Vol 26, No 4, April 2020

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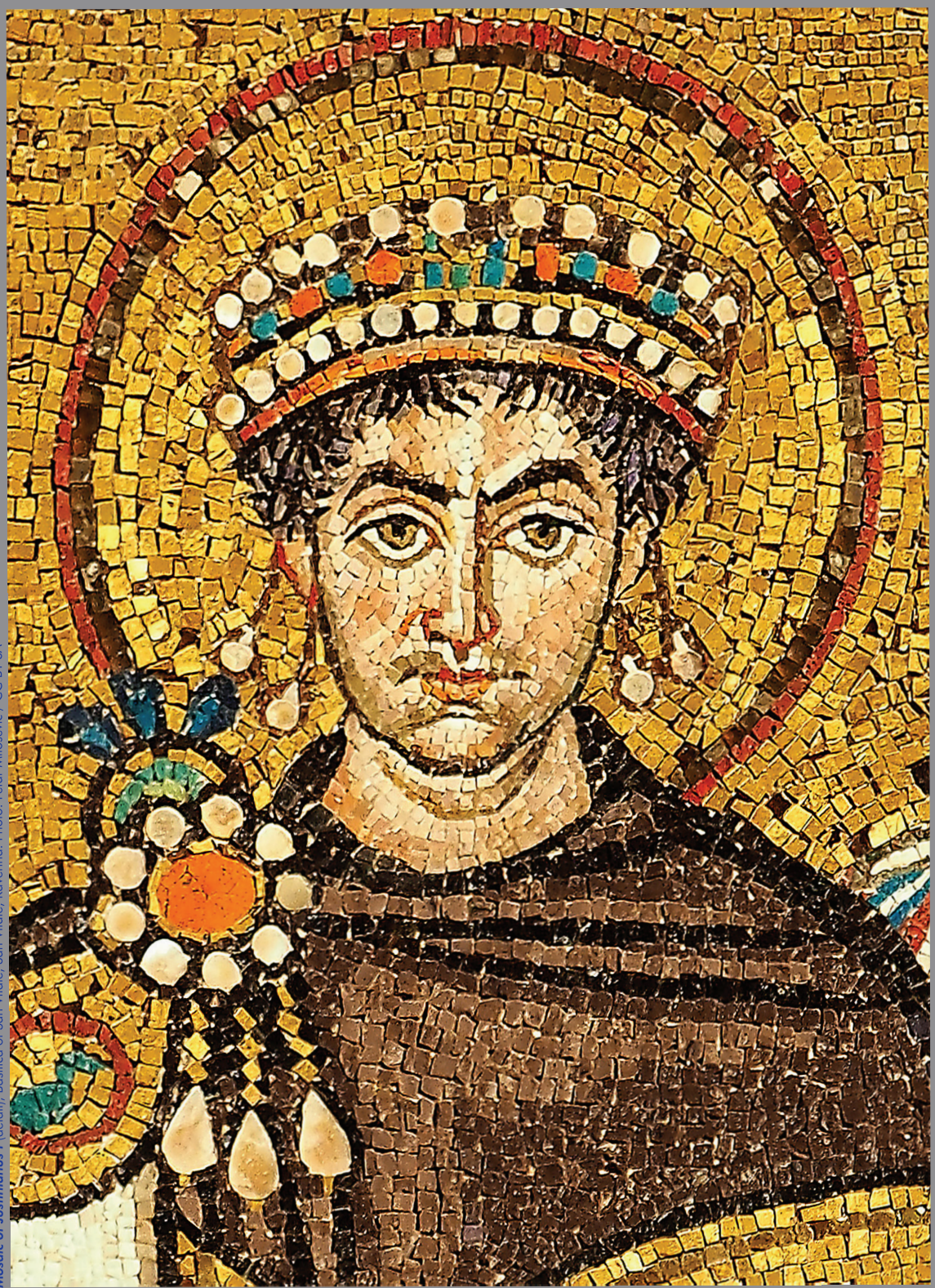

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EMERGING INFECTIOUS DISEASES

ISSN 1080-6040

Pages 641–832

EMERGING INFECTIOUS DISEASES®

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

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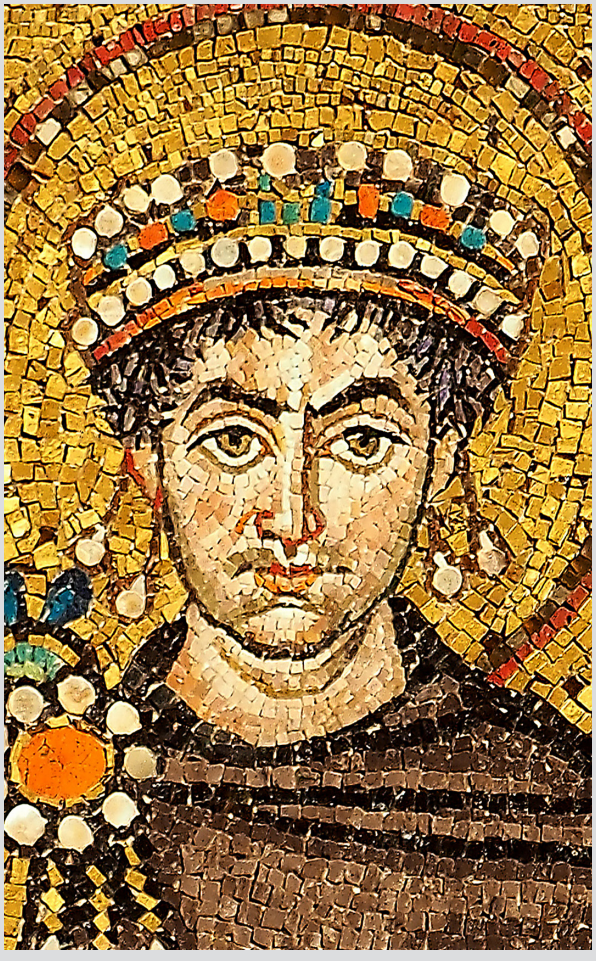

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Mosaic of Justinianus I (detail), Basilica of San Vitale, San Vitale, Ravenna. Photo: Petar Milošević / CC BY-SA

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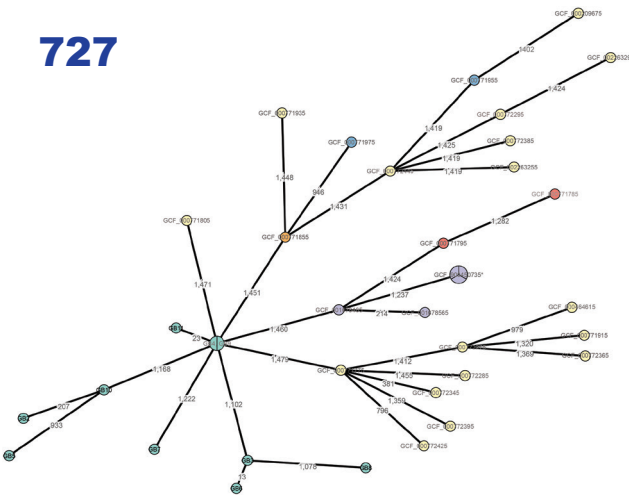

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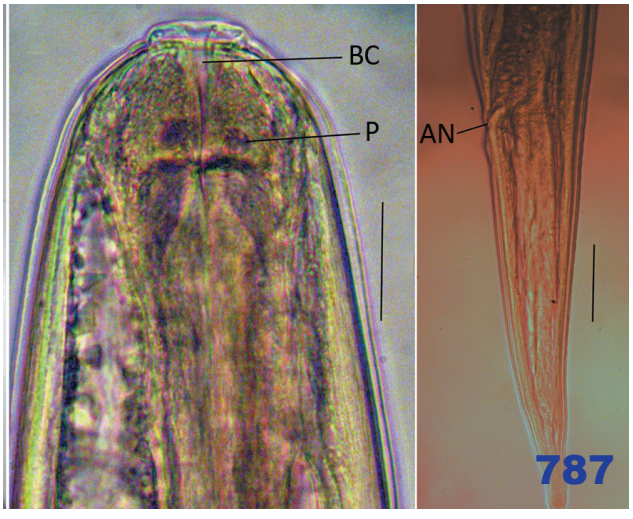

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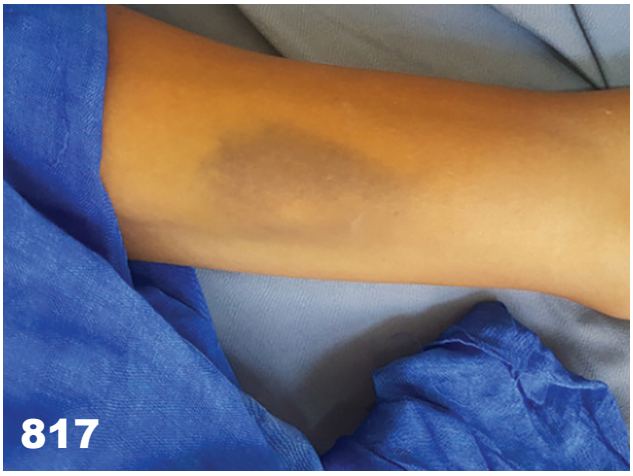

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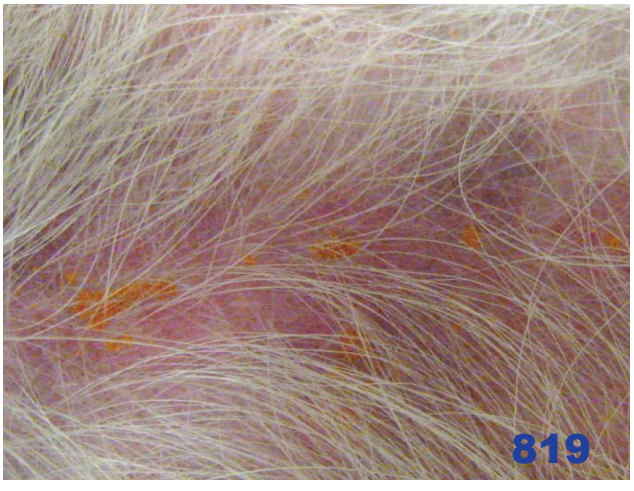

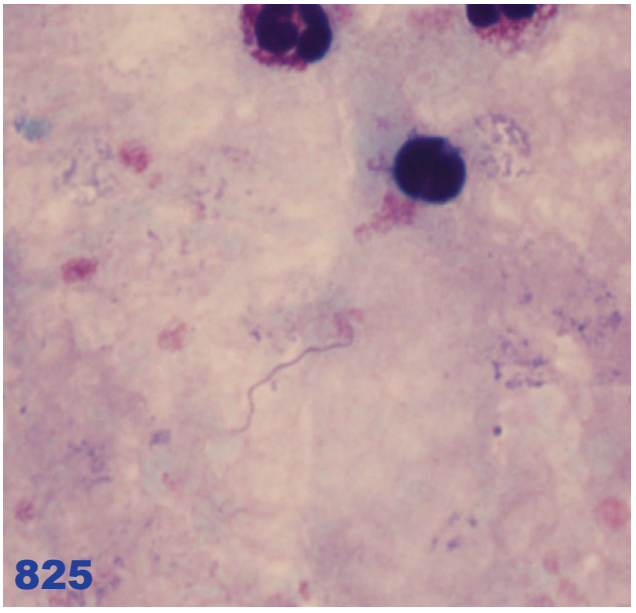

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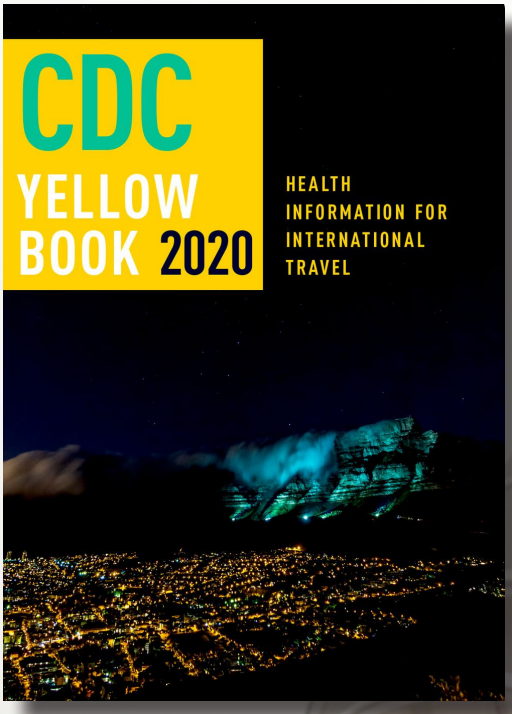

