,*15*), which suggests that the severity of HAdV-associated disease might also be attributable to population or individual host differences in susceptibility. Although patient 1 did not have an unusual medical history, patient 2 had concurrent conditions that might have contributed to severity of disease.

The described cases provide another example of the fact that adenoviruses can cause severe influenza-like respiratory disease in immunocompetent adults, and together with numerous case reports, highlight the need for retaining testing for adenovirus infection in the differential diagnosis of the etiology of acute respiratory disease. Our study confirms the value of restriction enzyme analysis (conducted by using gel electrophoresis of digested DNA or in silico digestion of complete genomic sequences) as a powerful approach for discrimination of intratypic HAdV genetic variability for molecular epidemiology studies.

HAdV7-associated respiratory disease has been shown to be preventable by oral vaccination with a live

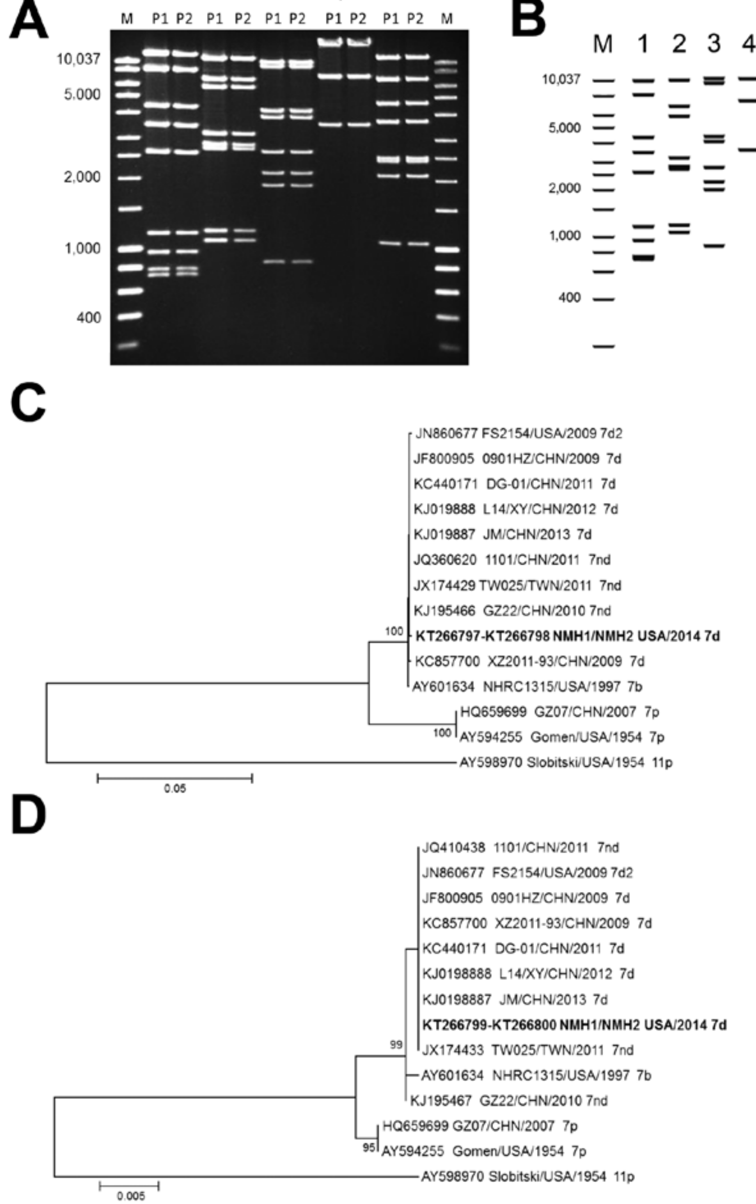

Figure 3. Enzyme and phylogenetic analysis of human adenovirus 7 isolates from 2 patients in Illinois, USA, 2014, and comparison isolates. A) Restriction enzyme analysis of genomic DNA from human adenovirus 7 isolates from patients 1 and 2 (P1 and P2, father and son). Lane M, molecular mass marker. Values on the left are in basepairs. B) Virtual restriction enzyme analysis of 6 complete genomic sequence of adenovirus strain DG01 from China. Lane m, Molecular mass marker; lane 1, *BamH*I; lane 2, *Bcl*I; lane 3, *BstE*II; lane 4, *Hpa*I; lane 5, *Sma*I. Values on the left are in basepairs. Phylogenetic analysis of C) hexon (HVR1–7) and D) fiber genes of adenovirus 7 strains from Asia and North America. Strains isolated in this study are indicated in bold. Phylogenetic trees were inferred by using the neighbor-joining method with 1,000 replicates. Bootstrap values are shown at branching points, and values <70% are not shown. Evolutionary distances were computed by using the Kimura 2-parameter method. There were 14 nt sequences and 1,509 positions in the final dataset for hexon gene analysis and 978 positions for fiber gene analysis. Analyses were conducted in MEGA6 (*10*). Hexon and fiber genes of human adenovirus 11p (strain Slobitski) were used as references. Scale bars indicate nucleotide substitutions per site.

nonattenuated virus formulation currently licensed for exclusive use among military personnel (http://www.cdc.gov/vaccines/hcp/vis/vis-statements/adenovirus.html). Licensing of this vaccine for use among civilians, or the design of next generation noninfectious vaccines, merits consideration for efficient intervention to prevent severe adenovirus-associated acute respiratory disease in the general population.

This report describes 2 cases of severe respiratory disease in adults associated with the apparent recent emergence of genome type 7d in the United States in 2014. In the possible scenario of a dispersion of this strain as a prevalent causative agent of acute respiratory disease requiring hospitalization, genome typing will be a valuable asset to surveillance and outbreak investigation efforts.

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history of adenovirus infections in transplant recipients, and viral pathogenesis.

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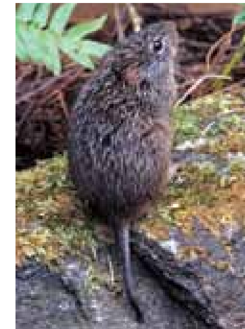
Rat Lungworm Expands into North America

Emily York, integrated pest management specialist at the Sam Noble Museum of Natural History, discusses the rat lungworm expansion in North America.



Cotton rat, *Sigmodon hispidus*. Photo courtesy Public Health Image Library.

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Hypervirulent *emm59* Clone in Invasive Group A *Streptococcus* Outbreak, Southwestern United States

David M. Engelthaler,[1] Michael Valentine,[1] Jolene Bowers, Jennifer Pistole, Elizabeth M. Driebe, Joel Terriquez, Linus Nienstadt, Mark Carroll, Mare Schumacher, Mary Ellen Ormsby, Shane Brady, Eugene Livar, Del Yazzie, Victor Waddell, Marie Peoples, Kenneth Komatsu, Paul Keim

The hyper-virulent *emm*59 genotype of invasive group A *Streptococcus* was identified in northern Arizona in 2015. Eighteen isolates belonging to a genomic cluster grouped most closely with recently identified isolates in New Mexico. The continued transmission of *emm*59 in the southwestern United States poses a public health concern.

Several cases of invasive group A *Streptococcus* (GAS) disease were detected in January 2015 in a northern Arizona hospital. A substantive percentage of the cases were associated with a homeless shelter and a local jail; outbreak case-patients were predominantly male and Native American. Other studies have shown an increase in infection risk for invasive GAS in Native American/First Nations populations (*1*,*2*), and outbreaks within this population in Arizona have been previously documented (*3*). Whole genome sequence analysis determined that the hypervirulent subtype *emm*59 was present among the first cases analyzed in early 2015. *emm*59 is known to have caused a nationwide outbreak of invasive GAS in Canada during 2006–2009 (*4*,*5*), and cases and outbreaks have been reported in the United States (*6*–*8*).

The Study

We identified isolates for sequencing from 29 invasive GAS cases diagnosed in patients in a northern Arizona hospital during January–July 2015 and randomly selected an additional 99 GAS isolates from a repository of >2,000 Arizona GAS isolates collected during 2002–2006 (no isolates from patients in Arizona were available for 2007–2014). Four additional isolates from central Arizona identified in 2015 were included in the analysis (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/22/1/15-1200-Techapp1.pdf). All isolates were grown on 5% sheep blood tryptic soy agar plates (Hardy Diagnostics, Santa Maria, CA), and incubated at 37°C with 5% CO_2. DNA was extracted by using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) following manufacturer's protocol. Genomic DNA libraries were prepared by using the Nextera XT library prep kit (Illumina, San Diego, CA) and sequenced with paired-end reads (250 bp) on an Illumina MiSeq instrument, as previously described (*9*). The finished genome of the *emm*59 Canadian clone MGAS15252 (GenBank accession no. CP003116) and high-quality publicly available sequence-read data from 44 US isolates, from NCBI short read archive (BioProject #PRJNA194066), were included in the subsequent phylogenetic analyses. The final core genome (all nucleotide loci found in all genomes) for single-nucleotide polymorphism (SNP) detection was 1,636,024 bp (98.6% of reference).

We used NASP SNP analysis pipeline (http://tgennorth.github.io/NASP/) for whole-genome SNP typing as previously described (*10*). SNP matrices were developed for both the whole species and the *emm*59-only analyses. We used MEGA version 5.2.2 software (*11*) to generate maximum parsimony phylogenetic trees. Regions of high SNP density were identified as possible regions of recombination and were further analyzed for impact on the consistency index. Genomes were assembled by using UGAP (https://github.com/jasonsahl/UGAP). GAS *emm* subtypes were assigned by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), querying the study genome assemblies against the Centers for Disease Control and Prevention's (CDC) *emm* type-specific sequence database (http://www.cdc.gov/streplab/m-proteingene-typing.html). We resolved dual *emm*-type hits using CDC's *emm* typing Sanger sequencing primers (http://www.cdc.gov/streplab/protocol-emm-type.html) as a BLAST query and noting hit locations.

We identified 18 of the 29 contemporary northern Arizona isolates as subtype *emm*59; the remaining isolates

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were composed of 6 additional *emm* types: *emm*1 (n = 2), *emm*5 (n = 2), *emm*58 (n = 1), *emm*81 (n = 2), *emm*83 (n = 1), *emm*89 (n = 2), and *emm*94 (n = 1). The 99 historical and 4 contemporary background Arizona isolates included 25 distinct *emm* types (online Technical Appendix Table). No *emm*59 isolates were identified in this background set, and none had been previously reported in Arizona.

The 18 Arizona *emm*59 cases occurred during January–July 2015 (Table). An *emm*59-only phylogenetic analysis demonstrated the apparent presence of multiple lineages of *emm*59 in the 2015 Arizona isolates (Figure 1). A distinct clone consisting of 14 of the 18 *emm*59 isolates were separated from each other by only 0–4 SNPs, genomically supporting the presence of an ongoing outbreak; ≥8 of these patients were epidemiologically linked to physical contact, cohabitation, or both with 1 other person (data not shown). The additional *emm*59 isolates make up additional lineages separated from one other by 8–28 SNPs. No recombination was identified among the Arizona isolates. A relatively large number of SNPs and indels were seen within an approximate 23-kilobase region (Figure 1). This region has been previously reported to contain mutational hotspots associated with virulence (*12*,*13*). Considering the presumptive positive selective force on this region, SNPs within the region were not included in the final phylogenetic analysis.

Table. Epidemiologic data for 18 case-patients with invasive *emm*59 group A *Streptococcus* infection, Arizona, USA, 2015*

Category	Value
Race	
American Indian or Alaskan Native	15 (83)
White	3 (7)
Sex	
F	4 (22)
M	14 (88)
Mean age, y (range)	40 (26–79)
Clinical information	
Cellulitis	7 (39)
Necrotizing fasciitis	5 (28)
Sepsis	9 (50)
Risk factors	
Injury	7 (39)
Alcohol abuse	10 (56)
Homeless	8 (44)
Living in shelter	5 (28)
Local jail term within ≈1 mo. of diagnosis	6 (33)

*Values are no. (%) patients except as indicated. Epidemiologic data based on available information.

When compared with all other publicly available US *emm*59 isolate genomes, nearly all the genomes identified in the United States were closely related to each other and to the Canadian clone MGAS15252; individual isolate SNP branch lengths ranged from 0 to 10 (Figure 2). The Arizona outbreak isolates were separated from 2 New Mexico isolates by 4 and 5 SNPs each; these isolates fell within the overall Arizona clade and were subsequently included

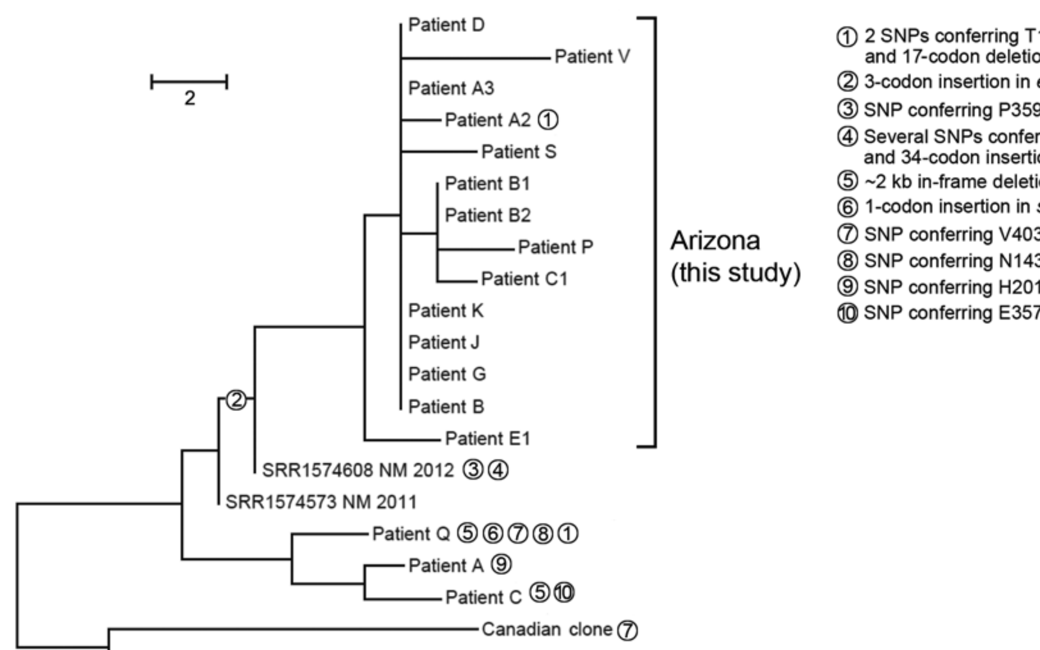

Figure 1. Phylogenetic single-nucleotide polymorphism (SNP) tree of *emm*59 isolates from a northern Arizona hospital displaying distribution of mutations in a 23kb positively selected region during invasive group A *Streptococcus* outbreak, southwestern United States. Maximum parsimony tree of all SNP loci (n = 58) in *emm*59 isolates (n = 18) from Arizona, 2 recent New Mexico isolate genomes, and the Canadian clone reference isolate MGAS15252. Consistency index = 1.0. Branch lengths represent numbers of SNPs between isolates; unit bar is in the figure. Numbered circles distinguish lineages of selected mutations in *scpA, enn, sfbl, mga, sfbx,* and *sof* genes in a 23-kb hotspot mutational region. Scale bar indicates SNPs.