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Stemming the Rising Tide of Human-Biting Ticks and Tickborne Diseases, United States

Lars Eisen

Ticks and tickborne diseases are increasingly problematic. There have been positive developments that should result in improved strategies and better tools to suppress ticks, reduce human tick bites, and roll back tickborne diseases. However, we equally need to address the question of who is responsible for implementing the solutions. The current model of individual responsibility for tick control evolved from a scenario in the 1990s focusing strongly on exposure to blacklegged ticks and Lyme disease spirochetes in peridomestic settings of the northeastern United States. Today, the threat posed by human-biting ticks is more widespread across the eastern United States, increasingly complex (multiple tick species and >10 notable tickborne pathogens), and, across tick species, more spatially diffuse (including backyards, neighborhood green spaces, and public recreation areas). To mitigate tick-associated negative societal effects, we must consider shifting the responsibility for tick control to include both individual persons and professionally staffed tick-management programs.

Ticks and tickborne diseases are distinctly on the increase in the United States (*1*,*2*). Congress responded to this growing problem by establishing a Tick-Borne Disease Working Group in 2016, as part of the 21st Century Cures Act (https://www.fda.gov/regulatory-information/selected-amendments-fdc-act/21st-century-cures-act), and the first biannual Tick-Borne Disease Working Group report was published in 2018 (*3*). Congress also recently passed the Kay Hagan Tick Act (https://www.congress.gov/bill/116th-congress/senate-bill/1657/text/is) to combat vectorborne diseases. Federal public health agencies have generated new strategic plans aiming to strengthen both research and operational capacity to more effectively counter the threat of ticks and tickborne diseases (*4*–*8*). The Entomological Society of America produced a position paper on tickborne diseases (*9*) and led the formation of a new coalition named the Vector-Borne Disease Network, which includes the Entomological Society of America and a wide range of scientific and medical societies, professional associations, and the 5 Centers for Disease Control and Prevention–funded Regional Centers for Excellence in Vector-Borne Diseases (*10*). These are all positive developments expected to contribute improved strategies and better tools to suppress ticks, reduce human tick bites, and roll back tickborne diseases. However, at the root of the growing problem with ticks and tickborne diseases lies the thorny problem of who will be responsible for implementing the solutions.

In the United States, national surveillance of reportable tickborne diseases is achieved through the National Notifiable Diseases Surveillance System (*11*). National surveillance of ticks and pathogens found in ticks was launched only recently as part of the Epidemiology and Laboratory Capacity for Prevention and Control of Emerging Infectious Diseases program of the Centers for Disease Control and Prevention, which provides funding to states, cities, and territories (*12*). The initial focus was on the blacklegged tick (*Ixodes scapularis*) (*13*), with planned expansion to include a wider range of human-biting tick species. Collectively, these national surveillance programs provide information on when and where humans are at greatest risk for exposure to ticks and tickborne pathogens at state and county scales. When risk has been defined in space and time, the next obvious question is how to most effectively suppress ticks, reduce human tick bites, and roll back tickborne diseases. *I. scapularis* ticks and Lyme disease in the northeastern region is perhaps the best example of just how intractable this problem is. In parts of this region, peak risk for exposure to nymphal ticks (the primary vectors of Lyme disease spirochetes to humans) is already clearly defined in space (e.g., shady and moist

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DOI: https://doi.org/10.3201/eid2604.191629

habitats in backyards, neighborhood green spaces, and recreation areas) and time (spring and early summer) (*14*,*15*). There is no question that every year will be a bad year for Lyme disease in the northeastern region. However, *I. scapularis* ticks and their associated pathogens persist in the environment and continue to cause human illness year after year (*2*,*16*). Potential solutions that have emerged over the past 2 decades include a wide array of approaches to prevent tick bites through personal protection measures or to suppress host-seeking ticks or disrupt enzootic pathogen transmission through environmentally based control methods, but evidence for their impact on human tick bites or illness is limited (*17*–*22*). Moreover, uptake of these solutions by the public remains weak because of limited acceptability of some methods with perceived risk to the environment, pets, or family members, as well as low willingness to pay, combined with the consideration that the lowest-cost methods (e.g., use of tick repellents and daily tick checks) require high levels of daily vigilance over several months each year (*18*,*23*–*25*).

The overall public health threat posed by ticks and tickborne diseases in the United States is steadily increasing to include new human populations because major vector ticks are expanding their geographic ranges (*14*,*26*–*29*), and we are still discovering new native tickborne human pathogens (*1*,*2*,*16*). For public health messaging, surveillance of ticks and their associated pathogens is especially useful at the leading edges of an expanding vector tick species range. Moreover, the negative effect of ticks on human health is expanding from long-recognized pathogen transmission and tick paralysis to also include an allergic response to red meat believed to be associated with previous bites by ticks, including the lone star tick (*Amblyomma americanum*) (*30*). Our most recent warning signal was the introduction and establishment along the Eastern Seaboard of an invasive tick species (the Asian longhorned tick, *Haemaphysalis longicornis*) with potential to negatively impact the cattle industry and perhaps also public health if this tick is found to commonly bite humans in the United States (*31*).

The negative societal effects of ticks and tickborne diseases in the United States, including a general feeling that family members are not safe during outdoor activities in the backyard and elsewhere, has reached the point where we need to rethink the basic concepts of how to counter this threat. We still need a human Lyme disease vaccine (*32*,*33*), and intriguing new tick and pathogen control and tick-bite prevention technologies are on the horizon (*3*,*19*,*20*,*34*,*35*). However, these technologies will still not address the major issue of who should bear the responsibility for implementing proven tick control and tickborne disease prevention solutions. As noted a decade ago by Piesman and Eisen (*36*): "Mosquito control is a community responsibility; tick control is an individual homeowner responsibility. This may explain why currently in the United States, several thousand people are dedicated to mosquito control, whereas only a few dozen are dedicated to public-health related tick control." Other investigators have more recently similarly noted the difference in how mosquitoborne and tickborne diseases are addressed in the United States and argued for a shift toward area-wide suppression of *I. scapularis* ticks and Lyme disease spirochetes (*37*,*38*). With these considerations in mind, the relentless increase in ticks and tickborne diseases in the United States raises 2 pointed questions that are addressed in more detail in the following sections: First, is it possible to turn the tide of tickborne diseases while control of ticks and their associated disease agents remain an individual responsibility or will this ultimately require a shift to also include a strong community-based effort? Second, can we develop local, professionally staffed programs capable of working with the public to reduce the risk for tick bites on both public and private land?

Shifting Sands of Ticks and Tickborne Diseases

The concept of tick control as an individual homeowner responsibility emerged, in part, from the knowledge gained about *I. scapularis* ticks, the Lyme disease spirochete (*Borrelia burgdorferi* sensu stricto), and tick encounter locations in the late 1980s and the first half of the 1990s, which made perfect sense at that time. Lyme disease was the near absolute focus among tickborne diseases, most of human infections occurred in the northeastern United States, and residential properties were pinpointed as the most common location for encounters with *I. scapularis* ticks in Lyme disease–endemic areas (*19*,*21*,*39*,*40*). Moreover, as is still the case, both broadcast application of residual acaricides to the vegetation and placement of rodent-targeted tick control devices require physical access for control to be implemented on private properties. The difficulty in accessing these residential high-risk environments presented (and still presents) a major impediment for development of community-driven tick control, and the main focus was therefore on devising tick suppression approaches intended for use in backyards and tick-bite prevention measures for personal protection (*19*). The notable exception was approaches targeting white-tailed deer, which

were recognized as dominant hosts for the adult life stage of *I. scapularis* ticks and potentially represent a weak link in the life cycle of the tick (*41*). With the exception of deer fencing, which can be used for single residential properties, deer-targeted tick control approaches (i.e., deer reduction or treatment of deer with topical acaricide) require area-wide implementation to be successful. There is broad consensus that the white-tailed deer is a main driver for the remarkable increase in *I. scapularis* ticks in the northern parts of the eastern United States over the past 40 years (*17*,*19*,*42*,*43*). However, fierce debate continues about the specific thresholds required to be reached for either deer reduction (achieving a sufficiently low deer density) or topical treatment of deer with acaricides (achieving a sufficiently high level of treatment coverage in the deer population) to suppress *I. scapularis* tick populations to the point where we also see an effect on human tick bites and tickborne diseases (*17*,*19*,*43*–*45*). Despite promising results in some studies (*43*,*45*), neither deer reduction nor topical treatment of deer with acaricides has, to date, been widely used operationally to control *I. scapularis* ticks.

In the 25 years since control of human-biting ticks in the United States evolved into an individual homeowner responsibility, the sands of ticks and tickborne diseases have shifted dramatically, and we are no longer facing the same problems as in the 1990s. Although there is empirical evidence that *I. scapularis* tick bites still result most commonly from tick encounters on residential properties in suburban/exurban settings of the northeastern United States (*46*), ongoing spread and population increase of this tick across the northern part of the eastern United States might have resulted in a more spatially diffuse risk for tick encounters as the density of host-seeking *I. scapularis* ticks reached levels across the landscape where even activities of limited duration (compared with the time spent in your own backyard) increasingly results in tick encounters. A recent systematic review and meta-analysis on spatial risk factors for *I. scapularis* tick bites and *I. scapularis* tick–associated diseases in eastern North America concluded that risk occurs in backyards, as well as in neighborhood green spaces and public lands used for recreation (*47*).

Expanding ranges of other human-biting vector ticks contribute to a changing risk scenario for tick bites. Jordan and Egizi (*48*) reported that during 2006–2016, the vector tick species most commonly collected from humans and submitted to a passive tick surveillance system in New Jersey shifted from *I. scapularis* to *A. americanum*. Both *A. americanum* ticks and the Gulf Coast tick (*A. maculatum*) are spreading northward from their previous core ranges in the southeastern United States (*27*–*29*), and we now also have the invasive *H. longicornis* tick to contend with along the Eastern Seaboard, as far north as New York state (*31*).

Lyme disease is still by far the most commonly reported tickborne disease in the eastern United States, where 2 primary causative agents (*B. burgdorferi* sensu stricto across the eastern half and *B. mayonii* in the upper Midwest) are transmitted by *I. scapularis* ticks (*2*). However, several other tickborne illnesses, as well as co-infections with Lyme disease, are on the rise and increasingly recognized as serious health threats. These illnesses include conditions caused by viral, bacterial, and parasitic pathogens transmitted by *I. scapularis* ticks (*Anaplasma phagocytophilum*, *Babesia microti*, *B. miyamotoi*, *Ehrlichia muris eauclairensis*, and Powassan virus), *A. americanum* ticks (*E. chaffeensis*, *E. ewingii*, Bourbon virus, and Heartland virus), and *A. maculatum* ticks (*Rickettsia parkeri*) (*1*,*16*,*27*,*28*).

In contrast to the situation in the Northeast and upper Midwest, *I. scapularis* ticks are only a minor public health threat compared with *Amblyomma* ticks in the Southeast. Moreover, the potential involvement of *A. americanum* ticks in red meat allergy is concerning because this notorious human-biter is not only abundant in the Southeast but also expanding its range north and thus affecting new human populations (*28*,*29*). Finally, the American dog tick (*Dermacentor variabilis*) remains a threat across its wide geographic range as a vector of the agents causing Rocky Mountain spotted fever (*R. rickettsii*) and tularemia (*Francisella tularensis*) (*1*). Other vector tick species similarly are public health concerns in the Rocky Mountain region and the far western United States, including the western blacklegged tick (*I. pacificus*), the Rocky Mountain wood tick (*D. andersoni*), the Pacific Coast tick (*D. occidentalis*), the brown dog tick (*Rhipicephalus sanguineus*), and *Ornithodoros* spp. soft ticks (*1*).

The strategies devised 2 decades ago to address *I. scapularis* ticks and Lyme disease spirochetes on residential properties in the Northeast are not necessarily well suited to address the current broader, more complex, and spatially diffuse threat of ticks and tickborne diseases in the United States. There is hope that a badly needed human Lyme disease vaccine will be found, but this will only solve 1 part of the overall problem with tickborne pathogens and it will not have any effect on tick populations. Because no silver bullets are on the near horizon to broadly address the increasing threat of ticks and tickborne diseases in the United States, we must reassess the problem and consider new shorter-term solutions.

One reasonable assessment, based on the experience over the past 25 years and the steadily worsening problem, is that the responsibility for tick and pathogen control must be shifted to include both individual persons (responsible for their own properties and use of personal protection measures) and local public health programs with professional staff (responsible for public outreach, assistance to homeowners with selection of appropriate tick control options, and control of ticks and tickborne pathogens in high-use risk areas, such as neighborhood green spaces and picnic areas and hiking trails on public lands). This 2-pronged concept for responsibility should be accompanied by a 2-pronged spatial concept: first, making the backyard a safe, tick-free zone; and second, achieving area-wide suppression of ticks and tickborne pathogens to reduce the risk for tick encounters in other high-use environments.

Need for Local and Professionally Staffed Integrated Tick-Management Programs

Basic differences in the biology of vector mosquitoes and vector ticks drive the selection of methods and implementation schemes to control these pests. In the United States, local risk associated with tickborne pathogens tend to be predictable both in space and time (across years and seasonally), whereas the local intensity of transmission of mosquitoborne viruses fluctuates dramatically among years and builds over the warm time of the year when mosquitoes are active. This advantage for tick control is counteracted by the fact that mosquito control can focus initially on known larval development sites and then, as needed based on surveillance data, move to a space spray emergency measure not requiring physical access to residential properties. For ticks, every year brings a seasonally predictable emergency situation, risk habitats are diffuse and include both private and public lands, and current options for area-wide tick suppression are limited and have weak evidence bases for impact on human tick bites and disease (*20*). Even control of ticks, such as *I. scapularis* and *A. americanum*, in backyards is problematic because we have a poor understanding of how effectively host-seeking ticks are suppressed across the full extent of a residential property through broadcast of synthetic acaricides, natural acaricides, or fungal control agents by homeowners or commercial pest control companies. A large-scale study that limited application of synthetic acaricide to include only a barrier zone along the lawn–woods ecotone on residential properties did not find the observed suppression of host-seeking ticks within this treated portion of the residential properties to result in reduced human tick bites for the residents (*49*). To more effectively suppress ticks in the environment and reduce human tick bites and tickborne diseases, we need to invest in studies to optimize the effect of existing technologies, as well as stimulate the development of novel approaches.

Nevertheless, elements of organized mosquito control can be used as building blocks for an integrated tick-management program. Well-functioning mosquito management programs are based on the principles of integrated pest management (striving to protect the human population from mosquito bites and mosquitoborne disease agents while at the same time minimizing the impact of pesticides on the environment) and staffed with professionals experienced in public outreach, mosquito biology, pesticide use, and operational surveillance and control concepts. Expanding the activities of existing mosquito management programs to also include ticks (*50*) provides an economy of scale compared with the alternative of having separate community-supported mosquito- and tick-management programs. Specific benefits from building tick responsibilities into an existing mosquito management program might include shared use of existing office/laboratory space, laboratory equipment, and vehicles; presence of professionals already skilled in morphologic vector identification and knowledgeable about basic principles for vector surveillance and control; presence of licensed and highly experienced pesticide applicator personnel; and presence of personnel with previous experience of public outreach for vector-related issues. Regarding access to experienced personnel, effective control of ticks, in backyards or elsewhere, requires control products targeting host-seeking ticks or ticks on host animals to be implemented by persons with a solid understanding of tick biology (e.g., to ensure that the product is applied to the environment in a manner that maximizes contact with host-seeking ticks), the nature of the acaricide product used (e.g., the frequency of acaricide applications needed to provide sustained control over the tick season), and the limitations of the application equipment (which, for example, can effect penetration into microhabitats in which ticks are found). Another potential benefit from strengthening the linkages between mosquito and tick control is an increased involvement by industry in tick control solutions through the already existing interface between industry and the American Mosquito Control Association. A better defined market for tick control products should stimulate industry to invest in new solutions.

The most productive way of exploring the concept of integrated tick-management programs would be (well-funded) demonstration projects focused on geographic locations with strong existing mosquito management programs and severe problems with a wide range of tick species and tickborne diseases. Such an effort is guaranteed to be challenging because it needs to include development of tick-specific knowledge and acquisition of tick-specific equipment; development, implementation, and evaluation of a locally appropriate, standardized tick/pathogen surveillance scheme to address key knowledge gaps, if they exist, for human-biting ticks of local concern and their associated pathogens; development, implementation, and evaluation of a public outreach program to raise local awareness of spatially and seasonally variable risk for exposure to locally found ticks and tickborne pathogens; and development, implementation, and evaluation of schemes for suppression of locally found human-biting ticks on high-use portions of public lands (e.g., along hiking trails, and in and around camp sites, picnic areas, and playgrounds) and on private properties in conjunction with homeowners and using different tick suppression models (e.g., by tick-management program personnel; through contracts with licensed pest control operators from the tick-management program and with oversight by tick-management program personnel; or through homeowner incentives leading to tick suppression executed either by the homeowner or a licensed pest control operator). The lessons learned from such demonstration projects to establish integrated tick-management programs staffed by public health professionals would greatly improve our ability to produce specific and realistic guidance for best management practices.

Moreover, selection of specific tick and pathogen suppression methods to include for either backyard control or area-wide tick management will be challenging because the evidence base for existing approaches is reasonably strong for acarologic outcomes (density of host-seeking ticks and pathogen-infected, host-seeking ticks) but extremely weak for human-based outcomes (human–tick encounters and human illness) (*19–21,45,49*). Initial evaluations of tick and pathogen suppression schemes in an integrated tick-management program would focus on acarologic outcomes; if these were deemed successful, subsequent evaluations should progress to also include human-based outcomes. One major downstream outcome would be improved guidance for best management practices for tick suppression and reduction of human tick bites based on real-world scenarios, which will need to account for local variation in tick species of public health concern needing to be addressed (e.g., only *I. scapularis* ticks, only *A. americanum* ticks, or both species). Cost assessments would be critical to clarify the resources needed to either build ticks into an existing mosquito management program or build an integrated tick-management program from the ground up in settings lacking existing mosquito management programs. Finally, the need for adequate funding for operational tick management cannot be overstated; tick management cannot be incorporated into an existing mosquito management program as an unfunded activity or mandate, and a stand-alone tick-management program equally will require substantial and sustained funding.

Acknowledgments

I thank the members of the Tick Biology, Ecology, and Control Subcommittee of the 2020 Tick-Borne Disease Working Group for stimulating discussions about human-biting ticks and tickborne diseases.

About the Author

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Ecology and Epidemiology of Tickborne Pathogens, Washington, USA, 2011–2016

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Release date: March 13, 2020; Expiration date: March 13, 2021

Learning Objectives

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

- Describe tickborne pathogens in Washington state, according to surveillance data integrating human case histories and detection of pathogens in field-collected ticks
- Determine detection of pathogens in field-collected ticks in Washington state during 2011 to 2016, according to surveillance data
- Identify clinical and public health implications of detection of tickborne pathogens and diseases in Washington state, according to surveillance data

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DOI: https://doi.org/10.3201/eid2604.191382

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Tickborne diseases are rare in Washington, USA, and the ecology of these pathogens is poorly understood. We integrated surveillance data from humans and ticks to better describe their epidemiology and ecology. During 2011–2016, a total of 202 tickborne disease cases were reported in Washington residents. Of these, 68 (34%) were autochthonous, including cases of Lyme disease, Rocky Mountain spotted fever, tickborne relapsing fever, and tularemia. During May 2011–December 2016, we collected 977 host-seeking ticks, including *Ixodes pacificus*, *I. angustus*, *I. spinipalpis*, *I. auritulus*, *Dermacentor andersoni*, and *D. variabilis* ticks. The prevalence of *Borrelia burgdorferi* sensu stricto in *I. pacificus* ticks was 4.0%; of *B. burgdorferi* sensu lato, 3.8%; of *B. miyamotoi*, 4.4%; and of *Anaplasma phagocytophilum*, 1.9%. We did not detect *Rickettsia rickettsii* in either *Dermacentor* species. Case-patient histories and detection of pathogens in field-collected ticks indicate that several tickborne pathogens are endemic to Washington.

Tickborne infections are the most common cause of vectorborne disease in the United States (*1*). Disease epidemiology is complex, the result of many causes, such as spatiotemporal variation in infected vectors, human behavior, reservoir host abundance, and climate variation (*1*,*2*). Compared with highly endemic areas such as the upper Midwest and northeastern United States, for the state of Washington, the epidemiology and ecology of tickborne diseases is not well characterized. Washington is divided into 39 counties spread across multiple, distinct ecosystems; the diverse ecology presents a range of potential vector habitats.

Human Lyme disease cases, with and without travel outside the state within the exposure period, have been confirmed in Washington (*3*). *Borrelia burgdorferi* sensu stricto (s.s.)–infected *Ixodes pacificus* ticks have been documented in far western North America, including California, USA, and British Columbia, Canada (*4*–*6*). In addition, a recent study in Washington documented canine seroprevalence of 3.8 cases/1,000 dogs (*7*). *Anaplasma phagocytophilum* has been reported in small mammals (*8*,*9*) and in vector ticks in California (*10*); clinical cases of anaplasmosis have been reported in dogs from California to British Columbia (*7*,*11*,*12*).

Rare cases of autochthonous babesiosis have been reported in Washington, 3 caused by *Babesia duncani* and 1 caused by a *B. divergens*–like organism (*13*–*15*). Evidence of *D. albipictus* ticks as the vector for *B. duncani* has only recently emerged (*16*).

Three of 11 *Ixodes* tick species (*I. pacificus*, *I. angustus*, and *I. spinipalpis*) reported from Washington are known or suspected vectors for tickborne diseases (*17*). *I. pacificus* ticks are frequent human-biters, and the species is an established vector of *B. burgdorferi* s.s. and *A. phagocytophilum* and a putative vector of *B. miyamotoi* (*18*). *I. angustus* ticks can experimentally transmit *B. burgdorferi* s.s. and might play a role in the spirochete's enzootic cycle (*19*,*20*); likewise, *I. spinipalpis* ticks might play a role in the natural maintenance of *B. burgdorferi* sensu lato (s.l.) (*21*).