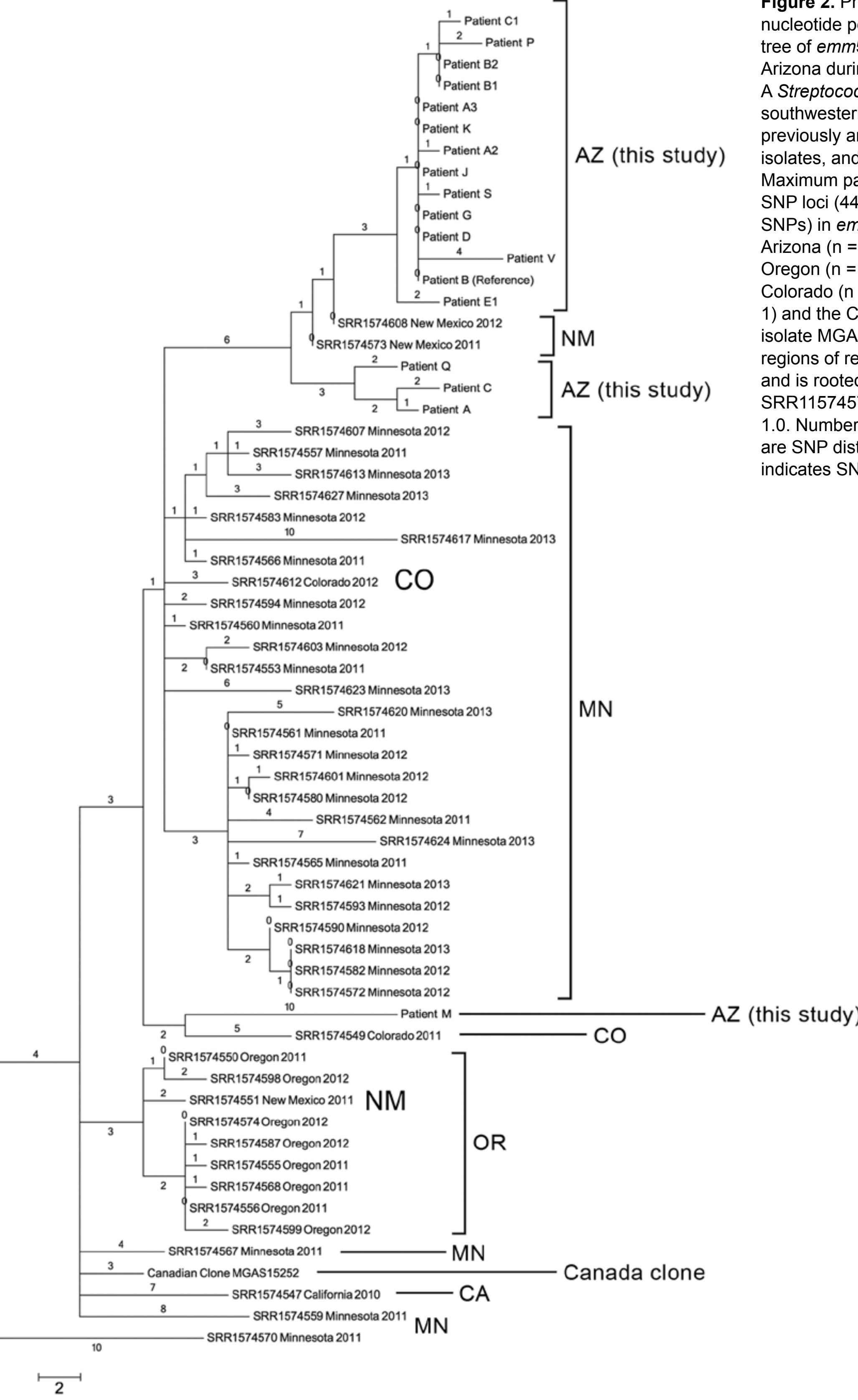

Figure 2. Phylogenetic single-nucleotide polymorphism (SNP) tree of *emm*59 isolates from Arizona during invasive group A *Streptococcus* outbreak in the southwestern United States, previously analyzed US *emm*59 isolates, and the Canadian clone. Maximum parsimony tree of all 177 SNP loci (44 parsimony informative SNPs) in *emm*59 isolates from Arizona (n = 18), Minnesota (n = 29), Oregon (n = 8), New Mexico (N = 3), Colorado (n = 2), and California (n = 1) and the Canadian clone reference isolate MGAS15252. Tree has regions of recombination removed and is rooted with Minnesota isolate SRR11574570. Consistency index = 1.0. Numbers above branches are SNP distances. Scale bar indicates SNPs.

in the Arizona-only phylogenetic analysis (Figure 1). Conversley, the isolate from patient M appears more distant from the larger Arizona population. The Arizona clades, with the exception of that of the isolate from patient M, all appear to arise from the large Minnesota polytomy. The previously estimated 1.3–2.1 SNPs/year mutation rates for GAS (*14*,*15*) further support the Arizona outbreak as being caused by a single clone, likely originating from New Mexico and being spread over 6–12 months.

Conclusions

The *emm*59 subtype of GAS, the etiologic agent of a substantial nationwide outbreak of invasive GAS in Canada during 2006–2009 (*4*), is now present in Arizona, causing at least 1 outbreak of epidemiologically and genomically linked cases and several additional epidemiologically unrelated cases. The lack of *emm*59 in background isolates in Arizona from the previous decade, along with its low genetic diversity, suggests that *emm*59 emerged recently in Arizona. Following the *emm*59 epidemic in Canada, this subtype was subsequently seen in a few US states; a retrospective analyses of the Centers for Disease Control and Prevention Active Bacterial Core surveillance (ABCs) system (http://www.cdc.gov/abcs/reports-findings/survreports.pdf) identified 40 US *emm*59 isolates during 2000–2009 (*6*) and an additional 67 isolates during 2010–2012 (*7*). Of note, only 5 (of the 40 *emm*59 isolates from 2000–2009 (2 from Minnesota, 2 from California, and 1 from Oregon) appeared to be closely related to the Canadian clone (defined by the authors as being separated by <16 SNPs) (*6*); in contrast, all of the strains from the 2010–2012 survey appeared to be more closely related to the Canadian clone. The more recent ABCs analysis identified an increasing number of southwestern isolates, including 4 from Colorado and 6 from New Mexico (*7*), although no outbreaks were specifically described in these states (Arizona is not included in the ABCs system). Similar to this outbreak study, Olsen et al. (*7*), in an analysis of 60 MN *emm59* isolates from case-patients with identified race, determined that 25 (42%) were from Native Americans; of 5 isolates from New Mexico in that study, 3 were from Native Americans.

Given the apparent distal nature of the Arizona/New Mexico isolates to the Minnesota population in our study, it is reasonable to propose an unidentified epidemiologic relationship between these case populations. However, caution must be used in drawing conclusions regarding the relationships of isolates from disparate geographic regions because only limited comparable sequence data from previous *emm*59 studies in the United States (*7*) were publicly available to compare to the Arizona isolates. Epidemiologic investigations, along with healthcare provider and patient education activities, are ongoing in Arizona to further determine the extent of the current outbreak and the associated risk factors and to help mitigate effects and limit or prevent further spread to at-risk populations.

Dr. Engelthaler is an associate professor with the Translational Genomics Research Institute in Flagstaff, AZ. His research interests are in advancing epidemiology and clinical response through applied infectious disease genomics.

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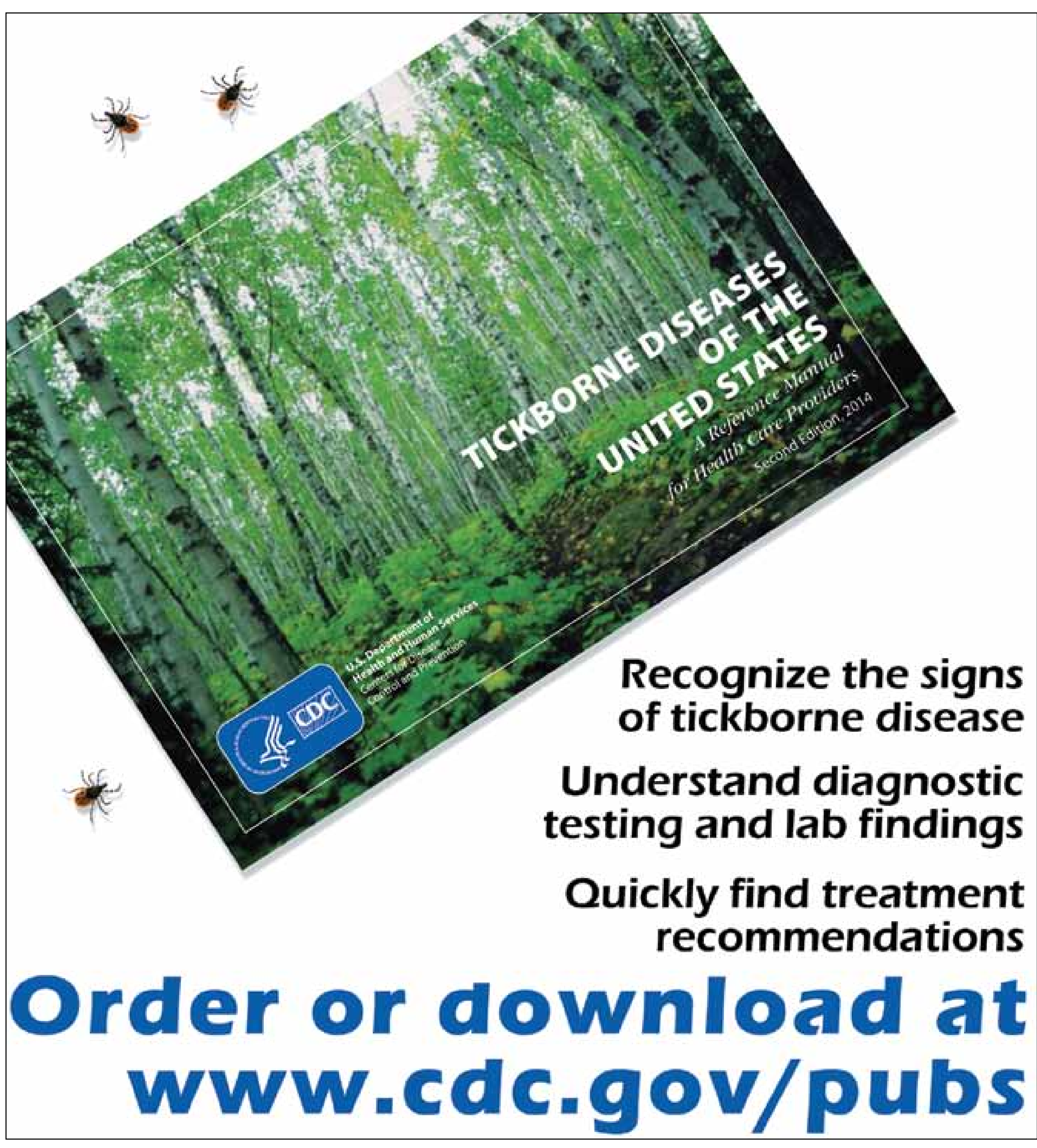

Leishmania infantum Infection in Blood Donors, Northeastern Brazil

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To the Editor: *Leishmania infantum* is endemic to northeastern Brazil. It is responsible for visceral leishmaniasis (VL), a major emerging health problem in urban areas. Transmission occurs predominantly by the *Lutzomyia longipalpis* sand fly, but transfusion-associated VL, caused by *L. infantum*, has been reported from southern Europe and, by *L. donovani*, on the Indian subcontinent (*1*,*2*). Most *L. infantum* infections are asymptomatic (*3*), raising concern that the parasite could be present in donated blood from otherwise healthy residents in areas to which it is endemic (*4*).

VL caused by *L. infantum* is endemic to 20 of Brazil's 27 states; an annual average of 3,553 cases occur nationwide, with 54% of all cases reported from Brazil's northeastern region. The state of Ceará historically ranks first or second in number of cases; an annual average of 467 cases were reported during the last decade (*5*). Thirty-eight percent of cases were reported from Fortaleza, the capital, where 28.4% of the state's population resides. Over a 10-year-period, 277 (7.8%) persons with VL have died, and 109 (39%) of VL-related deaths have occurred in Fortaleza.

Sixty-nine percent of blood donors for Ceará reside in Fortaleza. To determine the prevalence of *Leishmania* infection among healthy blood donors, we tested blood donated to the State of Ceará Public Blood Bank. Compulsory serologic testing was also done for *Trypanosoma cruzi* (Chagas disease), hepatitis B and C, *Treponema pallidum* (syphilis), human T-cell lymphotropic virus types 1 and 2, and HIV-1 and -2. In the blood bank, 60% of units are centrifuged to separate the buffy coat in preparation for platelet separation. During May–November 2011, we randomly selected 431 buffy coats and tested them for *Leishmania* spp. by ELISA and PCR. To separate plasma from cells, 10 mL of buffy coat was centrifuged in Ficoll-Hypaque (Histopaque -1077, Sigma-Aldrich, São Paulo, Brazil). We tested plasma for leishmanial IgG by ELISA using a modified protocol of Evans et al. (*6*). IOC/L2906 *L. infantum* strain (MHOM/BR/2002/LPC-RPV) was used as the source of promastigote antigens. In addition, DNA was extracted from the mononuclear cell preparation, and PCR was performed with primers 150 (5′-GGG[G/T]AGGGGCGTTCT[G/C]CGAA-3′) and 152 (5′-[G/C][G/C][G/C][A/T]CTAT[A/T]TTACACCAACCCC-3′) that target the 120-bp conserved region of the *Leishmania* kDNA minicircle present in all *Leishmania* spp (*7*). As a positive control, kDNA was extracted from *L. amazonensis* promastigotes, strain BA-125 (MHOM/BR/87), characterized by PCR and isoenzymes (*8*). All PCR-positive samples were purified and sent to Ludwig Biotec (Alvorada, Brazil) for sequencing by ACTGENE-Molecular Analysis. The Federal University of Ceará Ethics Committee approved this study.

Buffy coats from 57 (13.2%) serum samples from 431 donors were positive for leishmanial IgG, and 20 (4.6%) were positive for *Leishmania* spp. DNA. Sequencing of all PCR-positive samples confirmed the *Leishmania* genus. Three donors tested positive by both ELISA and PCR. Overall, the prevalence of leishmanial infection was 17.1% of blood donors. Eighty of the 431 units tested positive for ≥1 of compulsorily screened infections and were rejected. Of the remaining 351 that were negative for co-infection, 43 (12.2%) were positive for leishmanial IgG and 15 (4.3%) for *Leishmania* spp. DNA. Two donors were positive for both by ELISA and PCR. The prevalence of *Leishmania* infection among blood units accepted for transfusion was 16%.

The results demonstrate a surprisingly high prevalence of *Leishmania* infection in blood donors in Fortaleza, several times higher than that other diseases for which blood is screened (Figure). In a recent study in Salvador, Brazil (*9*), 5.4% of blood donors had leishmanial antibodies, of which 68% were positive by its PCR targeting kDNA amplification.

The percentages of antibody- or PCR-positive units capable of transmitting *Leishmania* and the outcomes are unknown. Viable *Leishmania* might not be in the blood of all PCR-positive donors, and even when present, the inoculum might be reduced by removal of infected circulating mononuclear phagocytes in the buffy coat, or parasites might be affected by steps involved in preparation or storage. However, if we consider units that test positive by PCR as being potentially infectious, the number of recipients at risk is of substantial concern. For example, in 2011, there were 99,933 blood donations to the State of Ceará Public Blood Bank. After compulsory screening for the other bloodborne pathogens, 93,238 units were accepted for transfusion. Extrapolating from the PCR-positive rate of 4.3%, a total of 4,009 recipients possibly were exposed to infection. Further studies are needed to

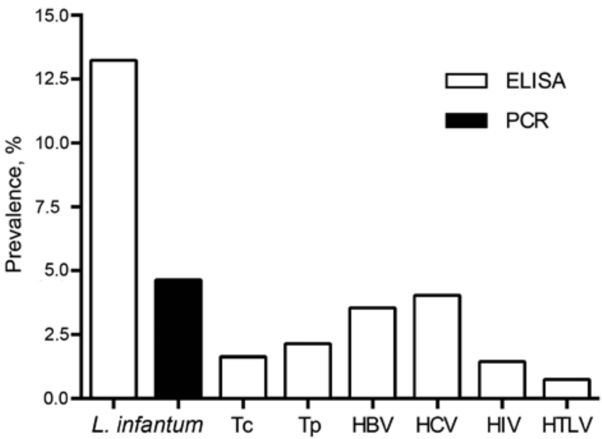

Figure. Comparison of the prevalence of *Leishmania infantum* as tested by PCR and ELISA and of other infections compulsorily tested in 431 blood donors in Fortaleza, state of Ceará, northeastern Brazil. HBV, hepatitis B virus; HCV, hepatitis C virus; HTLV, human T-cell lymphotropic virus; Tc, *Trypanosoma cruzi*; Tp, *Treponema pallidum*.

determine whether recipients of blood from donors who are PCR positive and/or leishmanial antibody positive become infected with *L. infantum*. Persons with advanced AIDS or other immunosuppressive conditions seemingly would be at greatest risk for VL.

In Brazil, legislation requires that all blood for transfusion be tested for *T. cruzi* ., hepatitis B and C, *T. pallidum*, human T-cell lymphotropic virus types 1 and 2, and HIV-1 and -2. As additional information becomes available, screening for *L. infantum* also might be advisable to reduce the possibility of the recipient becoming infected, developing VL, and possibly being a reservoir of infection in the community (*10*), particularly in Ceará and other regions where the prevalence of *L. infantum* infection is high.

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Morbillivirus and Pilot Whale Deaths, Canary Islands, Spain, 2015

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To the Editor: Four strains of cetacean morbillivirus (CeMV; family *Paramyxoviridae*, genus *Morbillivirus*) have been detected in the global cetacean population: porpoise morbillivirus (*1*), dolphin morbillivirus (*2*), pilot whale morbillivirus (PWMV) (*3*), and Longman's beaked whale morbillivirus (*4*). In addition, 2 novel

CeMV sequences or strains isolated from the Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) and the Guiana dolphin (*Sotalia guianensis*) have been recently reported in the Southern Hemisphere (*5,6*).

Pilot whales are known to be susceptible to 2 strains of CeMV, PWMV, and dolphin morbillivirus (*3,7,8*). Only 2 deaths of whales have been reported to be caused by PWMV: 1 long-finned pilot whale (*Globicephala melas*) (*3*) and 1 short-finned pilot whale (*G. macrorhynchus*) (*8*). We report deaths of 3 short-finned pilot whales caused by PWMV in the northeastern Atlantic Ocean along the coast of the Canary Islands, Spain.