Competent reservoirs for *B. burgdorferi* s.s., including deer mice (*Peromyscus maniculatus*), western gray squirrels (*Sciurus griseus*), and several *Tamias* spp. chipmunks, are found in Washington (*22*–*25*). *P. maniculatus* deer mice have been found infected with *B. burgdorferi* s.l. in western Washington (*22*). Although not recognized as human pathogen reservoirs, lizards are notable blood-meal hosts for immature *I. pacificus* ticks (*26*,*27*) and 3 lizard species are found in Washington: northern alligator lizard (*Elgaria coerulea*), southern alligator lizard (*E. multicarinata*), and the western fence lizard (*Sceloporus occidentalis*) (*25*,*28*; C.S. Arnason, Biology of the western black-legged tick, *Ixodes pacificus*, (Cooley and Kohls, 1943): a potential vector of Lyme disease in south coastal British Columbia [master's thesis], Vancouver: Simon Fraser University; 1992). Both *E. multicarinata* and *S. occidentalis* lizards are zooprophylactic against *B. burgdorferi*.

Autochthonous cases of Rocky Mountain spotted fever (RMSF) were reported in Washington each year until the 1940s (*29*). To date, there is no published evidence of *R. rickettsii* in ticks collected in Washington. Tularemia is prevalent throughout the Northern Hemisphere and occurs in many animal species (*30*). Recent *Francisella tularensis* antibody detections were reported from wildlife in Idaho (*31*). Up to 10 cases of tularemia are reported each year in Washington (*29*). *D. andersoni* and *D. variabilis* ticks, both competent vectors of *R. rickettsii* and *F. tularensis*, occur in the state (*18*,*32*). The brown dog tick, *Rhipicephalus sanguineus*, a known vector of RMSF in the southwest, is also reportedly present (*33*,*34*).

Borrelia hermsii, the causative agent of tickborne relapsing fever (TBRF), occurs in Washington and is vectored by *Ornithodoros hermsi*, a soft tick (family Argasidae) typically found in rodent nests (*35*,*36*). TBRF is the most commonly reported autochthonous tickborne disease in Washington; up to 12 cases are reported annually (*29*). The first documented evidence of canine infection with *B. hermsii* was reported in a dog with travel to Chelan County, Washington (*37*). *B. hermsii*–positive *O. hermsi* ticks have also been documented in Washington (*38*).

Human cases of Lyme disease, anaplasmosis, ehrlichiosis, babesiosis, spotted fever rickettsioses

(including RMSF), TBRF, and tularemia are reportable to local health jurisdictions in Washington. However, clinical underrecognition and underreporting of disease are suspected. To clarify the epidemiology of tickborne diseases in Washington, we analyzed locally acquired cases and tick surveillance data. Our objectives were to describe tickborne disease epidemiology among autochthonous human cases in Washington during the study period, as well as Ixodid vectors and pathogen detections in ticks collected in Washington.

Materials and Methods

Human Case Identification

Human tickborne disease cases are identified through mandatory, but passive, reporting to local health jurisdictions from Washington healthcare providers and laboratories testing Washington residents. We reviewed all cases of anaplasmosis, ehrlichiosis, Lyme disease, babesiosis, TBRF, RMSF, and tularemia reported during 2011–2016. To ensure comparability over time, we reclassified cases to the Council for State and Territorial Epidemiologists case definitions as of 2017. Confirmed and probable cases were included for each condition. Reclassifications were required for Lyme disease, babesiosis, and tularemia. Local health jurisdictions interviewed cases in the year of report to determine clinical course, travel history, and most likely exposure location. Cases were classified as locally acquired (in-state), out-of-state acquired, or unknown exposure location based on a standardized definition. We evaluated frequency distribution of demographic variables for each condition with locally acquired cases.

Tick Surveillance

Washington State Department of Health (DOH) staff conducted weekly or biweekly tick drags during March–October at 15 sites in 5 counties in western Washington that were identified as having suitable tick habitat, public access, and relative proximity to DOH offices, thus allowing frequent monitoring. We sampled 7 sites regularly for ≥2 years and 8 sites for 1 year. Sampling was also performed in 2 counties deemed most likely exposure locations for locally acquired Lyme disease cases reported during 2011–2016. We conducted surveillance using tick drags, the most effective sampling method for both *Ixodes* and *Dermacentor* ticks. Lack of resources and capacity prevented us from including Argasid tick surveillance as part of this study. We sampled by dragging a 1 m^2 piece of flannel on the ground along either a 30-m transect or for 30 minutes in a plot created in a specific vegetation type. We inspected drags for ticks every 3–6 meters. We also obtained ticks from partners in 15 counties who found unattached, unfed ticks on themselves and reported GPS collection locations.

Upon collection, we speciated ticks using standard taxonomic keys, then stored them in vials of 95% ethanol at 4°C (*17*,*39*,*40*). We submitted specimens to either the Laboratory of Medical Zoology, University of Massachusetts–Amherst (Amherst, MA, USA); the Centre for Disease Control, British Columbia (Vancouver, BC, Canada); or the US Centers for Disease Control and Prevention (Fort Collins, CO, USA) for pathogen testing.

DNA Extraction and Molecular Identification

Pathogen testing varied by laboratory and over time; groups of ticks were tested by different laboratories for different pathogens. Testing of *Ixodes* and *Dermacentor* ticks followed each laboratory's protocols (*33*,*41*–*43*). *Ixodes* ticks were tested for *A. phagocytophilum*, *B. burgdorferi* s.s. and s.l., *B. miyamotoi*, *B. mayonii*, *Babesia* spp., *B. microti*, *Ehrlichia muris*–like agent, Powassan virus, Heartland virus, Colorado tick fever virus, and Bourbon virus. *Dermacentor* ticks were tested for *F. tularensis*, *R. rickettsii*, Powassan virus, Heartland virus, Colorado tick fever virus, and Bourbon virus. *B. burgdorferi* s.l. detected in ticks tested before 2015 were not subspeciated.

Results

During 2011–2016, a total of 202 cases of tickborne disease were reported in Washington residents; because of reclassification, this number does not match what is reported in Centers for Disease Control and Prevention notifiable condition data. Of these cases, 68 (34%) were autochthonous: Lyme disease (16 cases), RMSF (2 cases), TBRF (25 cases), and tularemia (25 cases). Yearly counts of locally acquired tickborne disease cases were low; <20 cases were reported annually (Figure 1). Tularemia and TBRF were the most frequently reported autochthonous tickborne diseases. All TBRF exposures were in eastern Washington, most in Okanogan and Spokane counties, whereas tularemia cases were broadly distributed (Figure 2). Low numbers (2–6 cases) of locally acquired Lyme disease were reported each year; for each case, no travel outside Washington during exposure periods was reported. We determined likely exposure locations based on exposure to tick habitat or known tick bite if travel to multiple counties occurred during the exposure period; these cases involved 12 counties in both eastern and western Washington.

Only 2 probable cases of RMSF were reported; both met the minimum IgG detection value. One

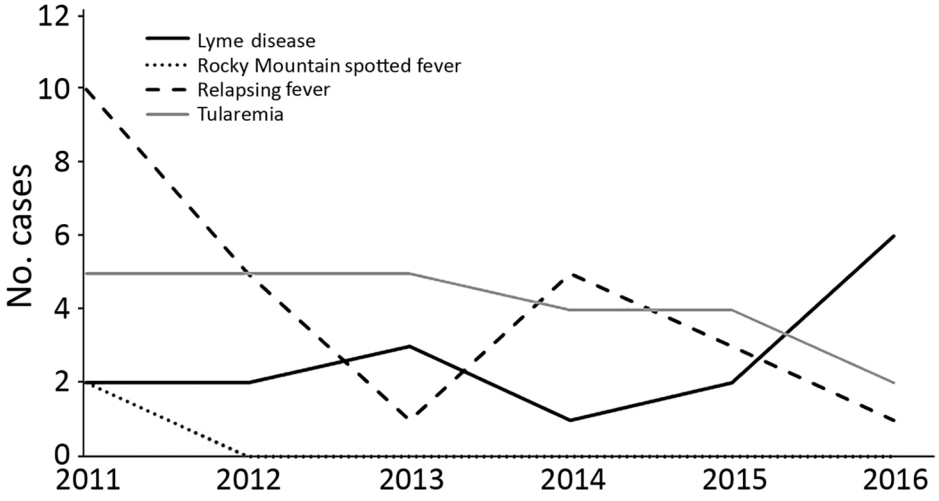

Figure 1. Locally acquired cases of tickborne diseases, Washington, USA, 2011–2016.

case-patient experienced fever, lymphadenopathy, and a single ulcerated lesion. The second case-patient reported fever and myalgia with no rash and reported a known tick bite; however, the tick was detected after symptom onset. Both patients reported likely exposure in north central Washington.

Tickborne diseases cases were reported throughout the year; the highest case counts occurred during April–October. Lyme disease cases in May, tularemia cases in July, and TBRF cases in September.

Lyme disease was the most commonly reported imported tickborne disease, and overall case counts of imported Lyme disease increased over the study period (Figure 3). Low numbers of travel-associated anaplasmosis, babesiosis, RMSF, and TBRF were reported. Two cases of blood transfusion–associated babesiosis were reported, 1 in 2014 and 1 in 2015. The blood donors in each case were Washington residents with travel history to babesiosis-endemic states (Massachusetts and Connecticut). No human cases of

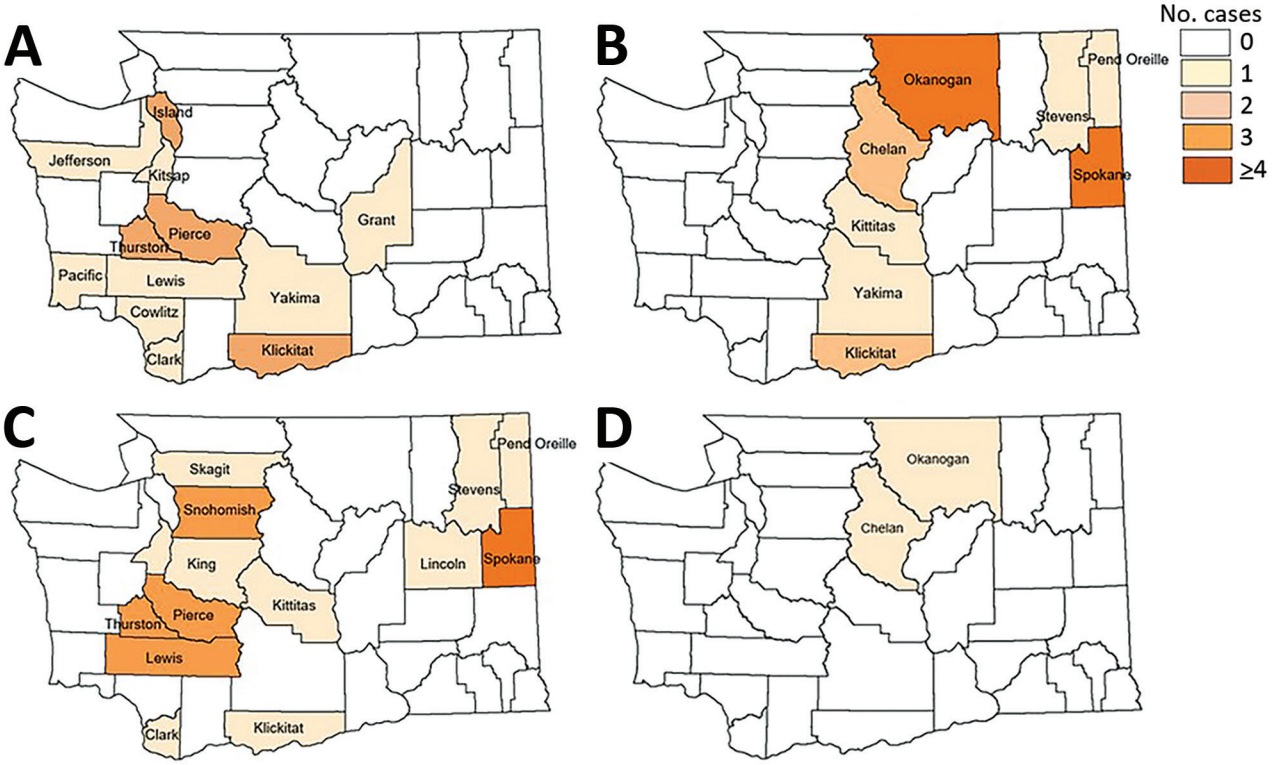

Figure 2. Counties of likely exposure for autochthonous human tickborne disease cases, Washington, USA, 2011–2016. A) Lyme disease; B) tickborne relapsing fever; C) tularemia; D) Rocky Mountain spotted fever.