During mid-January–May 2015, a total of 3 whales (animals 1, 2, and 3) were found dead along the coasts of the Canary Islands (Table). Complete standardized necropsy was performed for all whales. Tissue samples from animals 1 and 2 were collected and fixed in 10% neutral-buffered formalin for histologic and immunohistochemical analyses (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/22/4/15-0954-Techapp1.pdf). Immunohistochemical analysis was performed (brain, intestine, lymph nodes, lung, kidney, adrenal gland, uterus, ovary, testis, and spleen) by using a monoclonal antibody against the nucleoprotein of canine distemper virus (CDV-NP; VMRD, Inc., Pullman, WA, USA) (*7*). Samples of lung, pulmonary lymph nodes, larynx, laryngeal tonsil, intestine, spleen, and brain were frozen (-80°C) for virologic analysis.

Grossly, the most remarkable findings in animal 1 were severe suppurative rhinitis, with clogged nasal passages by the accumulation of large quantity of purulent material, otitis media, sacculitis, and laryngitis. Severe diffuse epithelial hyperplasia and hyperkeratosis was observed along the upper respiratory tract and keratinized stomach. Animal 2 had severe proliferative dermatitis and cheilitis, and severe, suppurative, laryngeal tonsillitis. Animal 3 had advanced autolysis, which precluded pathologic analysis.

Histologically, moderate, multifocal, bronchointerstitial pneumonia, severe suppurative tonsillitis and systemic lymphoid depletion were identified in animals 1 and 2. Severe nonsuppurative meningoencephalitis with neuronal and glial cell degeneration and necrosis, microgliosis and syncytial cells were observed in animal 2.

Immunohistochemical analysis showed morbillivirus antigen in the bronchiolar epithelium, type 2 pneumocytes, and alveolar multinucleate cells. Syncytia from lymph nodes, laryngeal tonsil, spleen, and intestine also showed positive immunolabeling for morbillivirus. Epithelial tropism caused by the virus was suggested by identification of viral antigen in epithelia of the lung, larynx, keratinized stomach, intestine, kidney, urinary bladder, epididymis, and endometrial glands. In addition, intense immunolabeling was detected in neurons (soma, dendrites, axon hillock, and axons) and glial cells, primarily throughout the cerebral gray matter of animal 2.

Molecular detection of CeMV was performed by a using a 1-step reverse transcription PCR for a 426-bp conserved region of the phosphoprotein gene (*7*). All tested samples from animals 1 and 2 and a laryngeal tonsil sample from animal 3 showed positive PCR results. Because co-infections with herpesvirus and morbillivirus were observed during morbillivirus epizootics in seals in 1988 and dolphins in 2006–2007, we also tested the same tissue for herpesvirus by conventional nested PCR (*9*). Herpesvirus DNA was detected in all samples from animal 1 except

Table. Characteristics for 3 short-finned pilot whales stranded along the Canary Islands, Spain, 2015*

Animal no., age/sex	Total body length, cm	Weight, kg	Date stranded	Location	Decomposition code	Main pathologic findings	IHC-positive result	PCR-positive result
1, J/F	202	119	Jan 14	Fuerteventura	Moderate autolysis	Dermatitis, suppurative rhinitis, paranasal sinusitis, otitis media, air sacculitis, laryngitis, laryngeal tonsillitis, BIP, hyperkeratotic gastritis, lymphoid depletion	Lung, larynx, laryngeal tonsil, lymph nodes, spleen, adrenal gland, keratinized stomach, intestine, uterus, ovary	Lung, **larynx, pulmonary lymph node, laryngeal tonsil, spleen, intestine**
2, C/M	168	75	May 15	Fuerteventura	Fresh	Dermatitis, cheilitis, suppurative laryngeal tonsillitis, BIP, encephalitis	Lung, laryngeal tonsil, lymph nodes, spleen, kidney, testis, epididymis, intestine, adrenal gland, urinary bladder, brain	Lung, pulmonary lymph node, laryngeal tonsil, intestine, brain
3, A/M	448	900	May 20	Tenerife	Advanced autolysis	Advanced autolysis	None	Laryngeal tonsil

*IHC, immunohistochemical analysis; J, juvenile; BIP, bronchointerstitial pneumonia; C, calf; A, adult. Tissues that were positive for herpesvirus and morbillivirus simultaneously are indicated in bold.

lung, although no specific lesions compatible with this infectious agent were observed.

A pool containing all morbillivirus-positive PCR amplicons for animals 1 and 2 (GenBank accession nos. KT006289 and KT006290), a PCR amplicon for the brain sample from animal 2 (GenBank accession no. KT006291), and a PCR amplicon for the larynx from animal 3 were sequenced. A BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) showed that amplified samples were nearly identical to reference PWMV sequences (GenBank accession nos. AF200817 [*3*] and FJ842381 [*8*]). The sequence obtained from animal 3 was too short and degenerated to be accurately classified as CeMV, although it showed high homology with PWMV and porpoise morbillivirus.

It has been proposed that pilot whales might be enzootically infected with CeMV (*10*). These whales might be responsible for maintaining and transmitting CeMV over long distances or to other odontocetes. No die-offs have been observed in these species. However, an outbreak of a lethal morbillivirus infection in long-finned pilot whales caused by a dolphin morbillivirus strain occurred in the Mediterranean Sea during the end of October 2006–April 2007 (*7*).

Results of this study support the previous hypothesis that pilot whales have a species-adapted morbillivirus but indicate that lethal infections are not as rare as previously believed (*3*). The tropism of the virus in these cases, the high number of multinucleated syncytial cells, and the severity of the lesions resemble the acute systemic symptoms observed in dolphins infected with morbillivirus (*2*). Thus, pilot whales in the northeastern Atlantic Ocean could be at risk for infection, especially in one of the main pilot whale–watching regions between La Gomera and Southern Tenerife Islands in the Canary Islands, which has >700,000 visitors each year.

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Serogroup-specific Seasonality of Verotoxigenic *Escherichia coli*, Ireland

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To the Editor: Globally, an increasing number of serogroups of verotoxigenic *Escherichia coli* (VTEC) have been reportedly associated with human illness. The best known is serogroup O157; the World Health Organization also recognizes VTEC O103, O111, O145, and O26 as having the potential to cause severe disease (*1*). The increasing number of non-O157 VTEC infections is cause for concern. In general, human infections with VTEC are reportedly more common in late summer; the European Centre for Disease Control and Prevention reported that the number of cases across the European Union peaks each year during July–September (*2*). Similarly, the United States reported that the number of VTEC O157 cases peaks in late summer (*3*).

Ireland is now one of the countries with the highest incidence of VTEC infection (*2*). In Ireland, statutory notification of VTEC infection became mandatory in 2004. In common with surveillance internationally, the focus was initially on VTEC O157; since then, testing and surveillance for non-O157 VTEC have improved substantially as a result of increased awareness and availability of diagnostic methods for non-O157 detection. Non-O157 VTEC were first reported in Ireland in 1999 (*4*), and surveillance data indicated that only 14% of VTEC notifications in 2004 compared with 75% in 2014 were caused by non-O157 VTEC.

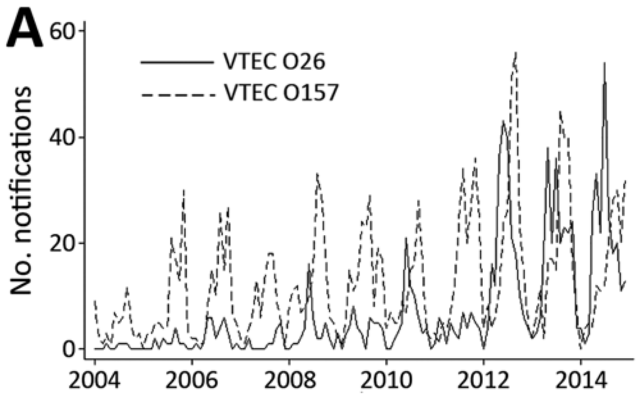
In the notification dataset for Ireland, the 2 primary VTEC serogroups (O26 and O157) over many years have seemed to differ in their seasonality; VTEC O26 notifications generally peaked ≈2 months earlier than VTEC O157 notifications (Figure, panel A). This earlier incidence peak for VTEC O26 has become progressively more consistent as the number of reported VTEC O26 notifications has risen. A study by Rivero et al. also suggested that non-O157 human infections may not exhibit the same seasonal variation observed for VTEC O157 (*5*).

In this study, we compared the seasonality of the 2 strains by using national notification data for 2004–2014 (n = 2,569 notifications for O157 and O26). We estimated the timing of the seasonal peaks (phase of seasonality) for each of the serogroups, and the difference between the 2 phases, by using times series quasi-Poisson regression, fitting terms for temporal trend, and a sine wave with a period of 12 months for seasonality and for interaction by serogroup. We compared the phase shifts of the 2 serogroups by using the Wald test. To rule out the possibility that the observed distributions were influenced by the occurrence of a limited number of outbreaks, we reanalyzed the data for sporadic cases alone and, because risk factors for VTEC infection have been shown to vary by age (*6*), separately for patients <5 years of age and for older child and adult patients.

The number of predicted cases peaked in July for VTEC O26 and in September for VTEC O157; the 2-month difference in phase (seasonality) by serogroup was significant ($p<0.0001$) (Figure, panel B). The difference in seasonality remained significant ($p<0.0001$) for sporadic cases alone; the predicted 2-month difference in seasonality was the same. The serogroup-dependent seasonality also remained when the data were analyzed separately for patients <5 years of age (predicted difference in phase 2 months, $p<0.0001$) and $\geq$5 years of age (predicted difference in phase 1 month, $p<0.0001$).

A significant increasing annual trend was also observed, in particular for VTEC O26. However, this increase is probably, at least in part, artifactual because of increased availability and more widespread use of clinical diagnostic tests for non-O157 VTEC in later years.

One possible explanation for the difference in seasonality is that the primary animal reservoirs for the 2 serogroups could differ. Cattle and sheep have been identified



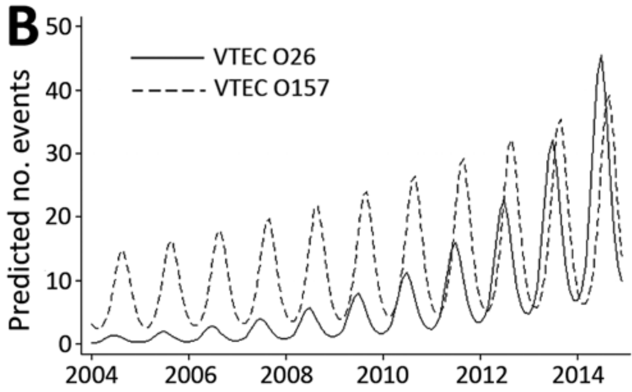


Figure. Verotoxigenic *Escherichia coli* (VTEC) O157 and VTEC O26, Ireland, 2004–2014. A) Seasonal distribution of notifications. B) Predicted seasonal distribution. Data source: Computerised Infectious Disease Reporting System (https://www.hpsc.ie/Notifiable Diseases) in Ireland, as of June 24, 2015. Predicted number of cases by month were derived from a cyclical quasi-Poisson model after trend and seasonality and interaction by serogroup were accounted for.

as carriers of O157 and O26 strains in Ireland (*7*,*8*). In Germany, cattle density has been shown to be significantly associated with human VTEC O157 incidence but only marginally associated with O26 incidence (*9*); the same study showed no association between cattle density and VTEC O91 infection, indicating that not all serogroups necessarily share the same reservoirs. Alternatively, animals of the same species may be preferentially colonized with different serogroups at different times of the year or at different developmental ages. Other possible explanations could be variation in survival characteristics between the 2 strains, which results in a different seasonal distribution in the environment, or specific human behavior (e.g., seasonal food) resulting in more frequent exposure to sources of VTEC O157 and VTEC O26 at different times of the year.

The consistent differences in seasonality identified here between the 2 most common VTEC serogroups suggest the existence of noteworthy underlying differences in disease etiology between the strains. Further exploration is recommended.

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New Delhi Metallo-β-Lactamase-1–Producing *Klebsiella pneumoniae*, Florida, USA[1]

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To the Editor: New Delhi metallo-β-lactamase (NDM)–producing *Enterobacteriaceae* have swiftly spread worldwide since an initial report in 2008 from a patient who had been transferred from India back home to Sweden (*1*). Epidemiologically, the global diffusion of NDM-1 producers has been associated with the Indian subcontinent and the Balkan region, which are considered the primary and secondary reservoirs of these pathogens, respectively (*1*). However, recent reports suggest that countries in the Middle East may constitute another potential reservoir for NDM-1 producers (*1*). More than 100 NDM-producing isolates have been reported in the United States, most of

[1]Preliminary results from this study were presented at the 54th Interscience Conference on Antimicrobial Agents and Chemotherapy, September 5–9, 2014, Washington, DC, USA.

which were associated with recent travel from the Indian subcontinent (*2*,*3*). We report an NDM-1–producing *Klebsiella pneumoniae* strain that was recovered from a patient who had been transferred from Iran to a hospital in Florida, United States.

The patient was a 72-year-old woman with diabetes who had suffered a hip fracture while residing in Iran. After fixation of the bone failed, the patient underwent hip replacement, which was complicated by dislocation and an infected hematoma. She was transferred to a hospital in Florida in February 2014 for further care. The wound culture collected upon arrival grew *K. pneumoniae* K351. The patient underwent surgical debridement, implant removal, and placement of an antimicrobial spacer for prosthetic joint infection. She was treated with tigecycline; however, the wound did not heal, and she underwent debridement with removal of the spacer and placement of antimicrobial beads.

K. pneumoniae K351 from the patient was resistant to all β-lactams tested, including carbapenems, and highly resistant to aminoglycosides and fluoroquinolones, retaining susceptibility only to tigecycline and colistin. PCR and sequencing revealed the presence of β-lactamase genes bla_{NDM-1}, $bla_{CTX-M-15}$, bla_{SHV-12}, and bla_{TEM-1} and 16S rRNA methyltransferase genes *rmtC* and *armA*. The strain sequence type (ST) was ST147, which is one of the predominant NDM-producing *K. pneumoniae* lineages and has been reported in many countries (*3*,*4*). Conjugation experiments using broth and filter mating methods did not yield any *Escherichia coli* J53 transconjugants with bla_{NDM-1}, despite repeated attempts. Plasmids of K351 were extracted by using the standard alkaline lysis method and used to transform *E. coli* TOP10-competent cells. An *E. coli* transformant harboring plasmid pK351 grew on Mueller-Hinton agar plates supplemented with 200 μg/mL of ampicillin. The transformant exhibited resistance to all β-lactams, including carbapenems and aminoglycosides; this resistance could be attributed to the presence of bla_{NDM-1} and *rmtC* in plasmid pK351, which was confirmed by PCR.

pK351 was fully sequenced on a PacBio RS II sequencing instrument (Pacific Biosciences, Menlo Park, CA) and annotated (GenBank accession no. KR351290) (*5*). pK351 is 106,844 bp in length, has an average GC content of 55.4%, and encodes IncFIB and IncFII-like replication proteins, with IncFIB belonging to B36 according to the replicon sequencing typing scheme (*6*). pK351 is most closely related (98% coverage and 99% identity) to 3 bla_{NDM-1}-carrying plasmids pKOX_NDM1, pRJF866, and pNDM-Ec1GN574 (GenBank accession nos. NC_021501, KF732966, and KJ812998, respectively) (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/22/4/15-1176-Techapp1.pdf). Compared with the 3 plasmids, pK351 is missing a 4,086-bp region between insertion sequence (IS) IS*1* and IS*903*-like mobile elements, probably due to IS*1*-mediated deletion. In addition, the region containing gene *ccdBA* between gene *resD* and an IS*1* remnant is replaced by a region encoding 2 hypothetical proteins in pK351. The remainder of pK351 exhibits 99.95% identity to the 3 related plasmids. The immediate genetic environment of bla_{NDM-1} in pK351 is identical to that in the 3 related plasmids, encompassing bla_{NDM-1} itself and the downstream sequence, flanked by 256-bp direct repeats (*7*).

Plasmids pKOX_NDM1 and pRJF866 were found in a *K. oxytoca* strain from Taiwan and a *K. pneumoniae* ST11 strain from Shanghai, China, respectively (*7*,*8*). *K. oxytoca* (pKOX_NDM1) was isolated from a patient from Taiwan who underwent surgery in Jiangxi, China. *K. pneumoniae* ST11 (pRJF866) was isolated from a patient in a burn unit in Shanghai just after a highly related NDM-1–producing *K. pneumoniae* ST11 strain was isolated from another patient in the same unit who had traveled to Jiangxi Province (*8*). pNDM-Ec1GN574 was detected in an *E. coli* strain from a patient previously hospitalized in India before being admitted to a community hospital in Canada (*9*). Identification of highly similar bla_{NDM-1}–carrying plasmids in various strain lineages and species in different locales suggests extensive horizontal transfer of these plasmids among broad-range hosts. Acquisition of these plasmids by globally distributed, multidrug-resistant *K. pneumoniae* lineages (ST11 and ST147) is of grave concern.