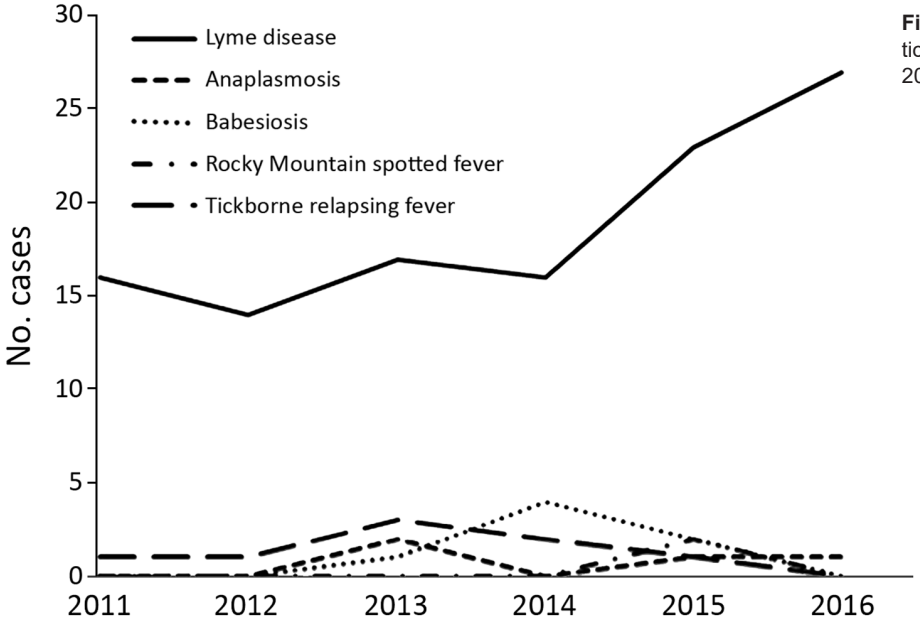

Figure 3. Travel-associated cases of tickborne diseases, Washington, USA, 2011–2016.

B. miyamotoi infection were reported in Washington residents during this period.

We identified no statistically significant differences in age or gender distribution between case-patients with locally acquired tickborne disease and those with imported cases or unknown exposure history. Among autochthonous cases, 43% of patients were female and 57% male; patient ages ranged from 7 to 91 years (median 49 years). Patients with imported cases were 39% female and 61% male; ages in this group ranged from 3 to 87 years (median 49 years).

During 2011–2016, we collected 977 unfed, host-seeking ticks from 53 sites in 19 counties (Appendix Tables 1, 2, http://wwwnc.cdc.gov/EID/article/26/4/19-1382-App1.pdf): *I. pacificus* (n = 438), *I. spinipalpis* (n = 236), *I. angustus* (n = 99), *I. auritulus* (n = 5), *D. andersoni* (n = 151), and *D. variabilis* (n = 46). Two *Ixodes* larvae were unspeciated. The 3 primary vector species, *I. pacificus*, *D. andersoni*, and *D. variabilis*, were active predominantly during the spring; 576/635 (91%) ticks were collected during March–May. Most ticks collected were adults: 100% *D. andersoni* and *D. variabilis* and 96% (420/438) *I. pacificus*.

We detected *B. burgdorferi* s.s. in 14/354 (4.0%) *I. pacificus* ticks (Table). However, detections were from only 3 of 5 counties where *B. burgdorferi* subspeciation was conducted: Clallam, 11/121 (9.1%); Klickitat, 2/117 (1.7%); and Yakima, 1/3 (33.3%) (Figure 4). In addition, we detected *B. burgdorferi* s.l. in 16/421 (3.8%) and *B. miyamotoi* in 10/227 (4.4%) *I. pacificus* ticks and *A. phagocytophilum* in 5/258 (1.9%) *I. pacificus* ticks. Six *I. pacificus* ticks were co-infected with 2 pathogens: 4 with *B. burgdorferi* s.s. and *B. miyamotoi*, 1 with *B. burgdorferi* s.s. and *A. phagocytophilum*, and 1 with *Borrelia* spp. and *A. phagocytophilum*. We also found *A. phagocytophilum* in 1/234 (0.4%) *I. spinipalpis* ticks. We detected *B. burgdorferi* s.l. in 4/235 (1.7%) *I. spinipalpis* ticks and in 1/99 (1.0%) *I. angustus* ticks. We did not detect *R. rickettsii* or *F. tularensis* in any field-collected *Dermacentor* ticks.

Discussion

Although Washington is considered a low-incidence state for tickborne diseases, our results indicate that vector populations in this state are infected with several disease-causing agents. Tickborne pathogens

Table. Pathogens detected in unfed, field-collected *Ixodes* species ticks, Washington state, 2011–2016

	No. positive/no. tested (%)							
	I. angustus		*I. pacificus*			*I. spinipalpis*		
Pathogen	Adult	Nymphs	Adult	Nymphs	Larvae	Adults	Nymphs	Larvae
Anaplasma phagocytophilum	0/79	0/16	5/240 (2.1)	0/17	0/1	1/4 (25.0)	0/122	0/108
Borrelia species*	1/82 (1.2)	0/16	4/361 (1.1)	0/17	0/1	1/5 (20.0)	1/122 (0.8)	0/108
Borrelia burgdorferi sensu lato	1/83 (1.2)	0/16	22/403 (5.5)	0/17	0/1	1/5 (20.0)	3/122 (4.1)	0/108
B. burgdorferi sensu stricto	0/41	0/4	14/340 (4.1)	1/14 (7.1)	0/0	0/3	0/63	0/0
B. miyamotoi	0/38	0/4	10/211 (4.7)	0/16	0/0	0/2	0/67	0/0

*Not differentiated to species.

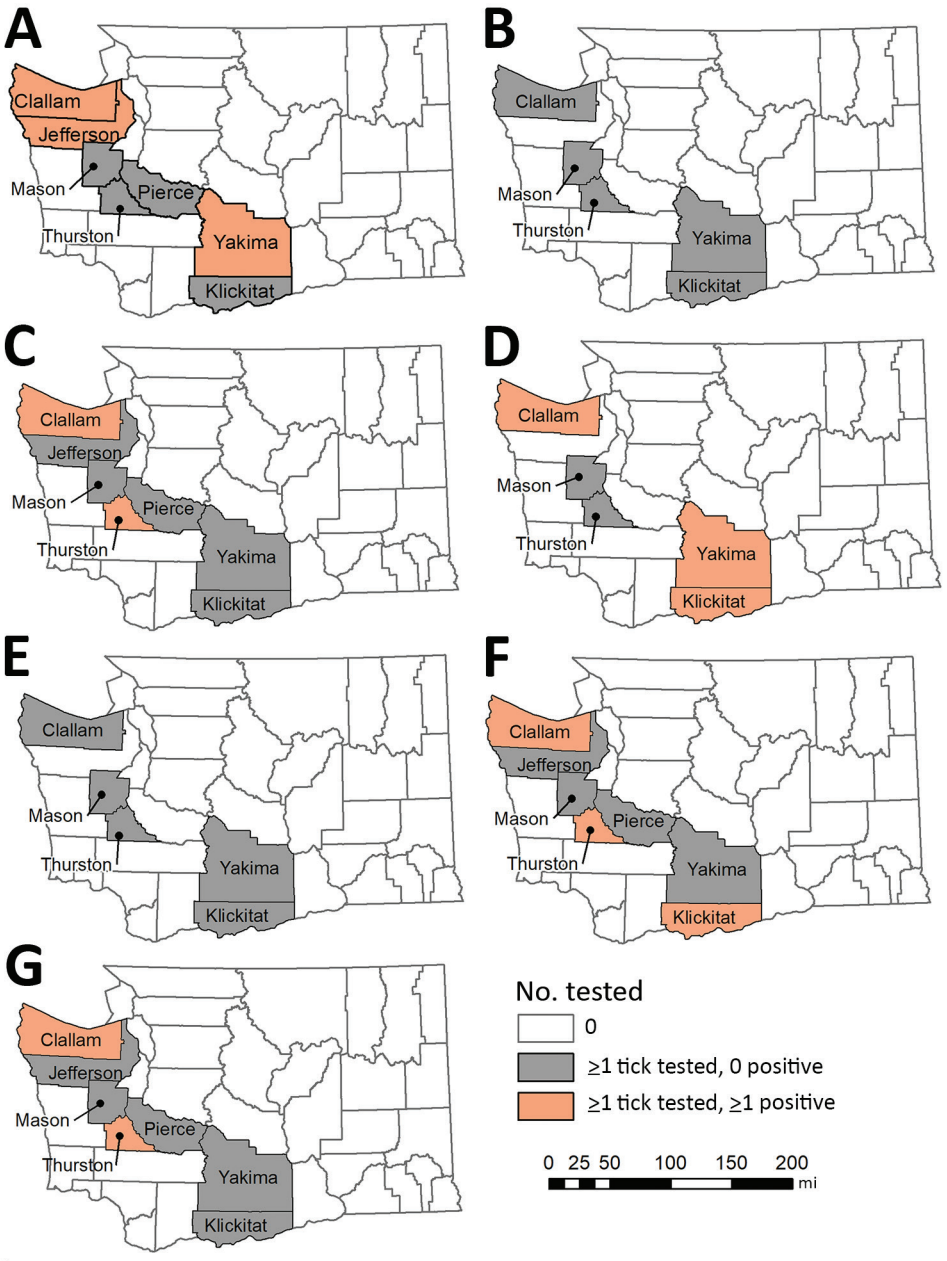

Figure 4. Counties with collection and testing of unfed, field-collected *Ixodes pacificus* ticks and pathogen detections, Washington, USA, 2011–2016. A) *Anaplasma phagocytophilum*; B) *Borrelia bissettiae*; C) *B. burgdorferi* sensu lato; D) *B. burgdorferi* sensu stricto; E) *B. lanei*; F) *B. miyamotoi*; G) *Borrelia* species.

now considered endemic in at least some areas of Washington include *B. burgdorferi* s.s., *B. hermsii*, *B. miyamotoi*, and *F. tularensis*. Evidence exists for the presence of *A. phagocytophilum*, *B. duncani*, and *R. rickettsii*, but further information is needed to assess risk. Human and animal healthcare providers should be aware of the possible risk of these diseases in their patients and should be vigilant for consistent symptoms paired with exposure histories.