The epidemiology of NDM-1–producing *Enterobacteriaceae* continues to evolve. The case reported here was imported to the United States upon patient transfer from Iran (*10*). The unusual path for this NDM-1–producing *K. pneumoniae* supports the hypothesis that the Middle East might be an additional reservoir for NDM producers.

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Ritual Slaughter as Overlooked Risk Factor for Brucellosis

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To the Editor: The current rates of animal and human brucellosis in southern Israel are unacceptably high (*1*). Unsupervised livestock rearing, smuggling of herds, and the dissolution of Israel's "test, slaughter, and compensate" program for small ruminants in 1997 have generated a large, uncontrolled animal reservoir in the region. The Bedouin Arab inhabitants who live in close proximity to herds and consume unpasteurized dairy products are disproportionately affected. Outbreaks from parts of Israel that were previously free of brucellosis are reported with increasing frequency. Moreover, 2 decades after supposed elimination of bovine brucellosis, a cattle herd adjacent to Bedouin grazing areas was found to be highly infected with *Brucella melitensis* (*2*).

We report 5 cases of severe brucellosis in patients from a community whose members typically do not raise herds and do not consume unpasteurized dairy products. The patients were Ethiopian-born Jews who exhibited fever and either respiratory signs or radiologic evidence of new pulmonary findings (Table). The true nature of the infection only became evident when *B. melitensis* was identified from blood cultures. Directed questioning revealed that all patients participated in ceremonial slaughter of sheep that were purchased from Bedouin owners in southern Israel.

The diagnostic pitfalls encountered by the medical staff are exemplified in the case of patient 1 (Table). In May 2014, this 68-year-old man was admitted to a hospital in southern Israel, with a 14-day history of fever, cough, and night sweats. His medical history was notable for asthma and previously treated pulmonary tuberculosis (TB). Computed tomography scan of his chest showed a new 1.5-cm^2 apical lung lesion with irregular borders. Laboratory evaluations were negative for rickettsiae, *Coxiella burnetii*, HIV, *Plasmodium* spp., and *Mycobacterium tuberculosis*. Four days after admission, gram-negative coccobacilli grew from his blood culture. *Brucella* IgM titer was 1:1,920. The patient received appropriate treatment for 6 weeks (Table) and recovered. *Brucella* IgM titers dropped to 1:40. Four months later, he returned to the infectious diseases clinic exhibiting fever, chills, and clinical and sonographic evidence of epididymo-orchitis. *Brucella* IgM titers rose to 1:1,240. Treatment was resumed for 3 months. In addition, a thoracoscopic biopsy of the lung lesion was performed to rule out malignancy. Pathologic examination of the biopsy specimen revealed a focus of fibrosis, with giant cells surrounded by lymphocytes (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/22/4/15-1192-Techapp1.pdf). After treatment, the patient's symptoms resolved and titers returned to low levels.

The long symptom-to-diagnosis interval (range 21–97 days) for patients in this report is alarming (Table). Treatment delays are associated with increased focal complications and relapse rates (*3*). Further, high case-fatality rates, allegedly due to low physician awareness, were reported in a largely immigrant cohort of brucellosis patients in Germany (*4*).

Several circumstances might have led to the failure to include brucellosis in the initial differential diagnosis for these patients, even in a disease-endemic region. First, we can assume that physicians are unfamiliar with the ceremonial slaughter central to the celebrations of Ethiopian Jews.

Table. Characteristics, treatment, and outcomes of patients with brucellosis who engaged in ritual slaughter, Israel*

Pt no.	Age, y/sex	Clinical features	Laboratory findings at admission	Chest imaging	Alternative diagnoses	Fever-to-diagnosis interval, d	Complication	Treatment and outcome
1	68/M	Cervical neck pain; cough; night sweats; 38°C	Hb, 11.9 mg/dL; leukocytes, 16.3 × 10^3 /μL; AST, 63 U/L; ALT, 118 U/L	CT: apical lung finding, new onset	Asthma exacerbation; lung malignancy	21	Focal lung lesion; relapse: epididymo-orchitis; suspected osteomyelitis C6: increased uptake bone scan	STR/2 wk, dox + cipro/6 wk; relapse: rising *Brucella* titers + epididymitis; same 3 drugs/12 wk; recovery
2	70/M	1st admission: fever; productive cough	Hb,14.9 mg/dL; leukocytes, 12 x 10^3 cells/μL; platelets, 136 x 10^3/μL; AST, 61 U/L; ALT, 47 U/L	Chest radiograph: retrocardial infiltrate	Asthma exacerbation; bronchitis	NA		Inhalatants: IV solumedrol, then oral prednisone
		2nd admission: hypothermia: 35.7°C; pulse oximetry, 94% on room air	Hb: 12 mg/dL; leukocytes, 2.8 x 10^3 cells/ μL, 3.7 x 10^3 cells/μL; platelets, 38 x 10^3/μL; Na, 129 meq/L; AST, 134 U/L; ALT, 100 U/L	CT: multiple RUL pulmonary nodules; mediastinal lymphadenopathy	TB	92	Sepsis	STR/2 wk, dox/6 wk: recovery
3	45/M	Fever; prolonged headache	Hb, 11.7 mg/dL; AST, 58 U/L; ALT, 102 U/L; Na, 133 meq/L; ESR, 70 mm Hg/h	Chest radiograph: diffuse bilateral pulmonary nodules rule out miliary TB	TB; cryptococcal meningitis	21	Suspected discitis C5–6 per MRI	Genta/wk, dox + cotrim/12 wk; recovery
4	55/M	Cough; fever; low back pain	Hb, 11.8 mg/dL; AST, 86 U/L; ALT, 120 U/L; ESR, 90 mm Hg/h; CRP, 92.6 mg/L	Chest radiograph: peribronchial thickening	Pneumonia (rx cefuroxime); temporal arteritis	28		Genta/2 wk, dox/6 wk; persistent low back pain
5	49/F	Cough; fever	Hb, 9.5 mg/dL; leukocytes, 3.7 x 10^3 cells/μL; platelets, 116 x 10^3/μL	Chest radiograph: no pathologic changes; CT: no pathologic changes	TB; infective endocarditis caused by *Actinobacillus ureae*	90		Genta/2 wk; dox + rif/7 wk; recovery

*Pt, patient; lab, laboratory; Hb, hemoglobin; leukocyte: leukocytes; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CT, computed tomographic scan; C6, cervical vertebra 6; STR, streptomycin; dox, doxycycline; cipro, ciprofloxacin; NA, not available; IV, intravenous; Na, sodium; RUL, right upper lobe; TB, tuberculosis; ESR, erythrocyte sedimentation rate; MRI, magnetic resonance imaging; genta, gentamicin; cotrim, cotrimoxazole; CRP, C-reactive protein; rx, prescription; rif, rifampin.

The tradition includes slaughtering, skinning, and eviscerating a sheep, followed by mincing of the sheep meat. This venerated ritual is performed by trained members of the Ethiopian community and supervised by the spiritual leader, the *Kes* (*5*). Second, patients were consistently reluctant to disclose their participation in ceremonial slaughter to medical staff. Third, the managing physicians considered differential diagnoses for febrile respiratory illness in line with the patients' Ethiopian origins: reactivation of TB or chronic pulmonary disease exacerbation (*6*). Patients 3 and 5 had a history of prolonged cough at admission; patient 3 had chest radiograph results suggestive of miliary TB. Finally, for patient 5, *Actinobacillus ureae* was initially but erroneously identified as the cause of bacteremia.

All patients in our study had clinical or radiologic evidence of lung involvement. Causality between exposure to *Brucella*-infected aerosols and pulmonary manifestations of brucellosis has been demonstrated in animal models. After an aerosol challenge with *B. melitensis*, animal lungs have shown perivascular inflammation as well as microgranulomas (*7*). In a study of hunters infected with *B. suis*, in which 38% had respiratory symptoms, aerosol spread or conjuctival innoculation was considered the most likely route of infection (*3*).

Aerosol exposure during slaughter could be linked to the pulmonary manifestations of brucellosis observed in these patients. The granulomatous changes in the lung biopsy specimens of patient 1 are typical of lung involvement in brucellosis (*8*). The patients in this report did not use protective gear during contact with animal parts, which inevitably increased their risk for infection through direct or aerosol contact (*9*).

This reports illustrates an unsuspected mode of brucellosis transmission in an area with soaring brucellosis

rates: transmission from infected animals to persons clandestinely engaging in ritual slaughter; specifically, an Ethiopian Jewish community. Physicians in countries receiving immigrants should be aware of ceremonial practices that place patients at risk for zoonoses. The severe respiratory manifestations that ensued following aerosol exposure to animal blood or secretions suggest that brucellosis with pulmonary involvement after inhalation of *Brucella*-infected aerosols might be more common than previously documented.

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Follow-up of Ebola Patient, 2014–2015

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To the Editor: The 2014–2015 epidemic of Ebola virus disease (EVD) in West Africa affected 23,666 persons and caused 14,603 deaths (*1*). The World Health Organization (WHO) declared the epidemic a public health emergency (*2*). Although Ebola virus is transmitted by unprotected physical contact with infected persons, published reports about which body fluids are infected or the risk for fomite transmission are few (*3*). For most cases, virus was detected by reverse transcription PCR (RT-PCR) of clinical (saliva, feces, semen, breast milk, tears, nasal blood, skin swab) and environmental specimens (*4*). Earlier reports of the follow-up of recovered patients stated that viral RNA was detected by RT-PCR for up to 33 days in vaginal, rectal, and conjunctival swab samples from 1 patient and up to 101 days in seminal fluid from 4 patients. Infectious virus was detected in 1 seminal fluid sample 82 days after disease onset (*4,5*).

Attendees at the Eighth Meeting of the WHO Advisory Group on the EVD Response (*1*) discussed potential risk factors, including hidden chains of transmission and sexual transmission, and determined the following criteria. A country can declare “interruption of transmission” when 42 days have elapsed since the last diagnosis of a case. A country can declare that the “outbreak has stopped” when test results from the last case are negative twice or after another 90-day interval. For determining a cutoff for finally declaring the strategy and criteria for elimination, extensive follow-up on infectivity of semen in Ebola survivors is needed.

We report follow-up of a man who recovered from EVD and was monitored for 165 days after he was declared Ebola-free. The 26-year-old man from India returned to New Delhi, India, from Liberia on November 10, 2014, with a certificate from the government of Liberia stating that he was “cured” of Ebola. Because EVD is considered an exotic disease in India, he was placed in isolation at the Airport Health Organization quarantine center at Indira Gandhi International Airport, New Delhi (*6*). Serum and semen samples were collected and sent to the National Centre for Disease Control (NCDC), New Delhi, and the National Institute of Virology (NIV), Pune, India. The serum was negative by

Table. Results of attempted real-time RT-PCR and virus isolation from semen of Ebola virus disease survivor, 2014–2015*

Sample no.	Days declared Ebola-free†	RT-PCR C_t		Cell lines used for virus isolation	Passage							
					1		2		3		4	
		NCDC‡	NIV§		CPE	C_t	CPE	C_t	CPE	C_t	CPE	C_t
1	45	17	22.5	Vero CCL81	None	28.5	None	None	None	None	None	None
				Vero E6	None	31.5	None	None	None	None	ND	None
2	64	21	24	Vero CCL81	None	None	None	None	None	None	ND	ND
				Vero E6	None	32.5	None	None	None	None	ND	ND
3¶	77	22	ND	NA	NA	NA	NA	NA	NA	NA	NA	NA
4¶	98	26	ND	NA	NA	NA	NA	NA	NA	NA	NA	NA
5¶	111	27	ND	NA	NA	NA	NA	NA	NA	NA	NA	NA
6	125	30	34	Vero CCL81	None	None	None	None	ND	ND	ND	ND
				Vero E6	None	None	None	None	ND	ND	ND	ND
7	141	28	38	Vero CCL81	None	None	None	None	ND	ND	ND	ND
				Vero E6	None	None	None	None	ND	ND	ND	ND
8	165	28	35.0	Vero CCL81	None	None	None	None	ND	ND	ND	ND
				BHK-21	None	None	None	None	ND	ND	ND	ND

*C_t, cycle threshold; CPE, cytopathic effect; NA, not applicable; NCDC, National Centre for Disease Control, New Delhi, India; ND, not done; NIV, National Institute of Virology, Pune, India; RT-PCR, reverse transcription PCR.
†Primers from http://www.crcf.sn/wp-content/uploads/2014/09/CDC-Ebola-International-Lab.pdf.
‡Primers from the Centers for Disease Control and Prevention, Atlanta, Georgia, USA.
§Primers from (7).
¶Samples received at NCDC only.

real-time RT- PCR ([*7*]; http://www.cdc.gov/vhf/ebola/diagnosis/index.htm) and positive (titer 1:400) by IgG ELISA (*8*). However, the semen was positive for Ebola viral RNA by real-time RT-PCR. A viral RNA extraction kit (QIAGEN, Valencia, CA, USA) was used to extract RNA from samples. Real-time RT-PCRs were set up in a Bio-Rad real-time PCR machine (Model C1000 Touch CFX96; Hercules, CA, USA) by using a SuperScript III Platinum One-Step qRT-PCR kit with ROX (Invitrogen, Carlsbad, CA, USA).

As a confirmatory measure, partial nucleoprotein gene (1216 nt), partial viral protein (VP) gene (337 nt), and the intergenic region near the VP gene along with VP polyA tail (383 nt) from the semen sample were amplified by RT-PCR (*9*). These sequences (GenBank accession nos. KT191140–KT191142) showed 100% similarity with *Zaire ebolavirus* isolate EBOV/DML14077/SLe/WesternUrban/20150630. To avoid cross-contamination, we used a γ-inactivated Ebola Reston virus strain as a positive control. The RT-PCRs were set up in a Bio-Rad thermal cycler (C1000) by using SuperScript III One-Step RT-PCR System with Platinum-Taq-DNA-Polymerase (Invitrogen).

Ebola virus isolation attempts were made from the semen sample in VeroE6 cells, Vero CCL81, *Pipistrellus* bat embryo, and BHK-21 cell lines at NIV. No isolate was obtained. Cycle thresholds of 28.5 and 31.5 were observed in Vero CCL81 and Vero E6 cells, respectively, in the first passage by real time RT-PCR, but no virus or cytopathic effect was detected in the subsequent 2 passages (Table).

According to WHO guidelines, the semen sample was transported from NCDC to a Biosafety Level 4 laboratory at NIV (for virus isolation). At the time of inoculation in cell culture, the sample had been subjected to a single freeze–thaw cycle. The sensitive nature of the virus may be why Ebola virus was not isolated. PCR positivity alone is not sufficient for considering a patient infectious for Ebola; however, because EVD is considered an exotic disease in India, we depended on real-time RT-PCR–based data for establishing EVD positivity.

Follow-up semen samples were positive by real time RT-PCR for up to 165 days after the patient was declared Ebola-free and were negative thereafter. Cycle thresholds of samples tested at NIV were 22.5 on day 45 after being declared Ebola-free, 24 on day 64, 34 on day 125, 38 on day 141, and 35.0 on day 165 (Table).

Clear criteria for elimination and declaration of the end of an outbreak are needed because any misinterpretation or miscommunication among the countries could negatively affect community confidence (*10*). Although we monitored the patient for 165 days, monitoring began ≈10 days after the patient had recovered. Ebola viral RNA persistence has been documented in a human semen sample for up to 10 months after the patient was declared Ebola-free (*11*). According to the data from this study, the current elimination period may need to be extended, and further studies on the infectivity of semen samples from recovered EVD patients are warranted.

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Sustained Elevated Cytokine Levels during Recovery Phase of Mayaro Virus Infection

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To the Editor: Mayaro virus (MAYV), a mosquito-borne alphavirus endemic to South America, causes a self-limiting febrile arthralgia syndrome closely resembling Chikungunya fever (*1*). MAYV has been detected increasingly as imported infections in international travelers returning to Europe and North America (*2*–*9*). Joint pain, the most prominent symptom, is often long-lasting (several months), sometimes incapacitating (*4*,*6*,*7*,*9*), and may recur (*8*). Arthralgia develops during the acute phase and symmetrically affects the wrists, ankles, and small joints of hands and feet. Joint swelling may occur initially, but permanent joint damage has not been described (*5*). The clinical disease and diagnostic procedures have been described (*1*–*9*), but immunologic parameters and their possible role in the clinical follow-up of patients (i.e., during the postacute long-lasting arthralgia period) remain to be investigated.

To further our knowledge of MAYV infection, we analyzed cytokine levels in serum samples from 6 travelers to South America who returned to Europe with Mayaro fever (MF). Two of the cases occurred during 2014; 4 occurred during 2011–2013 (*2*–*5*).

The 6 travelers comprised 2 men and 4 women who were 20–54 (median 36) years of age (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/22/4/15-1502-Techapp1.pdf). The 2 most recent cases occurred in spring 2014 in a 28-year-old female student and a 54-year-old male physician. Serologic testing was performed for both patients at the Bernhard Nocht Institute and confirmed by virus neutralization testing (*4*).

The student had traveled for 3 weeks in Ecuador, visiting rainforest villages and hiking in the jungle. During her

stay, she had experienced myalgia of the forearms, arthralgia of fingers and toes, subfebrile but elevated body temperatures, and maculopapular exanthema. On examination in Germany, the student had no clinical signs of disease, but she reported arthralgia of the ankles and hands. Laboratory test results showed a slightly increased C-reactive protein level (6.2 mg/L, reference value <5 mg/L); liver and kidney values and blood count were within reference ranges. MAYV indirect immunofluorescence assay showed positive IgM and IgG titers (1:320 and 1:2,560, respectively; cutoff for both was <1:20) (*3*). Acute MF was diagnosed. Two weeks later, follow-up serologic testing showed negative IgM but unchanged IgG titers. Arthralgia with stiffness lasted for 6 weeks.

The physician had traveled for 3 weeks through the jungle in Bolivia, during which time headache, myalgia, shivers, and fatigue developed, followed by foot arthralgia and maculopapular exanthema. On his return to Germany,

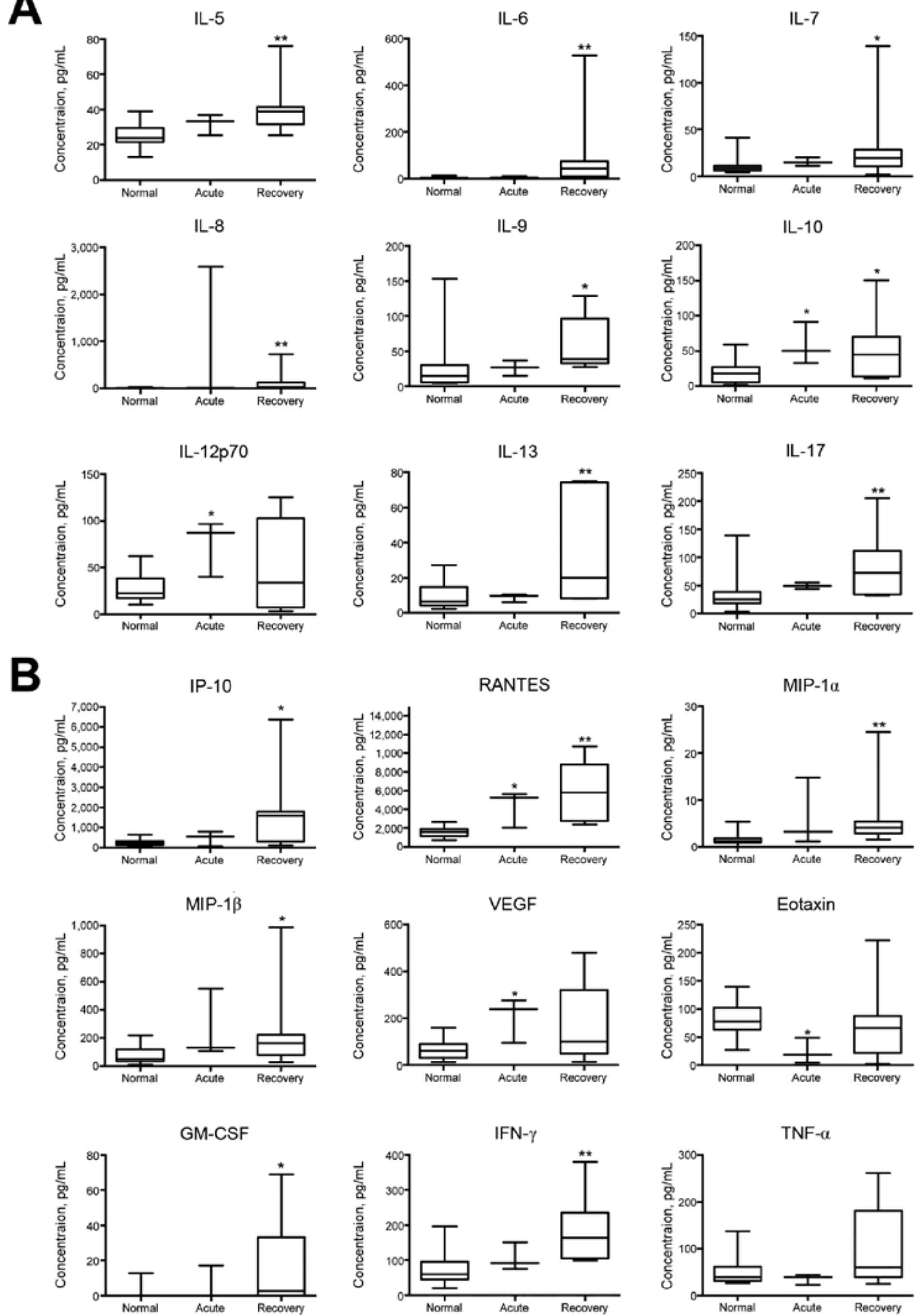

Figure. Changes in cytokine and growth factor levels in the acute and recovery phase of Mayaro fever. Box-and-whisker plots show median, upper and lower quartile, minimum, and maximum values. A) During the prolonged recovery phase, serum levels of interleukin (IL) 5–10, IL-13, and IL-17 were significantly elevated compared with levels for healthy controls. IL-10 levels were also significantly increased during the acute phase, as were IL-12p70 levels. B) Significantly increased serum concentrations of interferon-γ (IFN-γ)–induced protein 10 (IP-10), regulated on activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory proteins (MIP)–1α and –1β, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IFN-γ were detected in the prolonged recovery phase; significant elevations were also seen in the acute phase for RANTES and vascular endothelial growth factor (VEGF). Tumor necrosis factor α (TNF-α) concentrations showed a nonsignificant median decrease during the acute phase, whereas eotaxin levels were significantly decreased at that time. *p<0.05 and **p<0.01, versus values for healthy controls (Kruskal-Wallis test).

he was seen in an outpatient practice for persisting (2 months) bilateral foot pain. Laboratory test results showed C-reactive protein levels, and liver and kidney function values, and full blood count within reference ranges. MAYV indirect immunofluorescence assay was negative for IgM but positive for IgG (1:20,480). Postacute MAYV infection was diagnosed. Two months later, follow-up serologic testing showed unchanged titers. Arthralgia with pronounced morning stiffness lasted for 6 months.

After obtaining written consent from all patients, we subjected their serum samples to multiplex cytokine serum analyses (Bio-Rad Laboratories, Munich, Germany). Blood was drawn at different times after symptom onset (15–117 days). Serum samples were classified as acute (≤30 days after symptom onset, n = 3) or postacute (>30 days after symptom onset, with arthralgia, n = 8). Twenty serum samples from healthy blood donors were run in parallel. During the acute phase of MF, interleukin (IL) 10, IL-12p70, RANTES (regulated on activation, normal T cell expressed and secreted), and vascular endothelial growth factor concentrations for patients were significantly elevated compared with those for healthy controls (Figure). Furthermore, a significant decrease was noted for eotaxin levels during the acute phase of disease. In the postacute arthralgic recovery phase, concentrations of IL-5–10, IL-13, IL-17, IP-10 (interferon-γ–induced protein 10), RANTES, macrophage inflammatory proteins 1α and 1β, granulocyte-macrophage colony-stimulating factor, and interferon-γ were significantly higher than those for healthy controls. TNF-α concentrations showed a nonsignificant median decrease during the acute phase (Figure). No significant changes in either phase were demonstrated for IL-1b, IL-2, IL-4, basic fibroblast growth factor, granulocyte colony-stimulating factor, monocyte chemotactic protein 1, and platelet-derived growth factor β polypeptide levels (data not shown). Cytokine levels measured in the acute phase did not differ significantly from those measured in the recovery phase.

The most striking clinical feature of MF is long-lasting arthralgia, similar to that seen in chikungunya fever, which develops in the acute phase and persists thereafter. In the travel-associated cases, arthralgia lasted for 2 to >12 months. In the examined patients, the prolonged arthralgia recovery phase was paralleled by significantly increased proinflammatory cytokine levels, indicating ongoing inflammation, probably related to arthritis. Elevated levels of RANTES and IP-10 suggest T-cell recruitment, possibly reflecting virus persistence and replication, as described for the related Chikungunya virus (*10*). Thus, cytokine measurements may be helpful for monitoring patient symptoms, especially when signs of arthritis (swellings and redness) and elevated standard serum inflammatory parameters are no longer present. In our study, the picture of MF cytokine elevations paralleled those described for Chikungunya virus infection (*10*); these elevated levels may help to elucidate the pathogenesis of MAYV-induced arthralgia. More immunology data are required to complete this evolving picture of viral arthralgia syndromes.

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Enterovirus A71 Genogroups C and E in Children with Acute Flaccid Paralysis, West Africa

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To the Editor: Human enterovirus 71 (EV-A71) of the species e*nterovirus A*, genus *Enterovirus*, and family *Picornaviridae* is a serious public health threat because it can cause large outbreaks of hand, foot and mouth disease (HFMD). In addition, EV-A71 has been associated with severe and sometimes fatal neurologic complications that affect mostly infants and children and that range from aseptic meningitis and encephalitis to poliomyelitis-like acute flaccid paralysis (AFP) (*1*). EV-A71 has been classified into 7 genogroups, A–G, on the basis of the diversity of the nucleotide sequences of the viral protein 1 (VP1) capsid (*1,2*). Since 1997, increasing epidemic activity of genogroups B and C has been reported in the Asia-Pacific region and has caused large HFMD outbreaks with high rates of illness and death. Subgenogroup C2 was identified as the main causal agent associated with cases of fatal encephalitis in the devastating HFMD outbreaks of Taiwan in 1998 and Australia in 1999 (*1*). Since those outbreaks, C2 has been frequently reported in countries in Asia, Europe, North America, and South America and has been associated with neurologic complications and fatal infection (*1*). Although genogroups E and F were recently discovered in Africa, the epidemiology of EV-A71 has been largely unexplored in this continent. Only 4 AFP cases associated with EV-A71 infection have been reported in Africa (*2–5*), and the only outbreak caused by EV-A71 occurred in Kenya in 2000 among a small number of HIV-infected orphans and was attributed to genogroup C (*6*).

To investigate the circulation and genetic diversity of EV-A71 in West Africa, we retrospectively analyzed 236 nonpolio enterovirus (NPEV) isolates obtained through routine poliomyelitis surveillance activities at the World Health Organization's Regional Polio Laboratory in Senegal during 2013–2014. Following WHO guidelines (*7*), the laboratory received and processed stool specimens from 1,600 children with AFP from various West Africa countries. NPEV was found in isolates from most countries except Cape Verde (0/5 specimens): Gambia (8/64), Guinea-Bissau (8/49), Guinea (42/355), Mauritania (20/108), Niger (95/596), and Senegal (63/423). NPEV was initially characterized by amplification of the VP1 capsid protein coding region by using reverse transcription PCR and partial sequencing, as described (*8*). After molecular typing of all NPEV isolates, we identified 4 new EV-A71 isolates from 4 patients, 1 each from Guinea, Mauritania, Niger, and Senegal (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/4/15-1588-Techapp1.pdf). BLAST analysis (http://www.ncbi.nlm.nih.gov/) of the partial VP1 nucleotide sequences showed that the isolates from this study shared 96%–97% nt sequence identity with those of other EV-A71 isolates deposited in GenBank.

The complete VP1 nucleotide sequences of the 4 EV-A71 isolates were determined by using reverse transcription PCR (GenBank accession nos. KT818793–KT818796; online Technical Appendix Table 2). Sequences were aligned by using ClustalW (http://www.clustal.org). Phylogenetic investigation indicated that the EV-A71 strains detected in 3 of the 4 isolates (from Senegal, Guinea, and Mauritania) clustered within subgenogroup C2. The most closely related EV-A71 strains are not those previously reported from other Africa countries (Madagascar, Cameroon, Nigeria, and Central African Republic) but are strains found in isolates from France, Finland, United Kingdom, Spain, Germany and Netherlands, all isolated during 2006–2010 (Figure). The C2 clusters from Africa and Europe showed an average of 97.1% nt identity and 99.8% aa similarity. Within the Africa C2 cluster, the nucleotide and amino acid alignments displayed a substantial proportion of conserved positions: 862/891 (96.7%) and 297/297 (100%), respectively. Because most enteroviruses evolve at the rate of ≈1% nt substitutions per year in the VP1 region, the sequence divergence of ≈3% suggests that these enteroviruses had been circulating in the region for ≈3 years before they were detected. However, we cannot exclude the possibility that these isolates originated from multiple importation events from abroad, especially from countries in Europe. The fourth isolate (from Niger) clustered within genogroup E, which, before this study, included only 2 complete VP1 sequences from isolates from Central African Republic and Cameroon and an additional partial VP1 sequence from an isolate from Nigeria.

Comparison of complete VP1 amino acid sequences of all EV-A71 strains considered for the phylogenetic analysis showed that 107 (36%) of 297 aa sites were variable. None of the residues found in the variable sites of the Africa strains in our study corresponded to residues previously associated with genogroup C neurovirulent phenotypes (A170V, N31D, L97R, G145E and D164E) (*9,10*). The Niger E isolate showed specific residues (L24M, A170T) that differed from those of other genogroup E isolates.

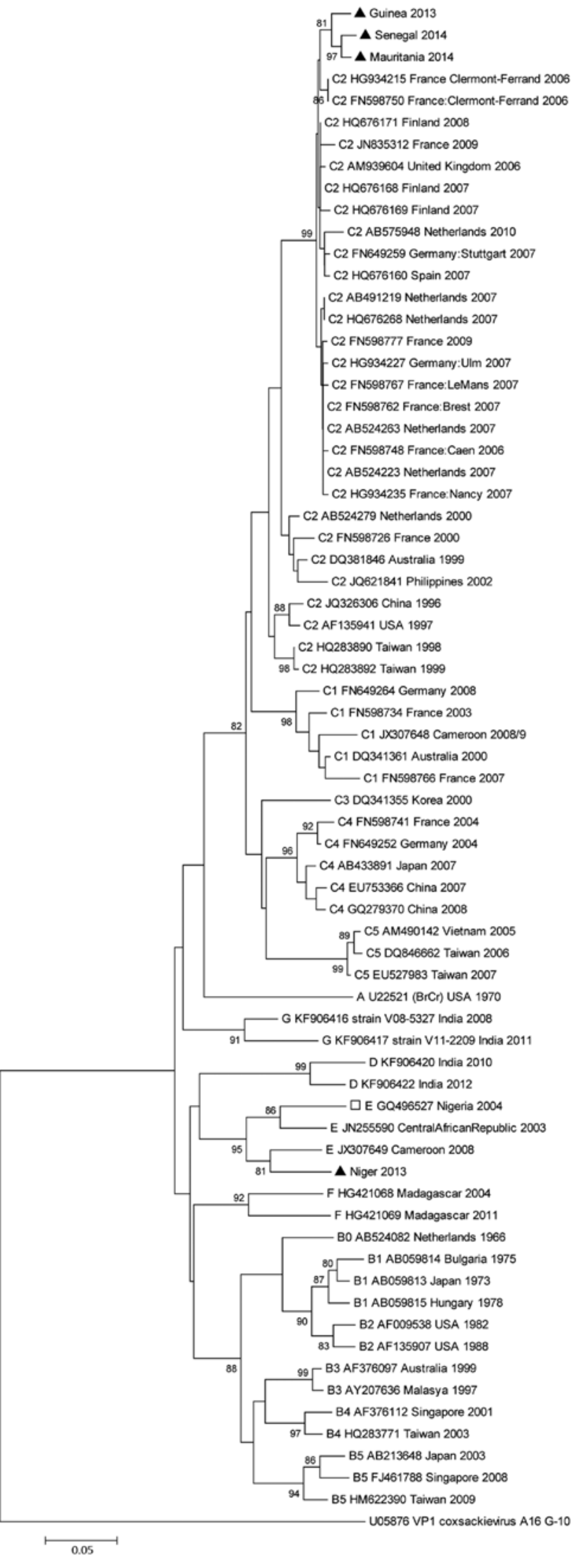

Figure. Phylogenetic tree created with the complete VP1 nucleotide sequences (891 bp in length) of enterovirus A71 isolated from 4 patients with acute flaccid paralysis in West Africa, the most similar nucleotide sequences identified by a search in GenBank by using BLAST (http://www.ncbi.nlm.nih.gov/), and a representative global set of enterovirus A71 sequences belonging to different genogroups and subgenogroups. The coxsackievirus A16 prototype G-10 sequence was introduced as the outgroup. The tree was inferred with a neighbor-joining method that used MEGA5 software (http://www.megasoftware.net/). Distances were computed by using the Kimura 2–parameter model. The robustness of the nodes was tested by using 1,000 bootstrap replications. Bootstrap support values >80 are shown in nodes. Black triangles indicate the 4 strains from this study. Open square indicates a partial sequence. Scale bar represents nucleotide substitutions per site. Abbreviations of virus names indicate genogroups or subgenogroups/GenBank accession number/origin/year of isolation, respectively. A color version of this figure is available online (http://wwwnc.cdc.gov/EID/article/22/4/15-1588-F1.htm).