Tick and human surveillance led to a common picture of increased activity in the spring. *I. pacificus* ticks infected with *B. burgdorferi* s.s., *B. miyamotoi*, or *A. phagocytophilum* were found in 4 of 9 surveyed counties. Human Lyme disease case-patients reported exposures in 12 counties. However, overlap in infected vector populations and human cases of Lyme disease occurred in just 2 counties, Klickitat and Yakima. In the remaining 10 counties, we found no positive detections of *B. burgdorferi* s.s. Field surveillance was not performed in 7 of these counties (Clark, Cowlitz, Grant, Island, Kitsap, Lewis, and Pacific); in 2 (Jefferson, Pierce), all field-collected *I. pacificus* tested negative or were not tested; and in 1 (Thurston), *B. burgdorferi* s.l. was detected, but not subspeciated. No autochthonous human cases of anaplasmosis were reported, so there was no overlap with infected vector

populations. Lack of systematic tick sampling in several of these counties, owing to their distance from DOH and resource capacity restraints, resulted in few or no unfed ticks collected, thereby limiting pathogen detection. Additional surveillance in these areas is needed to better describe the pathogen prevalence and potential for human–tick encounters.

The higher prevalence of *B. burgdorferi* s.l. (8.8%) and *B. burgdorferi* s.s. (9.1%) in *I. pacificus* ticks found in Clallam County suggests that ≥1 competent reservoir host exists in the area. The only zooprophylactic host in the area, the northern alligator lizard (*E. coerulea*), is uncommon at the sites where these ticks were collected, which might be a contributing factor to the higher pathogen prevalence. Alternatively, the small area sampled might be producing unstable prevalence estimates; additional sampling is needed to increase confidence in these findings. No human case-patients with Lyme disease reported exposure in Clallam County, possibly because of limited human–tick interaction in this area; further studies are needed to determine the most likely reservoir and to better describe human–vector interactions. All dually infected *I. pacificus* ticks also were collected in Clallam County.

In contrast to Clallam County, Klickitat County *I. pacificus* ticks had much lower prevalence of *B. burgdorferi* s.s., but 2 human patients with Lyme disease reported exposure there. The hotter, drier habitat of Klickitat County supports populations of all 3 Washington lizard species, which could be a contributing factor to why, despite the abundant tick population, the pathogen prevalence is lower.

The almost total lack of pathogen detection in field-collected *I. angustus* ticks suggests that this species plays little or no role in the maintenance or transmission of *B. burgdorferi* in Washington. This finding is confirmed by reports from California, Oregon, and Washington (*44*). Small numbers of both *I. spinipalpis* and *I. angustus* ticks have been found attached to humans in Washington and submitted to DOH for identification, but their role in pathogen transmission remains unknown.

B. miyamotoi was detected at a similar prevalence in *I. pacificus* adults as *B. burgdorferi* s.s., which is contrary to what has been found in other states, where prevalence of *B. burgdorferi* s.s. is often 10-fold higher than *B. miyamotoi* (*45*). No human cases of *B. miyamotoi* disease have been reported in Washington, which is likely attributable to a lack of clinical suspicion and testing but could also be attributable to *I. pacificus* ticks being a less efficient vector of *B. miyamotoi* than of *B. burgdorferi* s.s.

A. phagocytophilum has been reported from dogs, but not humans, in Washington. Strain variation of *A. phagocytophilum* with specific host tropism has been described (*46*–*48*); it is unknown whether the strain in Washington is not pathogenic to humans or whether the lack of detection in humans is the result of clinical underrecognition. *I. pacificus* ticks appear to play a primary role in maintaining this pathogen in nature, although *I. spinipalpis* ticks might play a minor role.

We detected no *Babesia* species in any of the ticks tested. A recent study implicating *D. albipictus* ticks as the probable vector of *B. duncani* suggests that the appropriate tick species was not tested.

Further, we found no detections of *R. rickettsii* or *F. tularensis* in unfed ticks, which is consistent with findings in other states and suggests that both these pathogens are very rare in vector populations. The presence of 2 nonpathogenic strains of *Rickettsia*, including *R. peacocki*, which is refractory to infection with and maintenance of *R. rickettsii*, suggests that *R. rickettsii* could be present only in focal areas, which is consistent with other findings (*49*). Very low or zero prevalence of *R. rickettsii* is supported by human case data; only 2 probable cases were reported during the study period. Whereas tularemia is relatively common, the transmission routes for *F. tularensis* are varied and not limited to tick vectors (*50*).

Several limitations exist with our study. Field surveillance was conducted at a small number of sites because of limited resources and efforts to determine temporal tick activity. This resulted in inconsistent and largely convenience-based tick surveillance coverage across the state. There remains a paucity of understanding of what specific reservoirs drive the maintenance of these pathogens in nature. However, several known, competent reservoirs for *B. burgdorferi* s.s. exist in counties where pathogens were detected in the tick population. Little is currently known about the epidemiology of *R. rickettsii* in Washington.

All human case reports described here arose from passive surveillance systems; locally acquired cases required positive laboratory results. Underdiagnosis and underreporting of tickborne disease are likely, as patients might not seek healthcare and healthcare providers might be unaware of the possibility. In addition, common laboratory tests might be negative early in the course of illness and true cases could be missed, particularly if serologic testing is ordered early, rather than nucleic acid detection tests. In contrast, many of the diagnostic tests used for tickborne diseases have poor specificity (e.g., Lyme disease antibody testing) and might cross-react with other species (e.g., *Rickettsia* testing). The

application of these tests in a low-incidence setting decreases their positive predictive value, and some of the cases included in this analysis likely represent false-positive results.

The same is likely true for many of the probable Lyme disease cases for which symptoms did not meet the clinical criteria set in the Council for State and Territorial Epidemiologists case definition or for which symptom information was not available. Detections of Lyme disease in 4 counties (Grant, Jefferson, Kitsap, and Lewis) were based on a single probable case each; additional evidence for *B. burgdorferi* s.s. in ticks in these counties would help lend certainty to these findings. With the exception of Grant County, submissions from veterinarians and the general public indicate that *I. pacificus* ticks are present in those counties. Similarly, both reported RMSF cases met the minimum cutoff value for IgG; based on clinical histories, these results were likely false-positive. Some misclassification of human case exposure location is probable because there is no way to determine exposure location with certainty. Finally, unknown tickborne disease pathogens could be present in Washington for which diagnostic tests are not available. As awareness of tickborne diseases spreads in the general population and among healthcare providers, we could see an increase in the number of cases as a result of improvements in diagnosis and reporting.

Strengths of this study include tracking tick collection methods and feeding status, which enabled stratification of tick data for analysis of only field-collected, unfed ticks. Submissions from host-collected ticks might not represent the true distribution in Washington, instead reflecting the host's travel history and potentially distorting estimates of prevalence. In addition, field surveillance drags were conducted at known sites and, in most cases, at multiple times during the year, providing a better picture of seasonal tick activity. Testing of individual ticks, as opposed to pooling, provided more exact information about pathogen prevalence in each site's tick population and allowed us to assess co-infection rates in individual ticks. All but a very few ticks were identified to species before testing.

We interviewed all human case-patients for exposure history, including travel, enabling us to distinguish travel-related cases from possible autochthonous cases, which is crucial to understanding tickborne disease burden in Washington. The analysis of human and tick data in tandem allowed for a more comprehensive picture of pathogen distributions and prevalence in Washington than analyzing either alone.

The true underlying rate of tickborne diseases in Washington remains unknown. Several human and animal pathogens found in tick populations are endemic to Washington, including *B. burgdorferi* s.s., *Babesia* spp., *F. tularensis*, *B. hermsii*, *A. phagocytophilum*, *B. miyamotoi*, and *R. rickettsii*; healthcare providers should be vigilant for symptoms of disease and exposure histories. The rarity of tickborne diseases creates a surveillance and diagnostic challenge; it is difficult to maintain awareness and clinical suspicion for these conditions in low-incidence settings. Surveillance data from field-collected ticks identified areas of potential human risk unidentified by existing human surveillance. Ongoing surveillance of both human cases and tick vectors is required to determine the true burden of disease and to improve public health prevention messaging to healthcare providers and the public.

Acknowledgments

We thank Ashlee-Rose Ferguson, Michelle Jodziewicz, Lauren Sherman, Susan Brush, and Eryn Farkas for their many hours of dedicated tick surveillance; Lillian Manahan for map figures; Kathleen A. Stigi for additional case review and documentation; our tick surveillance partners who supported this project through tick collection and submission; and all the local health jurisdiction and healthcare facility staff who supported this project through patient interviews and specimen collection.

This study was funded by Centers for Disease Control and Prevention Epidemiology and Laboratory Capacity grants.

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Imported Arbovirus Infections in Spain, 2009–2018

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To determine the epidemiologic and clinical characteristics of patients in Spain with imported arbovirus infections, we analyzed 22,655 records from a collaborative network for January 2009–December 2018. Among 861 arbovirus infections, 845 were monoinfections (456 [53%] dengue, 280 [32.5%] chikungunya, 109 [12.7%] Zika) and 16 (1.8%) were co-infections. Most patients were travelers (56.3%) or immigrants returning to Spain after visiting friends or relatives (31.3%). Median patient age was 37 years; most (62.3%) were women and some (28.6%) had received pretravel advice. Only 12 patients were immunosuppressed. Six cases (all dengue monoinfections, none in immunosuppressed patients) were severe. Since 2014, nondengue arbovirus infections increased; until 2016, chikungunya and Zika were most common. Imported arbovirus infections (mostly dengue) were frequently diagnosed, although increased chikungunya and Zika virus infections coincided with their introduction and spread in the Americas. A large proportion of cases occurred in women of childbearing age, some despite receipt of pretravel advice.

In recent decades, pathogenic flaviviruses (yellow fever virus, dengue [DENV], and Zika [ZIKV]) and the alphavirus chikungunya (CHIKV) have become the most common agents of emerging arbovirus diseases among humans. Their geographic distribution, epidemiologic patterns, and modes of transmission overlap considerably, and because of increased human travel and migration, outbreaks have been reported in non–arbovirus-endemic areas. Proposed drivers of virus epidemics include factors that may affect vector distribution, such as climate change, wars/social change, and poverty (decreased vector control measures) (*1*). Although some factors are unlikely to occur in non–arbovirus-endemic settings, such as Europe, emergence of these infectious diseases in these regions may result from viremic persons with imported infections and presence of competent *Aedes albopictus* mosquitoes (*2*). In nontropical areas, the potential international threat of these viruses is increased by the possibility of nonvectorborne transmission, such as transfusional, sexual, and vertical transmission (with possible severe fetal damage) (*3*). Travelers are at high risk for infection by vectorborne diseases and may contribute to the spread of imported diseases in non–disease-endemic areas (*4*).

Our objective with this study was to describe the epidemiologic and clinical characteristics of patients in Spain with a confirmed diagnosis of imported arbovirus infection. For data, we used the Red Cooperativa para el Estudio de las Infecciones Importadas por Viajeros e Inmigrantes (+Redivi) network (http://www.redivi.es), a specialized network for imported infectious diseases in Spain.

Methods

We based our analysis on a cohort included in the +Redivi network. The network initially comprised 4 centers; however, new centers have been included over the years. Currently, +Redivi comprises 22 health centers (1 primary care center and 21 hospitals, specialized and not specialized in travel medicine) in 8 regions of Spain that share a common online database in which new cases of imported infections are registered. A unique data collection sheet is used to gather information about patient demographics, trip characteristics (destination, return date, duration, type of traveler), receipt of pretravel advice, receipt of antimalaria antiprophylaxis (when applicable), reason for consultation, and final diagnosis variables. Because

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DOI: https://doi.org/10.3201/eid2604.190443

patients were deidentified at the time of study inclusion, we could not link data back to patients. Cases recorded were those in which the final diagnosis was considered to be an imported disease associated with travel (for symptomatic and asymptomatic patients). Returning travelers/immigrants could account for >1 case in the database if they had traveled >1 time and received a new diagnosis of an imported disease. +Redivi classified persons attending a first consultation as follows: immigrant (person living in Spain but born in any other country), VFR (visiting friends and relatives) immigrant (immigrant traveling back from his/her country of birth after visiting friends and relatives), VFR traveler (Spanish traveler returning from his/her first-degree relative's country of birth), and travelers (conventional international tourists returning from travel, expatriates, and missionaries).