Our findings highlight the presence of EV-A71 with a high degree of genetic diversity in patients with AFP in West Africa. Future studies about the burden of disease, epidemiologic features, and evolution of EV-A71 in this region of Africa are needed to implement appropriate public health measures.

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Hepatitis E Virus Prevalence among Blood Donors, Ouagadougou, Burkina Faso

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To the Editor: The safety of blood product use is continually improving, but blood transfusion remains a challenge in Africa, given the high prevalence of bloodborne pathogens (*1*). In Africa, the main serologic tests done to reduce blood transfusion risks are for HIV and hepatitis B and C viruses. However, unknown or emerging pathogens among the population of blood donors, such as hepatitis E virus (HEV), may also jeopardize transfusion safety.

HEV is emerging as a potential threat to blood safety. High rates of HEV IgG prevalence among blood donors have been found in studies in the United States (7.7%), England (13.5%), France (16.6%), and Spain (19.6%) (*2*,*3*). A study in Iran showed a prevalence of 14.3% (*4*), and a study in China showed rates of up to 22.7% (*5*). Cases of HEV transmission by transfusion or transplantation have been reported, and recent studies in France and England showed risk for HEV in donated blood ranging from 1/2,218 to 1/2,848 donations (*5*,*6*).

In Burkina Faso, the prevalence of HEV IgG has been reported as 11.6% among pregnant women during 2012. Prevalence is >70% among butchers, who form a population exposed to pigs, which are a reservoir for HEV (*7*,*8*). To determine whether HEV continues to circulate among human populations outside known at-risk populations, we investigated prevalence of HEV IgG and IgM in the blood donor population of Ouagadougou.

During June and July 2014, we recruited 1,497 first-time blood donors (398 women, 1,099 men) within the National Blood Transfusion Centre in Ouagadougou. Persons 17–65 years of age who weighed >50 kg were included (Figure, panel A). Candidate donors were excluded if they had previously received blood transfusions, had jaundice or clinical

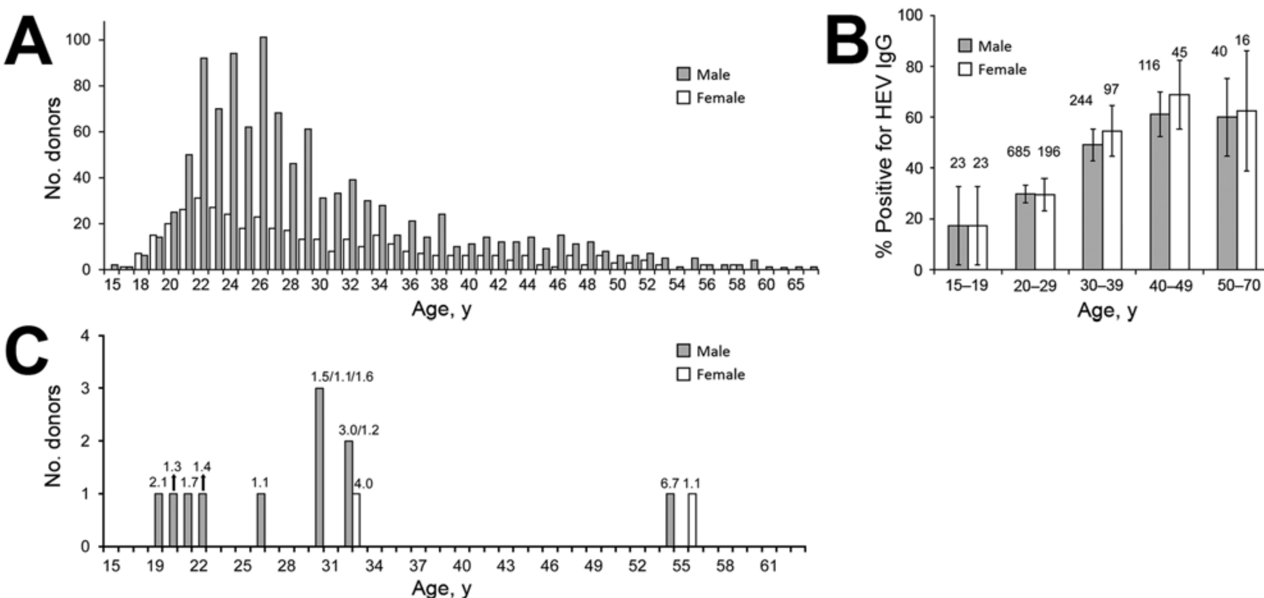

Figure. Age and sex distributions and HEV test results for blood donor population, Ouagadougou, Burkina Faso, 2014. A) All blood donors. Women: mean age 29.62 y, median 27 y, range 17–58 y; men: mean age 29.86 y, median 27 y, range 15–70 y. B) Donors whose samples were positive for HEV IgG. Numbers above bars indicate number of donors tested. Error bars indicate 95% CI for percentage in each category. C) Age and sex distribution of blood donors whose serum samples were positive for HEV IgM. Numbers on data bars are ratios of specific ELISA optical density to cutoff values (IgM index); ratios ≥1 are considered positive. Ratios are shown for each donor. HEV, hepatitis E virus.

signs of hepatitis, were pregnant, or had sexual contact with multiple partners. Demographic data collection was limited to age and sex, and residual serum specimens were anonymized as approved by the Ethics Committee of the National Blood Transfusion Centre. We used Dia.Pro IgG ELISA (Diagnostic Bioprobe Srl, Sesto San Giovanni, Italy) to detect HEV IgG; this assay uses HEV-specific synthetic antigens derived from open reading frame (ORF) 2 and ORF3 of all 4 HEV subtypes. We used Wantai ELISA (Wantai Biologic Pharmacy Enterprise Co., Ltd., Bejiing, China) to test 92 randomly selected samples for HEV IgG, which showed concordant results (data not shown) (*8*). We also used the Wantai ELISA for the detection of HEV IgM; this test has a sensitivity of 97.1% (95% CI 94.6%–98.5%) and a specificity ranging from 95.3% in serum samples from patients with acute hepatitis A to 100% in healthy donors (http://www.ystwt.cn/IFU/HEV/HEV-IgM_CE.pdf). The HEV IgM positive samples were tested twice for accuracy. All tests were performed according to the manufacturers' instructions; positive and negative controls were used in each plate.

The prevalence of HEV IgG was 39.0% (95% CI 36.5%–41.5%) by using Dia.Pro ELISA (Figure, panel B). This prevalence was twice that found in 2012 (*8*), but such wide variations were commonly found in Africa (*9*). In France, prevalence ranged widely, from 10% in the north to 52% in the south (*6*). HEV IgG prevalence increased significantly with age ($p<0.001$ by χ^2 test for trend) in both male and female donors, but age variation explained only partially the differences in the study population and those from a previous study (*8*). As described in France and other high-income countries (*4*,*6*), Traoré et al. found HEV genotype 3 in swine in Burkina Faso (*10*); thus, poor sanitation that disperses this oral–fecal transmitted virus might result in a high prevalence of HEV antibodies among the general population without causing epidemic illness that is more often associated to genotype 1.

Using the Wantai test, we found HEV IgM, a marker of recent infection, in samples from 2 women and 11 men in the blood donor population (1.9%, 95% CI 1.2–2.6% [Figure, panel C]). Samples from 7 men were positive for HEV IgG.

The HEV exposure prevalence we observed is similar to most of the published data from countries reporting endemic HEV and silent infection (*6,7*). IgM seroprevalence of 1.9% is indicative of low ongoing infection cycles, although no reference test is available (*2*). Our study was limited by the absence of HEV RNA screening to assess the presence of HEV particles and genotype in donated blood. However, HEV circulation is supported by 1) IgM signs of recent infection; 2) the commonality of silent infections with HEV, specifically genotype 3; and 3) another study that showed a clear, although rare, positive relationship between the number of IgM-positive samples and the number of HEV RNA-positive samples (*4*).

The risk for HEV infection through transfusions of donated blood emerged in West Africa in a similar way as described in European countries. Further assessment of the transfusion risk associated with HEV-positive donors will require an evaluation of HEV RNA in prospective donors and posttransfusion surveillance of occurrence of hepatitis.

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Porcine Deltacoronavirus, Thailand, 2015

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To the Editor: Porcine deltacoronavirus (PDCoV) was first reported in Hong Kong in 2012 and included the HKU15-44 and HKU15-155 strains (*1*). In early 2014, PDCoV was reported in pigs with diarrhea on swine farms in Ohio, USA (*2*), and later in other states (*2–5*). In April 2014, PDCoV strain KNU14-04 was reported in pigs in South Korea (*6*). A retrospective study in 2012 reported PDCoV strain S27 in Sichuan, China (*7*). Recently PDCoV strain CNJXNI2 has been reported in pigs with diarrhea in Jiangxi, China (*8*).

There are currently 28 complete PDCoV genomes from China, South Korea, and the United States available in GenBank. We report emergence of PDCoV infections on a commercial swine farm in Thailand.

In June 2015, we investigated reports of acute diarrhea in piglets, gilts, and sows on a swine farm. An outbreak occurred on a commercial swine farm (3,000 sows) located in the eastern province of Thailand. Clinical signs, including acute watery diarrhea, loss of appetite, and agalactia, were observed in gilts and sows in the breeding and gestation houses. Subsequently, piglets in farrowing houses had clinical signs (depression, fever, watery diarrhea, and severe dehydration). Although clinical signs were detected less frequently in fattening pigs in growth-finishing houses, PDCoVs were later detected from blood samples of fattening pigs.

The outbreak lasted 6 weeks (June 10–July 20, 2015). The mortality rate was 27.63% (829/3,000) in sows and 64.27% (2,892/4,500) in piglets but was lower than that usually observed for porcine epidemic diarrhea virus (PEDV) infection. A total of 865 (19.22%) piglets died and were culled during 10 production weeks. Postmortem examination of dead piglets showed emaciated animals and yellow pasty feces. Intestines and colons showed thin walls with a watery content and curdled milk. Histopathologic examination showed shortened and fused villi in the jejunum and ileum. An attenuated and vacuolated cytoplasm in enterocytes was also observed (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/22/4/15-1852-Techapp1.pdf) (*9*,*10*).

We examined 30 samples from the affected swine farm. Blood (n = 10), intestine (n = 8), lymph node (n = 2), feces (n = 6), and feed (n = 4) samples were collected for 2 day-old piglets and 17-, 19-, and 20-week-old fattening pigs. A total of 26 samples were positive for PDCoV by reverse transcription PCR (*2*) (online Technical Appendix Table 1). Because sick pigs had clinical signs similar to those of pigs with other swine virus diseases, all samples were tested for transmissible gastroenteritis coronavirus; PEDV; rotaviruses A, B, and C; porcine reproductive and respiratory syndrome virus; and circovirus. All test results were negative.

We selected 2 PDCoVs (S5011 and S5015L) for whole-genome sequencing and 14 PDCoVs for sequencing of spike (S), envelope (E), membrane (M), and nucleocapsid (N) genes and the 3′-untranslated region (UTR). Nucleotide sequences obtained were submitted to GenBank (online Technical Appendix Table 2).

Sequence analysis of the 2 PDCoVs from Thailand showed that their whole genomes had 99.98% nt identity (only 4 nt differences) with each other and highest nucleotide identities with PDCoVs from China (98.43% with AH2004). S gene sequences showed greatest diversity (99.97%–100% nt identities and 99.91%–100% aa identities) for PDCoVs from Thailand and 95.93%–96.68% with other reference PDCoVs, which is consistent with findings of previous report (*5*). In contrast, E, M, and N genes were conserved (100% nt identities for PDCoVs from Thailand and 99.19%–100% for E genes, 98.28%–99.07% for M genes, and 96.88%–97.81% for N genes with reference PDCoVs) (online Technical Appendix Table 3).

Phylogenetic analysis of the whole genome of PDCoVs from Thailand showed close relatedness with AH-2004, HKU15-44, S27-2012, and HKU15-155 virus strains from China. However, these viruses from Thailand were in a different subcluster than PDCoVs from the United States (Figure; online Technical Appendix Figure 2). PDCoVs identified in this study might represent a new variant of PDCoV because these 2 viruses have unique sequence characteristics: 3-nt (TCT) and 1-nt (A) deletions in the 5′-UTR, 6-nt (AGTTTG) and 9-nt (GAGCCAGTC) deletions in open reading frame 1a/b, and 4-nt (CTCT) insertion in the 3′-UTR (online Technical Appendix Table 4).

We identified PDCoV on a commercial swine farm in Thailand. Affected pigs had clinical signs of acute watery diarrhea, similar to those of pigs infected with PEDV, and had moderate illness and low mortality rates. PDCoVs were detected in symptomatic piglets, sows, and fattening pigs, although clinical signs in fattening pigs were least severe.

Swine farmers and veterinarians should be aware of PDCoV as another causative agent of watery diarrhea in pigs. Similar to PEDV, Wang et al. reported that sequence deletions, insertions, and mutations in PDCoVs in pigs might contribute to variant virus virulence (*2*).

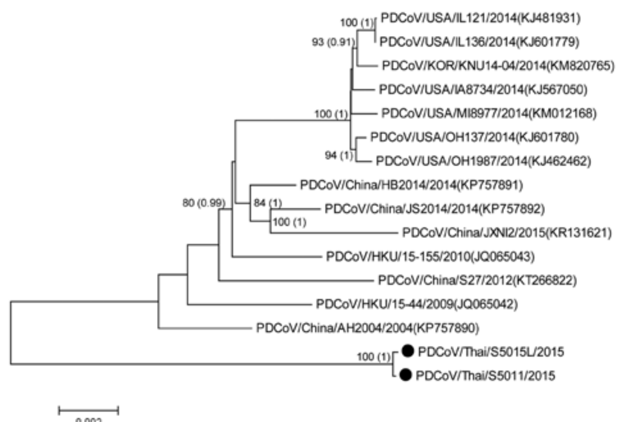

Figure. Phylogenetic analysis of whole-genome sequences of porcine deltacoronaviruses (PDCoVs), Thailand. Black circles indicate strains isolated in this study. The tree was constructed by using MEGA version 6.06 (http://www.megasoftware.net/) with the neighbor-joining algorithm and bootstrap analysis with 1,000 replications and BEAST (http://beast.bio.ed.ac.uk/) with Bayesian Markov chain Monte Carlo analysis of 5,000,000 generations and an average SD of split frequencies <0.05. Numbers along branches are bootstrap values (posterior probabilities). Scale bar indicates nucleotide substitutions per site.

Our findings might assist in development of diagnostic assays for differentiating PDCoVs in Thailand from PDCoVs in other countries. Because PDCoVs from Thailand were highly related to each other, PDCoV might have transmitted into Thailand by a single event. However, verification of this possibility would be difficult. Similar to the situation in the United States, PDCoV might be underdiagnosed in Thailand.

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Ebola Virus in Breast Milk in an Ebola Virus–Positive Mother with Twin Babies, Guinea, 2015

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To the Editor: Field clinicians working during the unprecedented Ebola virus disease (EVD) outbreak in West Africa, which began in December 2013, have been confronted with complex situations concerning mothers and breast-fed children in which one or both in a pair have tested positive for Ebola virus (EBOV) (*1*). More data, especially regarding virus shedding in breast milk, is critical to provide better care and guidance in future outbreaks. We report the case of a lactating EBOV-positive mother and her twin babies. The case is anonymously reported with the mother's consent. The study met the Médecins Sans Frontières Ethics Review Board and approved criteria for studies of routinely collected data.

In Guinea in 2015, a woman and her 4-month-old twins (baby 1 and 2) were registered as contacts after the woman's mother tested positive for EBOV (postmortem diagnosis by reverse transcription PCR [RT-PCR]). The woman and her babies, who were exclusively breast-fed, were followed daily by contact tracers. When baby 1 became febrile, the woman left her home to seek help from a traditional healer, bringing both twins with her. A few days later, baby 1 died and was buried without EBOV testing; according to the World Health Organization case definition, baby 1 was a probable EVD case-patient (*2*).

Eleven days after baby 1 died, the woman became sick; 5 days later, she was admitted to an Ebola treatment center. At admission (day 0), she had headache, loss of appetite, abdominal pain, joint pain, dysphagia, conjunctival injection, and myalgia but was afebrile. On day 1, a blood sample from the woman was positive for EBOV by RT-PCR (Xpert Ebola Assay, GeneXpert Instrument Systems; Cepheid, Sunnyvale, CA, USA) with a cycle threshold (C_t) of 32.5. Baby 2 tested negative for EBOV on day 1 and 72 hours later. Baby 2 was tested twice because he was considered at high risk for infection after being breast-fed for 6 days while his mother was symptomatic (i.e., until day 1 of her hospital admission).

On day 1, the woman was given convalescent-phase plasma from EBOV survivors; the treatment was given according to a compassionate-use protocol and was the standard process in this center at the time. On day 6, breast milk was sampled and tested positive for EBOV (C_t 21.6) (Table). The woman's clinical course was favorable; she remained afebrile during hospitalization, but mild symptoms persisted until day 5. The first convalescent-phase test, done on day 14, showed C_t values of 40.5 and 27.5 for blood and breast milk, respectively. On day 21, a second breast milk sample tested positive (C_t 32.7). On day 24, the woman was given cabergoline (0.5 mg 2×/d for 2 days) to cease lactation, after which no more breast milk samples could be collected. On day 29 after admission, she tested negative for EBOV in blood and urine and was reunited with baby 2. Serologic testing for baby 2 was done on day 23 and showed no sign of previous subclinical infection (ELISA, IgM, and IgG negative).

Many questions in this case remain unanswered, but our findings show the potential infectivity of breast milk for

Table. Overview of results from all Ebola virus RT-PCRs performed during hospitalization of breast-feeding mother of twin babies, Guinea, 2015*

Day after admission	Blood, C_t	Breast milk, C_t	Urine, C_t
1	32.5, glycoprotein	NT	NT
3	33.7, glycoprotein	NT	NT
6	NT	21.6, nucleoprotein	NT
14	40.5, nucleoprotein	27.5, nucleoprotein	NT
18	41.0, glycoprotein	NT	NT
21	40.3, nucleoprotein	32.7, nucleoprotein	NT
25	39.3, nucleoprotein	NT	NT
29	Negative, glycoprotein and nucleoprotein	NT	Negative, glycoprotein and nucleoprotein

*Testing performed by using the Xpert Ebola Assay (GeneXpert Instrument Systems, Cepheid, Sunnyvale, CA, USA). The lowest of the reported glycoprotein and nucleoprotein values are reported. C_t values <20 are highly positive, whereas C_t values >35 are weakly positive. C_t, cycle threshold; NT, not tested.

at least 26 days after EVD symptom onset and demonstrate a case in which a baby was not infected by breast milk from his EBOV-positive mother. However, it should be noted that the woman's breast milk was never tested while she was breast-feeding baby 2.

The literature on EBOV in breast milk of EBOV-positive patients is extremely scarce (*3*). In a previous study from the 2000 Sudan EBOV outbreak in Gulu, Uganda, breast milk from a convalescent-phase patient was sampled 15 days after symptom onset and tested positive for EBOV by RT-PCR and virus culture (*4*). Another study conducted in Guinea during the current outbreak, reported a mother–baby pair in which EVD developed in the baby 14 days after symptom onset in the mother, but breast milk from the mother sampled 17 days after symptom onset was negative by EBOV RT-PCR (*1*).

It is unclear whether infectious virus or defective particles are being secreted in breast milk. C_t values were consistently lower in breast milk than in blood when tested concomitantly, but in this case, breast milk samples were not collected until day 6. Our findings suggest that breast milk is infected by EBOV at a later stage of the disease than blood but then follows the expected replication kinetics observed in venous blood.

Considering the high EVD death rate, until further evidence is found, we recommend that EBOV-positive women stop breast-feeding immediately and that breast-feeding not be resumed until 2 negative RT-PCR tests of the breast milk have been confirmed. This suggestion is in line with the World Health Organization recommendation for testing semen in male EVD survivors (*5*). The public health risk for EBOV to remain in breast milk for at least 26 days after EVD symptom onset and for breast milk to possibly be infectious after a patient has cleared the virus from the blood should also be acknowledged.

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Chronic Infection of Domestic Cats with Feline Morbillivirus, United States

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To the Editor: Feline morbillivirus (FeMV) was first reported in Hong Kong, and mainland China in 2012 (*1*) and has been associated with tubulointerstitial nephritis, the histopathologic correlate of idiopathic chronic kidney disease (CKD); however, this association has not been proven by studies in FeMV-naive animals. In 2013, phylogenetically related strains were found in Japan, indicating broader geographic distribution in Asia (*2*). The lack of complete genome sequences for strains from other regions prevents assessment of the clinical relevance and genetic diversity of FeMV. Classical morbilliviruses, such as measles and canine distemper viruses, have a global distribution, suggesting that FeMV might be present elsewhere in the world (*3*). To confirm the presence of FeMV and assess its genetic diversity and infection patterns in the United States, we collected and analyzed urine samples from domestic cats.

We generated amplicons from 10 (3%) of 327 samples; 3 samples were from cats with CKD and 7 from cats without CKD. Sequencing results confirmed that these 493 bp amplicons correspond to unique strains of FeMV (*1*). FeMV^{US1} is 97% similar in the L gene amplicon sequence to FeMV^{776U} (*1*), whereas FeMV^{US5} is only 85% identical, making it very different to all previously identified FeMVs. We used these sequences to develop a pan-US primer set, priFeMV^{US}panL+ and priFeMV^{US}panL-, to amplify a highly conserved region (460 bp) of the L gene of the US strains (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/4/15-1921-Techapp.pdf). The results of these analyses demonstrated that FeMV is present outside of Asia.

In October 2013, we obtained the initial FeMV^{US1}-positive sample from a healthy 4-year-old male domestic shorthair cat (animal 0213). Approximately 15 months later, we obtained a follow-up urine sample from the still healthy cat, performed reverse transcription PCR (RT-PCR), and generated amplicons (online Technical Appendix Figure, panel A). Amplification and sequencing of the hemagglutinin (H) gene from the 2015 sample indicated that it was identical to that from the 2013 sample, suggesting that the cat was chronically infected. We developed a quantitative RT-PCR test by using L gene primers and a real-time probe (online Technical Appendix Table 2). Results indicated stable and comparable virus loads: 9.8×10^4 copies/mL in 2013 versus 7.8×10^4 copies/mL in 2015. This finding corroborates the view that cats can be chronically infected with FeMV and that the virus is persistently shed in urine.

We used primers to generate cDNA from clinical material and then determined the complete genome sequence of FeMV^{US1} (GenBank accession no. KR014147) by using RT-PCR and rapid amplification of cDNA ends. The major morbillivirus surface antigen is the H glycoprotein, and we used pan-FeMV H gene primer sets to detect additional viruses (e.g., FeMV^{US2}) (online Technical Appendix Figure, panel B). An indirect immunofluorescence assay was developed to screen serum samples for FeMV-specific antibodies. Antibodies to FeMV^{US1} were detected in fixed cells expressing FeMV H glycoprotein (positive up to 1:12,800 dilution), and antibodies to FeMV^{US5} were detected in nonpermeabilized cells (positive up to 1:6,400 dilution) (online Technical Appendix Figure 2). This result confirms that H

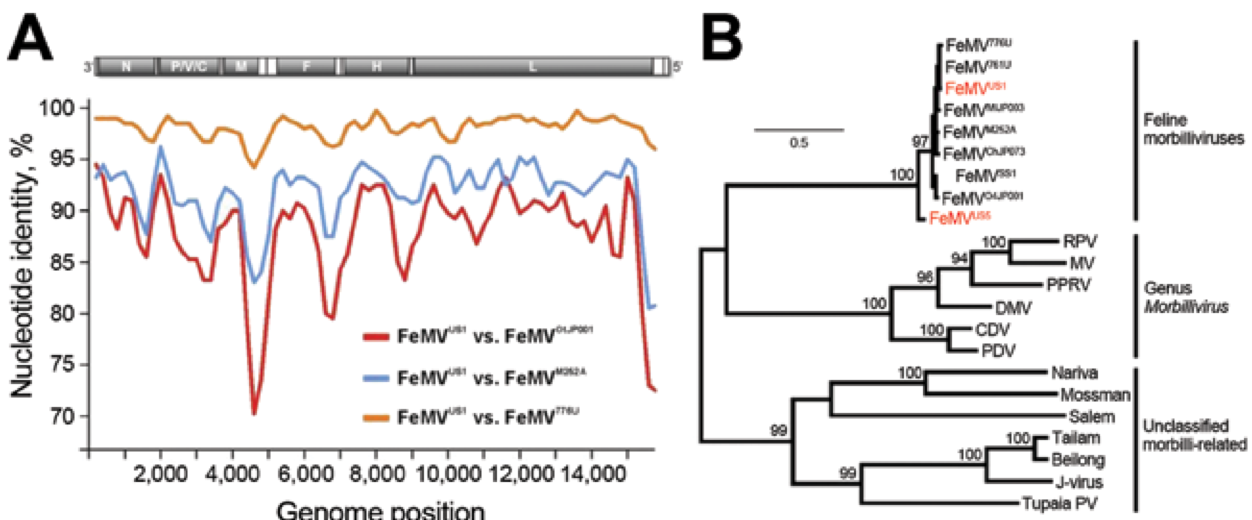

Figure. Phylogenetic analysis of feline morbillivirus (FeMV) whole genomes and hemagglutinin (H) genes collected from cats in the United States. A) Genomic sequence identity of FeMV^{US1}, compared with Asian strains, performed by using SSE V1.2 software (*4*) with a sliding window of 400 and a step size of 40 nt. B) Maximum-likelihood phylogeny of the translated H gene of FeMVs, the genus *Morbillivirus*, sensu strictu, and unclassified morbilli-related viruses was determined by using MEGA5 software (*5*) and applying the Whelan-and-Goldman substitution model and a complete deletion option. Numbers at nodes indicate support of grouping from 1,000 bootstrap replicates. Scale bar indicates substitutions per site.

glycoprotein–specific antibodies are present at high levels concurrent with the longitudinal detection of genomic RNA. A large-scale seroprevalence and cross-neutralization study is ongoing.

We used complete genome and H gene sequences in a comprehensive phylogenetic analysis. $FeMV^{US1}$ is closely related to viruses from Asia, highlighting the global distribution of FeMV (Figure, panel A). Compared with the sequence for the $FeMV^{776U}$ H gene, sequences for $FeMV^{US1}$ and $FeMV^{US5}$ were 98% and 81% similar, and the glycoproteins were 98% and 86% identical. The complete H gene of the most divergent US strain ($FeMV^{US5}$) clustered phylogenetically in a basal sister relationship with all other viruses from Asia and the United States (Figure, panel B), suggesting a long evolutionary association of FeMV in feline hosts.

Ecologic surveys continue to identify novel viruses that are homologous to known paramyxoviruses in many wildlife species, including bats and rodents (*6*). Investigating closely related viruses in domestic species is warranted, given the substantial number of animals that cohabitate with humans. Switches from natural to unnatural host species can result in enhanced pathogenicity (e.g., receptor switching has caused feline panleukopenia virus to infect dogs as canine parvovirus) (*7*). Given the high degree of antigenic relatedness of morbilliviruses, understanding evolutionary origins and trajectories and conferring cross-protection through immunization are critical. Although no evidence for FeMV transmission to humans or other animals exists, the propensity for noncanonical use of signaling lymphocytic activation molecule 1 F1 (CD150) should be investigated because epizootic transmission of morbilliviruses can occur (*8*).

The detection of FeMV sequences in a clinically healthy animal after 15 months is a novel and surprising observation but is consistent with the known propensity for morbilliviruses to persist in vivo (*9*). All known morbilliviruses cause acute infections, and the typical long-term clinical manifestations occur in the central nervous system, not the urinary system (*1*). These observations should prompt additional research because the prevalence of CKD in cats is high and because CKD decreases the quality of life of affected animals and is the ultimate cause of death for approximately one third of cats (*10*).

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Difficulties in Schistosomiasis Assessment, Corsica, France

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To the Editor: We would like to add some specification and clarification to the discussion regarding the diagnostics and case definitions for urinary schistosomiasis in travelers to Corsica, France (*1–3*). Evidence for a *Schistosoma*

haematobium infection typically depends on the detection of viable ova in the urine. However, in regard to *S. haematobium* infections acquired in Corsica, several ova excreted by the first 2 published case-patients (i.e., the 12-year-old boy and his father) exhibited atypical morphology (*4*). Therefore, we supplemented our morphologic study with a molecular study of miracidia by using cytochrome c oxydase mitochondrial DNA barcoding and the internal transcribed spacer 2 gene.

The results indicated that the schistosome responsible for the infection of the first case-patient reported in Corsica was *S. haematobium* that had been introgressed by genes of zoonotic *S. bovis* through a hybridization process. *S. bovis* is the cause of bovine intestinal schistosomiasis and uses the same intermediate host (*Bulinus truncatus* snails) that *S. haematobium* uses (*5*). Such interactions between *S. haematobium* and *S. bovis* have also been reported in Benin (*5*). These findings imply that the clinical course of case-patients and diagnostic test results might be affected by atypical schistosomiasis. Whereas the boy in our study experienced a clinically typical schistosomal infection of the bladder, his father and his siblings, who had identical histories of exposure, were seropositive for *S. haematobium* but were asymptomatic (*4*).

We recommend that clinicians treat any suspected case of *S. haematobium* infection, whether or not the patient excretes ova, given that the disease is potentially serious and the indicated drug for treatment (praziquantel) is safe. Epidemiologic analyses should take into account the role of zoonotic *S. bovis* infection and supplement parasitological investigations with molecular analyses (*5*,*6*).

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Correction: Vol. 21, No. 11

Cryptococcus was misspelled in the title of Climatic Influences on *Cryptococcus gattii* Populations, Vancouver Island, Canada, 2002–2004 (C.K. Uejio al.). The article has been corrected online (http://wwwnc.cdc.gov/eid/article/21/11/14-1161_article).

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In Memoriam: Sandy Ford (1950–2015)

Myron G. Schultz,[1] Alan B. Bloch

Sandra Lee Ford died April 11, 2015, at 64 years of age. To her children, she was a loving mother; to her co-workers, she was a caring human being and dedicated public health worker; to the world, she was a herald of the AIDS epidemic, playing a major role in alerting the world to the onset of this epidemic.

In January 1979, Sandy took a position in the Parasitic Diseases Division in the Centers for Disease Control, now the Centers for Disease Control and Prevention (CDC). Her job was to respond to requests for drugs that CDC provided through the Parasitic Diseases Drug Service (PDDS). These drugs had been unavailable in the United States. By complying with the Food and Drug Administration's rigorous requirements for investigational new drugs, CDC was able to import and dispense these drugs and fill a therapeutic gap. One of the 10 drugs in the armamentarium of the PDDS was pentamidine isethionate, which was intended to treat rare, imported cases of African trypanosomiasis. An unanticipated need for pentamidine was its use in treating *Pneumocystis carinii* pneumonia, now known as *Pneumocystis jiroveci* pneumonia or *Pneumocystis* pneumonia (PCP). Pentamidine had replaced sulfa drugs as the treatment of choice for PCP, which had been occurring mainly in infants and children with primary and secondary immune deficiencies and in adults receiving immunosuppressive drugs.

Demand for pentamidine was brisk. Sandy interacted skillfully with hundreds of clinicians across the United States to provide life-saving pentamidine to severely ill patients, and she kept very careful records. She relished the opportunity to help physicians, and she cared deeply about their ill patients. She wanted to be known as a drug technician, a title she coined for herself. On numerous occasions, she said how much she loved her job.

In early 1981, Sandy noticed an increase in the number of requests for pentamidine to treat cases of PCP. The cases were unusual because they were in adult male patients, a departure from the usual cases in children. In one instance, a New York physician asked for a second course of pentamidine to treat a patient with PCP who had not

Figure. Sandy Ford with vials of pentamidine for distribution to patients with *Pneumocystis* pneumonia.

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[1]Deceased.

responded to the first dose; these circumstances were unprecedented. Two weeks later, another New York physician told Sandy about 5 male patients who had both PCP and Kaposi sarcoma. Sandy learned that the sexual orientation for all these patients was homosexual. She knew that something unusual was occurring and informed her supervisor of this cluster of cases. In his bestselling book And the Band Played On, which chronicled the discovery of AIDS, Randy Shilts said, "That was how the thorough GS-7 drug technician in Room 161 of the Centers for Disease Control's Building 6 alerted the federal government to the new epidemic" (*1*). Simultaneously, clinicians in major medical centers were seeing, for the first time, homosexual patients with Kaposi sarcoma, PCP, cytomegalovirus infection, and other opportunistic infections. On June 5, 1981, CDC's Morbidity and Mortality Weekly Report described 5 severe pneumonia cases observed during October 1980–May 1981 in 3 Los Angeles hospitals (*2*). All 5 patients were young men with PCP, and the sexual orientation of all was homosexual. This publication was historic because it marked the beginning of awareness in the United States of a new, fearsome disease that was eventually given the name of acquired immunodeficiency syndrome (AIDS). The rest, as they say, is history.