We included in our analysis cases recorded in +Redivi during January 2009–December 2018. We identified patients with a diagnosis of arbovirus infection and compared 2 main populations: those with imported arbovirus infection and those with nonarbovirus infection. Acute/recent arbovirus infection was diagnosed for patients with exposure risk and a virus-positive PCR, positive IgM, evidence of seroconversion, or nonstructural (NS) 1 antigen detection for DENV. A diagnosis of ZIKV infection based on positive IgM required a finding of negative DENV IgM and vice versa or confirmation with a neutralization assay (not available at all centers). We classified cases as severe if there was evidence of shock and plasma leakage, severe hemorrhage, or organ impairment (according to the World Health Organization definition) (*5*). We performed a descriptive analysis to assess the distribution of these groups according to patient sex, age, type of case (immigrant, VFR, or traveler), time from arrival in Spain to medical consultation, immunosuppression, country of birth, duration of travel, date of first arrival in Spain, length of travel, date of return from travel, travel destination, and rate of pretravel advice receipt.

We expressed qualitative data as relative and absolute frequencies and quantitative data as median and interquartile ranges with 95% CIs. To compare categorical variables, we used χ^2 and Fischer exact tests when appropriate; to compare continuous variables, we used the Student *t* test (for data that were normally distributed) or the Mann-Whitney U test (for data that were not normally distributed). We used bivariate analyses to compare demographic characteristics and performed multivariable logistic regression to determine the association of exposures and outcomes. We obtained adjusted odds ratios (aORs) and 95% CIs and set the threshold for statistical significance as a 2-sided $p<0.05$.

Results

We analyzed 22,655 records from the +Redivi database; patients included 12,460 (55.0%) immigrants, 3,627 (16.0%) VFR immigrants, 414 (1.8%) VFR travelers, and 6,154 (27.2%) travelers. The most prevalent diagnoses in +Redivi were Chagas disease (18.7%), eosinophilia (5.4%), *Plasmodium falciparum* malaria (4.9%), latent tuberculosis (4.4%), schistosomiasis (4.1%), strongyloidiasis (3.9%), and arbovirus infections (3.8%). Among travelers, the second most prevalent diagnosis was arbovirus infections (8.2%), which followed acute nonspecific diarrhea (12.3%).

For a total of 861 (3.8%) cases (16 co-infections and 845 monoinfections), the final diagnosis was acute/recent arbovirus infection. Patients with monoinfections included 23 (2.7%) immigrants, 308 (36.5%) VFR immigrants, 21 (2.5%) VFR travelers, and 493 (58.3%) travelers. Arbovirus infections were mainly acquired in South America, Central America, and the Caribbean ($p<0.001$), except for travelers, who acquired most of their arbovirus infections (mostly dengue) in Southeast Asia (Tables 1, 2).

Regarding reason for medical consultation after travel, most patients with arbovirus infections reported fever, followed by myalgia. Six cases, all caused by DENV, were severe; no fatal cases were recorded. A small proportion of asymptomatic patients (53/845, 6.3%) sought a health examination and received a diagnosis of recent arbovirus infection.

Patients with arbovirus monoinfection were a median 37 years of age (interquartile range 30–46 years), and the proportion of women (62.2%; $p<0.001$) was higher than that of men. Of 526 women, 415 (78.9%) were in the age range to be fertile (15–50 years of age). Among patients with arbovirus monoinfection, only 12 (1.4%) were immunosuppressed (6 because of HIV infection, 3 because of medication, 3 because of other unspecified reason, and none because of transplant). Data on duration of travel and time elapsed from arrival in Spain to consultation are provided (Tables 1, 2). Regarding pretravel advice, 242 (28.6%) patients with an arboviral monoinfection had received advice.

The risk for arbovirus infection was not related to immunosuppression or duration of travel (Table 3). The risk was higher among female (aOR 1.40; 95% CI 1.21–1.61) than male patients and lower among patients who had received pretravel advice than among those who had not (aOR 0.65; 95% CI 0.55–0.77).

After 2014, cases of non-DENV arbovirus infection reported in +Redivi increased; CHIKV and

Table 1. Main characteristics of patients with arbovirus monoinfections included in study of arbovirus infections, Spain, 2009–2018*

Characteristic	Immigrants, n = 23	VFR immigrants, n = 308	VFR travelers, n = 21	Travelers, n = 493	p value
Median age (IQR), y	25 (14-48)	40 (34-48)	29 (11-37)	35 (29-43)	<0.001
Sex, no. (%)					<0.001
F	13(56.5)	240 (77.9)	10 (47.6)	263 (53.4)	
M	10 (43.5)	68 (22.1)	11 (52.4)	230 (46.7)	
Etiologic virus, no.					<0.001
Dengue	14	93	9	340	
Chikungunya	8	162	7	103	
Zika	1	53	5	50	
Region, no. (%)†					<0.001
Sub-Saharan Africa	4 (17.4)	12 (3.9)	2 (9.5)	42 (8.5)	
North America	0	0	0	2 (0.4)	
Central America and Caribbean	5 (21.7)	94 (30.5)	9 (42.9)	128 (26.0)	
South America	10 (43.5)	192 (62.3)	6 (28.6)	84 (17.1)	
South-central Asia	3 (13.0)	6 (2.0)	0	61 (12.4)	
Eastern Asia	0	0	0	2 (0.4)	
Southeast Asia	1 (4.4)	4 (1.3)	3 (14.3)	166 (33.7)	
Australasia	0	0	1 (4.8)	4 (0.8)	
Europe	0	0	0	2 (0.4)	
Median travel days, no. (IQR)	0	30 (27–55)	30 (21–75)	23 (15–50)	<0.001
Median weeks until first consultation, no. (IQR)	1.2 (0.6–4.3)	2 (0.9–5.8)	2.6 (1.3–4.4)	1.1 (0.4–3.6)	<0.001
Travel >30 d, no. (%)	0	135 (44.3)	10 (47.6)	164 (33.3)	0,.05
Pretravel advice, no. (%)	0	21 (6.9)	3 (14.3)	218 (44.3)	<0.001
Immunosuppression, no. (%)	1 (4.4)	4 (1.3)	2 (9.5)	5 (1.0)	0.008

*IQR, interquartile range; VFR, visiting friends and relatives.
†Data from Red Cooperativa para el Estudio de las Infecciones Importadas por Viajeros e Inmigrantes (+Redivi, http://www.redivi.es).

ZIKV were the most common arbovirus infections until 2016 (Figure). During the peak of the ZIKV outbreak (mainly December 2015–November 2016), there were 226 cases of arbovirus infections: 85 (37.6%) ZIKV, 82 (36.3%) DENV, 51 (22.6%) CHIKV, and 8 (3.5%) co-infections.

Dengue

A total of 456 (53%) DENV monoinfections were recorded; the proportion of infected women (262 women, 57.5%, p = 0.009) was higher than that of men. Of 262 women, 222 (84.7%) were 15–50 years of age, and of these, only 85 (38.3%) had received pretravel advice. Among patients with dengue infection, 3 had a previous diagnosis of HIV infection, 2 were immunosuppressed secondary to medication, and 1 was immunosuppressed for unspecified cause. The 6 patients with severe nonfatal dengue acquired the infection in South America (66.7%) and Southeast Asia (33.3%); none were immunosuppressed.

Chikungunya

A total of 280 (32.5%) CHIKV monoinfections were recorded; the proportion of women (198 women, 70.7%; p<0.001) was higher than that of men. Of 198 women, 136 (68.7%) were 15–50 years of age, and of these, only 23 (16.9%) had received pretravel advice. Among patients with CHIKV infection, 2 had a previous diagnosis of HIV infection and 2 were immunosuppressed (unspecified cause).

Zika

A total of 109 (12.7%) ZIKV monoinfections were recorded; the proportion of women (66 women, 60.6%; p = 0.128) was higher than that of men. Of 66 women, 57 (86.4%) were 15–50 years of age, and of these, only 7 (12.3%) had received pretravel advice. No cases in pregnant women were recorded. Among patients with ZIKV infection, 1 had a previous diagnosis of HIV infection and 1 was immunosuppressed secondary to medication.

Co-infections

At least 16 (1.8%) patients were co-infected with >1 arbovirus (some probable co-infections with positive serology results for DENV and ZIKV were also registered, but these cases could not be confirmed). DENV and ZIKV co-infection occurred in 10 patients and DENV and CHIKV co-infection in 4. Two patients were co-infected with CHIKV and ZIKV. Most patients were travelers (9/16, 56.3%) or VFR immigrants (5/16, 31.3%). The 10 DENV/ZIKV co-infections were acquired in South America (3), Central America and the Caribbean (3), Australasia (1), Southeast Asia (1), sub-Saharan Africa (1), and south-central Asia (1). The 4 DENV/CHIKV co-infections were acquired in South America (3) and Central America and the Caribbean (1). The 2 CHIKV/ZIKV co-infections were acquired in south-central Asia and South America. No co-infections in immunosuppressed patients were recorded.

Table 2. Main characteristics of patients with arbovirus monoinfections, by virus type, Spain, 2009–2018*

Characteristic	Dengue, n = 456	Chikungunya, n = 280	Zika, n = 109	p value
Median age (IQR), y	34 (29-43)	41 (34-52)	36 (30-43)	<0.001
Sex, no. (%)				0.001
F	262 (57.5)	198 (70.7)	66 (60.6)	
M	194 (42.5)	82 (29.3)	43 (39.4)	
Type of patient				<0.001
Immigrant	14	8	1	
VFR immigrant	93	162	53	
VFR traveler	9	7	5	
Traveler	340	103	50	
Region, no. (%)				<0.001
Sub-Saharan Africa	39 (8.6)	16 (5.7)	5 (4.6)	
North America	1 (0.2)	0	1 (0.9)	
Central America and Caribbean	89 (19.6)	94 (33.6)	53 (48.6)	
South America	105 (23.1)	139 (49.6)	48 (44.0)	
South-central Asia	58 (12.8)	11 (3.9)	1 (0.9)	
Eastern Asia	2 (0.4)	0	0	
Southeast Asia	156 (34.3)	17 (6.1)	1 (1.0)	
Australasia	3 (0.7)	2 (0.7)	0	
Europe	1 (0.2)	1 (0.4)	0	
Median no. travel days (IQR)	25 (15-36)	31 (25-66)	30 (20-59)	<0.001
Median no. weeks until first consultation (IQR)	1.1 (0.4-3.1)	3 (1.0-7.1)	1.2 (0.6-3.3)	<0.001
Travel >30 d, no. (%)	133 (30.1)	135 (50.2)	41 (38.0)	<0.001
Pretravel advice, no. (%)	175 (39.7)	55 (20.4)	12 (11.1)	<0.001
Immunosuppression (%)	6 (1.3)	4 (1.4)	2 (1.8)	0.919

*IQR, interquartile range; VFR, visiting friends and relatives.