Louis Pasteur famously said, "Chance favors the prepared mind." Chance placed Sandy as a drug technician in CDC's PDDS at a critical juncture in medical history. Although she had no formal training in medical science, she had a prepared mind and thought like an epidemiologist. When she comprehended that something unusual was occurring, she took appropriate action. In addition to her seminal role in the book And the Band Played On, Sandy was also portrayed in the movie by the same name and was the subject of many magazine and newspaper articles. A heroine of the AIDS epidemic, she continued her career at CDC until retiring in 2008 after 34 years of service. She did not capitalize on her notoriety and conducted her work and her relationships with dignity. She personified the woman in King Solomon's poem Eshet Chayil, which praises a woman of valor: "A woman of valor, who can find? Her worth is far above jewels…. She is robed in strength and dignity, and cheerfully faces whatever may come…. Wherever people gather, her deeds speak her praise" (Proverbs 31:10–31).

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Immunity

By William E. Paul, Johns Hopkins University Press, Baltimore, USA, 2015, ISBN: 978-1-42141-801-8 (hardback), ISBN: 978-1-42141-802-5 (ebook), Pages: 280; Price: US $29.95

Immunity, written by William E. Paul, MD, is a unique synthesis of autobiography and perspective on the history of immunology with easy-to-follow and informative explanations of key concepts. The author's death occurred in September 2015, just before publication of this book, his final manuscript. His discovery of interleukin-4 and his experiences as head of the Office of AIDS Research at the National Institutes of Health (Bethesda, MD, USA) are among the author's many outstanding achievements chronicled in the book. One chapter is devoted to Dr. Paul's previously described concept of the laws of immunology: universality, the idea that cells and products of the immune system are capable of recognizing an unlimited array of substances; tolerance, the notion that the response to self-antigens is controlled or eliminated; and appropriateness, the concept that the immune response is appropriate to the inducing pathogen.

Several figures in the book provide additional insights into this publication's distinctiveness: a photograph of one of Dr. Paul's mentors, Baruj Benacerraf, receiving the Nobel Prize; another photograph of a German 200-mark note featuring Paul Ehrlich, a Nobel Prize winner for his work in immunology; a photograph showing an Austrian 1,000-schilling note with the image of Karl Landsteiner, Nobel Prize winner for discovery of the immunology of blood groups; a diagram illustrating a peptide bound into the groove of a class I major histocompatibility complex molecule; and an electron microphotograph of a dendritic cell with adherent lymphocytes. The author was a major participant in remarkable advances in our understanding of immunology. For example, he states, "My professor of microbiology… was deeply skeptical about the function of lymphocytes…. Lymphocytes are so unprepossessing in appearance that many believed their chief function was to serve as nutrients for cells that were thought to be more important in antimicrobial responses…." Dr. Paul provides details of his discovery of the molecular structure and functions of interleukin-4, a critical regulatory factor for lymphocytes.

Furthermore, Dr. Paul offers insights on recent developments, such as the hygiene hypothesis of the genesis of allergic disease; inflammasomes; colony collapse disorder in honeybees; and clinical trials with dupilumab. He discusses these issues with the same profundity and clarity that he provides on his work in the frontiers of immunologic research early in his distinguished career. His response to the AIDS crisis provides an inspiring perspective for public health officials confronted with urgent challenges. He states, "In the end, I concluded that I had to take on the responsibility. The AIDS epidemic was such that one really didn't have a choice.... I was determined that while the planning process had to represent the best thinking in the field, it could not be too proscriptive. We didn't want to quash creativity, without which progress was sure to be hampered." Dr. Paul is recognized as being an important catalyst in the development of effective antiviral therapy and has been called a hero of the AIDS epidemic.

Few readers will not be impressed by Dr. Paul's energy, enthusiasm, and innovative spirit. This book will be relished by those who are captivated by the history of modern biologic and medical science.

Theodore M. Lee

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One Health: People, Animals, and the Environment

Edited by Ronald M. Atlas and Stanley Maloy, ASM Press, Washington, DC, 2014, ISBN: 978-1-55581842-5 (paperback); 978-1-55581843-2 (ebook), Pages: 318; Price: US $90.00

The One Health concept recognizes that the health of humans is connected to the health of animals and the environment. An interdisciplinary One Health approach involving human, animal, and environmental health partners worldwide is critical to address current public health issues, which include emerging infectious and zoonotic diseases. The book One Health: People, Animals, and the Environment reviews core concepts of One Health and highlights key One Health issues of public health importance.

The book comprises 5 sections. The first section covers the need for a One Health approach and reasons such an approach is important. Topics include the human–animal interface, ecologic approaches to studying zoonoses, and the role of wildlife in emerging infectious diseases. The second section covers zoonotic and environmental

drivers of emerging infectious diseases. This section includes an overview of the interconnectedness of human and animal pathogens for several timely One Health events and describes the emergence of influenza viruses across the species barrier; rabies control; foodborne diseases and transmission among humans, animals, and plants; environmental reservoirs of cholera; and the role of human activity on the spread of white-nose syndrome in bats. The rest of the book is devoted to causes behind the emergence of antimicrobial drug resistance and the need for disease surveillance that can identify pathogens crossing animal–human interfaces to provide early warning of new public health challenges and describes new technologies and approaches for public health surveillance, and the challenge of making One Health a reality. The editors share several examples of successful applications of the One Health approach to highlight the impact of collaboration between human, animal, and environmental health partners. Remaining challenges of implementing a One Health approach are also presented in the context of thwarting the threat of emerging infectious diseases. Throughout the book, the editors provide case histories of notable recent zoonotic infections, which provide real-world examples of implementing a One Health approach for diseases such as West Nile virus disease, hantavirus, Lyme disease, and severe acute respiratory syndrome.

The availability of texts describing the One Health approach is important, and this book provides a concise overview of One Health from the infectious disease perspective. Although this book focuses on the role of One Health specifically for emerging infectious and zoonotic diseases, it is a valuable introduction to the field of One Health. The book applies to a wide audience—physicians, veterinarians, environmental scientists, public health officials, policy makers, and students. It is a useful resource for those who want a better understanding of One Health and their role in the One Health movement. The One Health concept continues to gain recognition as a critical tool to address public health issues at the animal–human-ecosystem interface to have the biggest impact on improving health for all. In the words of the editors, "the One Health approach is simply too important to ignore."

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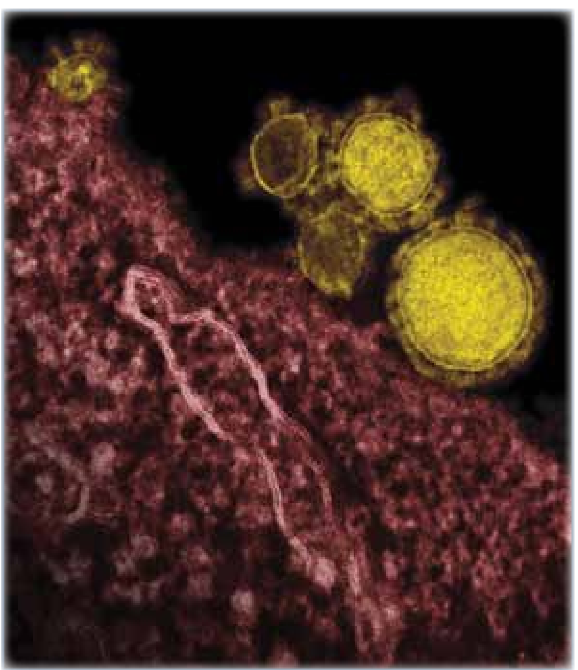
Unraveling the Mysteries of Middle East Respiratory Syndrome Coronavirus



Dr. Aron Hall, a CDC coronavirus epidemiologist, discusses Middle East Respiratory Syndrome Coronavirus

http://www2c.cdc.gov/podcasts/player.asp?f=8631627

Grant Wood (1891–1942), *Spring in the Country,* 1941. Oil on Masonite, 24 in x 22 1/8 in / 60.9 cm x 55.9 cm. Courtesy of the Cedar Rapids Museum of Art, Cedar Rapids, Iowa; Museum Purchase. 93.12

From Farm to Fable

Byron Breedlove

Grant Wood once quipped that "All the good ideas I ever had came to me while I was milking a cow." Though spurious, his comment underscores how the memories of farm life from his youth provided substance and inspiration for much of his artwork, particularly his landscapes. Wood was in fact born on a farm near Anamosa, Iowa, in 1891, and he lived there until his father died in 1901. His family moved to Cedar Rapids, Iowa, soon thereafter, when Wood was 10 years old.

In this month's cover image, Wood's *Spring in the Country,* a rolling patchwork of resplendent green Iowa farmland recedes to the distant horizon. Soaring clouds hover above the fields, swelling from the horizon till they meet the sky in the top third of the painting. As a plainly dressed woman prepares the soft ground with a hoe, a young man in overalls carefully takes another seedling from the basket next to him and presses it into its chosen spot. A ladle floats in the bucket of water, within easy reach for watering the young plants. Near a flowering tree, cresting a hill, a farmer relies on a pair of farm horses for tilling his fields on this warm early spring day. Also visible across this pristine panorama are more flowering trees, a small herd of white-faced cattle, an orderly row of fence posts, and other farms. These 3 people approach their day's labor with a relaxed ease.

One of the last works Wood completed before dying of pancreatic cancer hours from his 51st birthday, *Spring*

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in the Country, shares motifs with many of his other landscape paintings. It offers viewers a peaceful respite in this lush green, rolling farmland, a place free from signs of modernization or industry. James Dennis characterized those works as Wood's "idealized, fantasy Iowascapes." A second look confirms that power lines and telephone poles do not transect the landscape; no tractors, combines, or threshers thrum anywhere in this vast, pastoral countryside.

Notes from the University of Virginia American Studies Program state that "Although Wood's landscapes were received as idealized visions of America's heartland, a critical feature of the paintings is that they are pointedly not contemporary. America in the 1930s was in the grips of the machine age, erecting skyscrapers and building technological marvels like the Hoover Dam—yet machines do not enter into Wood's landscapes." Nor does Wood depict the sort of desperate toil and plight that would have been characteristic of the Great Depression from which the world was emerging.

While Wood was painting his final homages to the bygone simple farm life of his youth, agriculture was in the throes of major changes. Following a series of mechanical refinements during the 1930s that resulted in greater speed, power, and comfort, tractors began displacing farm horses and mules, though not outnumbering them until the early 1950s. Having reliable mechanical power in turn gave rise to related innovations and modifications to farm implements, enabling farmers to work faster and to farm more land.

Agriculture-related innovation during that time also led to the development of new varieties of crops and livestock, more effective pesticides, improved fertilizers, and better irrigation techniques. Among the dynamic, global forces that accelerated changes in food production and farming were the burgeoning expansion of mass transportation, shifts in population from rural to urban areas, and, of course, the demands and difficulties that resulted from World War II.[1] What were once local or regional enterprises now operated on national and global scales.

Farming became more specialized, and fewer commodities were grown and sold per farm. The pattern of ever more efficient food production and the advent of larger-scaled food processing and distribution has increased the risk and the scale of outbreaks of foodborne disease.

Though some foodborne diseases that were common near the start of the 20th century—during the time Wood was living on his family's farm—are now rare, others persist. Today there are also newly recognized and emerging pathogens, new toxins, and increasing problems with antibiotic resistance, leading to estimates from the World Health Organization that unsafe food is linked to the deaths of an estimated 2 million people annually from more than 200 food-related diseases.

Preventing, tracking, and investigating foodborne illnesses is an increasingly complex public health endeavor. It involves wide-reaching collaboration among agriculture and food processing industries, regulatory and public health agencies, and consumers, and its range can potentially be global. It is jolting to realize that Wood's nostalgic visual fables of farm life, with their white-faced cattle and farm horses, rolling green farm lands, and, country folks working under a warm spring sky, would in reality harbor many of the more than 250 bacterial, viral, and parasitic pathogens that today are known to cause foodborne illness.

[1]Researcher Katherine J. Curtis White cited a body of "literature investigating agricultural economics and population dynamics focuses on the 'pre-mechanization' period and the 'post-mechanization' period, with 1940 marking the decade of transition."

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EMERGING INFECTIOUS DISEASES™

Upcoming Issue

- *Rickettsia parkeri* Rickettsiosis, Arizona, United States
- Differences in Genotype, Clinical Features, and Inflammatory Potential of *Borrelia burgdorferi* sensu stricto Strains, Europe and United States
- Spectrum of Viral Pathogens in Blood of Returned, Malaria-Free, Ill Travelers from Canada
- *Plasmodium falciparum* K76T *pfcrt* Gene Mutations and Parasite Population Structure, Haiti, 2006–2009
- Emergence Potential of Bunyaviruses in Australia
- Acute Human Inkoo and Chatanga Virus Infections, Finland
- *Plasmodium falciparum* In Vitro Resistance to Monodesethylamodiaquine, Dakar, Senegal, 2014
- Astrovirus MLB2, a New Gastroenteric Virus Associated with Meningitis and Disseminated Infection
- Expansion of Shiga Toxin-Producing *Escherichia coli* by Use of Antibiotic Growth Promoters in Cattle
- MERS-CoV Outbreak at Tertiary Care Hospital, Jeddah, Saudi Arabia, 2014
- Expanded Geographic Distribution and Clinical Characteristics of *Ehrlichia ewingii* Infections, United States
- Projecting Periods of Delivery of At-Risk Babies after Zika Virus Disease Outbreaks
- Q Fever, Scrub Typhus, and Rickettsial Diseases as Common Causes of Febrile Illness in Children, Western Kenya
- Clinical, Virologic, and Epidemiologic Characteristics of Dengue Outbreak, Dar es Salaam, Tanzania, 2014
- Molecular Characterization of Canine Rabies Virus in Mali
- Two Fatal Cases of Septicemia Linked to Transmission of MRSA Clonal Complex 398 in a Hospital and Nursing Home, Denmark
- Increased Rotavirus Prevalence in Diarrheal Outbreak Precipitated by Severe Storms, Solomon Islands, 2014
- *Rickettsia sibirica mongolitimonae* Infection, France, 2010–2014
- Linkage to Care for Suburban Heroin Users with Hepatitis C Virus Infection, New Jersey, USA
- Severe Sepsis and Septic Shock Associated with Chikungunya Virus Infection, Guadeloupe, 2014
- Fatal Monocytic Ehrlichiosis in Woman, Mexico, 2013

Complete list of articles in the May issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

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

May 19–22, 2016
Clinical Virology Symposium
American Society for Microbiology
Daytona Beach, FL, USA
http://www.clinicalvirologysymposium.org/

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

December 3–8, 2016
ASLM
African Society for Laboratory Medicine
Cape Town, South Africa
http://aslm2016.org/

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to http://www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the “Register” link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association’s Physician’s Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Shiga Toxin 1–producing *Shigella sonnei* Infections, California, United States, 2014–2015

CME Questions

1. Your patient is a 32-year-old man in whom Shiga toxin 1 (Stx1)–producing *Shigella sonnei* infection is suspected. According to the surveillance study by Lamba and colleagues, which of the following statements about the epidemiologic features of recent emergence of Stx1-producing *S. sonnei* in the United States is correct?

A. In 2014, Stx1-producing *S. sonnei* infections were detected in New Mexico
B. All reported cases were epidemiologically linked to travel to Mexico
C. All isolates encoded stx1 and produced active Stx1, with pulsed-field gel electrophoresis (PFGE) revealing multiple PFGE patterns
D. In cluster 1 (mostly in San Diego), case-patients were less likely to be Hispanic than the general population

2. According to the surveillance study by Lamba and colleagues, which of the following statements about the clinical characteristics of recent Stx1-producing S. sonnei infections in the United States is correct?

A. All patients had bloody diarrhea
B. Half of patients had abdominal cramps
C. Three-quarters of patients required hospitalization
D. Case-patients younger than 5 years vs. those age 5 years and older were more likely to have fever (100% vs. 67%; $p = 0.054$) and bloody diarrhea (80% vs. 68%; not significant)

3. According to the surveillance study by Lamba and colleagues, which of the following statements about antibiotic resistance, treatment, and outcomes of recent Stx1-producing *S. sonnei* infections in the United States would most likely be correct?

A. The most common profile of antibiotic resistance included resistance to trimethoprim/sulfamethoxazole and susceptibility to ciprofloxacin and ampicillin
B. Most case-patients did not receive antibiotic treatment
C. One-quarter of case-patients treated with antibiotics had hemolytic uremic syndrome
D. Mortality rate was 10%

Activity Evaluation

1. The activity supported the learning objectives.

Strongly Disagree				Strongly Agree
1	2	3	4	5

2. The material was organized clearly for learning to occur.

Strongly Disagree				Strongly Agree
1	2	3	4	5

3. The content learned from this activity will impact my practice.

Strongly Disagree				Strongly Agree
1	2	3	4	5

4. The activity was presented objectively and free of commercial bias.

Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES®

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

* New infections resulting from changes or evolution of existing organisms.
* Known infections spreading to new geographic areas or populations.
* Previously unrecognized infections appearing in areas undergoing ecologic transformation.
* Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   * Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   * Reports laboratory and epidemiologic findings within a broader public health perspective.
   * Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   * Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   * Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   * Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Summary of Authors' Instructions

Author's Instructions. For a complete list of EID's manuscript guidelines, see the author resource page: http://wwwnc.cdc.gov/eid/page/author-resource-center.

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words in the main body of the text or include more than 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section comprises case series papers and concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.

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