Discussion

In recent years, imported emerging arbovirus infections have become a concern because of the increasing number of cases and the possibility of introduction and local transmission in non–arbovirus-endemic areas; several outbreaks have been documented in non–arbovirus-endemic areas, including several countries in Europe (*6*,*7*). We report data on ≈850 infections caused by arboviruses over a 10-year period (January 2009 to December 2018) registered at centers belonging to a specialized network for imported infectious diseases in Spain. Imported arbovirus infections were among the top 10 established diagnoses in this national network (despite the large proportion of immigrants included in the network, for whom acute arbovirus infection is an infrequent diagnosis); among travelers, arbovirus infection was the second most frequent diagnosis.

Most cases occurred in conventional travelers, followed by VFRs; the small proportion of cases in immigrants may reflect recent acute (nonviremic) infections. Among VFR immigrants, infection with DENV was relatively less frequent than infection with other arboviruses; whereas, for all other groups, the reverse was true. This distribution may reflect a hypothetical protected state if these patients had already experienced multiple DENV infections before immigration. On the other hand, some of the acute infections acquired by persons in this group may have resulted from waning or incomplete immunity to DENV because homotypic reinfections, as well as poor in vitro neutralizing activity of immune human serum for

Table 3. Analysis of variables potentially associated with arbovirus infection (dengue, Zika, and chikungunya virus), Spain, 2009–2018*

Variable	Univariate analysis		Multivariate analysis	
	OR (95% CI)	p value	aOR (95% CI)†	p value
Type of patient				
Immigrant	Referent	NA	Referent	NA
VFR immigrant	50.18 (32.80–76.78)	<0.001	47.15 (30.78–72.22)	<0.001
VFR traveler	28.89 (15.86–52.65)	<0.001	33.53 (18.20–61.77)	<0.001
Traveler	47.09 (30.96–71.62)	<0.001	45.18 (29.68–68.77)	<0.001
Age, y	1.01 (1.01–1.02)	<0.001	1.01 (1.00–1.01)	0.003
Female sex	1.46 (1.27–1.69)	<0.001	1.40 (1.21–1.61)	<0.001
Immunosuppression, yes/no‡	0.28 (0.16–0.49)	<0.001	0.61 (0.34–1.10)	0.099
Pretravel advice, yes/no‡§	0.68 (0.58–0.80)	<0.001	0.65 (0.55–0.77)	<0.001
Travel, d§	1.00 (0.99–1.00)	0.807	0.99 (0.99–1.00)	0.900

*aOR, adjusted odds ratio; NA, not applicable; OR, odds ratio; VFR, visiting friends and relatives.
†For type of patient, age, female sex, and immunosuppression.
‡Ratios reflect risk for those who answered yes compared with those who answered no.
§For travelers and VFR only.

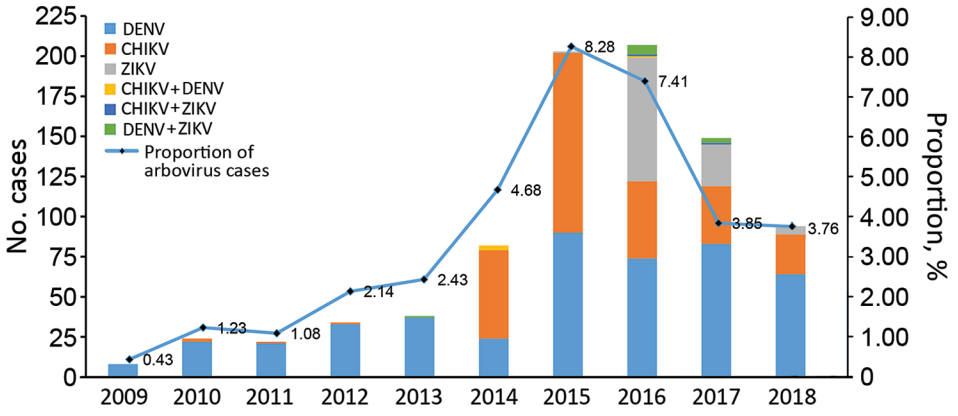

Figure. Proportion of arbovirus monoinfections and co-infections, by virus type and year, compared with total number of infections registered in the Red Cooperativa para el Estudio de las Infecciones Importadas por Viajeros e Inmigrantes (+Redivi, http://www.redivi.es) network, Spain, 2009–2018. A) Numbers of arbovirus infections; B) proportion of arbovirus infections compared with total number of infections registered.

different strains within a single serotype, have been reported (*8*,*9*). These issues could be further explored by investigating the arboviral serostatus of these patients before travel.

As expected, most cases overall were caused by DENV, although in recent years, and coinciding with the introduction of CHIKV and ZIKV in the Western Hemisphere, increased infections caused by CHIKV and ZIKV have been registered. In our study, CHIKV accounted for most of the arbovirus infections diagnosed during 2015, and ZIKV caused most of the arbovirus infections diagnosed during 2016, mirroring the epidemiologic situation and public concern at the time (ZIKV infection could account for some of the asymptomatic patients who may have sought care for reasons such as preconception or predelivery testing on return from travel) (*10*). Although the network's catchment area had increased over the years, the proportion of arbovirus infections compared with the total was considered, and increased arbovirus infections were observed during 2015–2017; a downward trend in 2018 reflected global epidemics and increased awareness of these infections.

We found an increased risk for arbovirus infection among women, possibly reflecting increased diagnosis of symptomatic infections. Previous studies have also described increased frequency of arbovirus infections in women compared with men, and female sex has been identified as a key risk factor associated with severe arthralgia caused by CHIKV infection (*11*–*13*). However, these findings are not consistent; other studies describing complicated infections have found that among patients with CHIKV infection admitted to an intensive care unit, the greater proportion were male (*14*). These issues, as well as the presence of other possible contributing factors such as underlying conditions, should be explored further.

As expected, having received pretravel information was a protective factor; however, a high proportion of patients did not seek pretravel advice, an area in need of improvement and identified in other studies (*15*). Although no vaccines are readily available for use in travelers, simple measures may limit the burden of some of these arbovirus infections, such as avoidance of mosquito bites and the correct diagnosis and education of patients who may transmit the infection to others (e.g., sexual transmission of ZIKV). Of note, many cases occurred in women of childbearing age (in some instances despite pretravel advice), an issue of concern given the association of ZIKV and possibly DENV infection with increased risk for congenital malformations (*16*,*17*). Although we found no records of ZIKV infections in pregnant women, cases of congenital ZIKV infection have been reported in Spain (*18*).

Available data about the outcome of arbovirus disease in immunosuppressed patients is limited. In our study, data were registered for 12 immunosuppressed patients with arbovirus disease; we found no association between infection and immune status, and none of the severe cases (all dengue) occurred in immunosuppressed patients. According to published data, CHIKV in solid organ transplant recipients seems to have a benign course and favorable outcome (*19*,*20*), whereas data for DENV in these patients are less clear. Case descriptions report uncomplicated outcomes, whereas a recent literature review reported a significantly higher incidence of severe dengue and a higher mortality rate among transplant patients with dengue (*21*–*23*). A recent report of DENV, ZIKV, and CHIKV infections diagnosed in hematopoietic stem cell transplant recipients and oncohematologic patients found prolonged viremia in those with DENV and viruria in those with ZIKV; the most common complication was thrombocytopenia, but all patients survived without sequelae (*24*). However, other case reports reveal less favorable outcomes and even atypical presentations: a fatal pseudotumoral form of

ZIKV meningoencephalitis in a heart transplant recipient has been reported (*25*).

With respect to arbovirus infection in HIV-positive patients, data are scarce, although favorable outcomes have been reported (*20*,*26*,*27*). In a study of 43 HIV-positive patients with confirmed ZIKV infection, no hospitalizations, complications, or deaths were found, and CD4 cell counts and HIV viral load did not differ after ZIKV infection (*28*). Regarding arbovirus infections in patients immunosuppressed for other reasons, available data are extremely limited and no firm conclusions can be drawn.

Of interest, we found 16 patients co-infected with multiple arboviruses, reflecting co-circulation and transmission by the same species of DENV, ZIKV, and CHIKV vector during the main outbreak periods in Latin America and the Caribbean. No cases were severe. During periods of intense transmission, co-infections are not infrequent or unexpected (*29*). A recent study that used reverse transcription PCR to investigate patients with acute undifferentiated febrile illness during the CHIKV outbreak in Haiti found that ≈10% of the children with CHIKV infection during the study period were infected with another arbovirus (ZIKV, DENV, or Mayaro virus) (*30*). These dual infections were diagnosed only after culture of the samples, and the authors concluded that that could suggest a low viral load of the co-infecting virus. In addition, the possibility of sequential infections acquired in areas of co-circulation has been previously noted, and sequential infections in patients returning from travel have been described (*31*). Further studies would be needed to determine whether co-infections, sequential infections, or both may be associated with a less favorable outcome.

Some study limitations include the inability to identify which case-patients were viremic at diagnosis. The database included confirmed diagnoses, but diagnosis of some cases may have been based on detection of virus IgM (with or without IgG; i.e., recent acute infections), and so the proportion of viremic patients who may be considered to potentially pose a risk for ongoing transmission in areas of Spain where competent vectors are present was not known. Because only imported cases were registered, possible secondary cases resulting from sexual transmission were not included, probably leading to underestimation of the real extent of imported disease. In some cases, cross-reactions between antibodies to the flaviviruses DENV and ZIKV may have posed a diagnostic challenge (neutralization assays were available only at some centers), and some cases of probable co-infections were excluded and considered monoinfections. Also, previous yellow fever vaccination was not recorded for most patients and was therefore not analyzed, although prior vaccination would be expected to yield possible false-positive IgG results and should not have interfered in the classification of cases as acute or recent infections (which was based on IgM, seroconversion, PCR, or NS1 antigen for DENV). Recent yellow fever vaccination may yield false-positive IgM results for DENV and ZIKV, but most of these infections in this series were acquired in regions where such vaccination was not required (Asia, Caribbean, Australasia). Basic information on main presenting symptoms was recorded, but more detailed information on individual symptoms, enabling a possible comparison with regard to presentation of each arbovirus infection, was not possible.

Despite study limitations, we present some aspects of a large series of imported arbovirus infections diagnosed at specialized centers in Spain. Those imported infections may pose a risk for further transmission in areas where competent *Ae. albopictus* mosquitoes are established, as illustrated by the recent diagnosis of autochthonous DENV and CHIKV infections in several southern European countries such as France, Italy, Croatia, and Spain (*2*,*32*). In 2018, the first 6 cases of locally acquired dengue were reported in Spain, 5 in patients who had traveled to Cádiz and Murcia in southern Spain and 1 in a patient from Barcelona (*2*,*33*). In 2019, an additional case of autochthonous DENV was diagnosed in Catalonia (*34*). The virus was probably introduced into these areas by viremic travelers. In past decades, *Ae. albopictus* mosquito populations have increased in areas of the European Union and are active in several provinces in mainland Spain. *Ae. aegypti* mosquitoes, the main vector for DENV transmission, have been introduced in areas of Europe, are present in Madeira (Portugal), and have been identified in the Canary Islands (Spain), although after control measures were implemented locally in 2017, no further detections in the Canary Islands were reported (*2*). Recently, probable sexual transmission of DENV in an area without documented presence of mosquito vectors was reported in Madrid, Spain (*35*).

These cases highlight the value of having a high index of suspicion and the need for rapid diagnosis of arbovirus infections, which may be aided in specific settings (e.g., emergency departments) by the availability of rapid diagnostic tests to detect specific antigens such as the NS1 DENV antigen. Early detection of imported infections in viremic patients could enhance the study of virus genomics through virus sequencing and phylogenetics, which may be used to investigate infectious disease outbreaks and contribute

to transmission chain tracking. Viremic patients could also be instructed to protect themselves from insect bites to prevent further transmission in areas where competent vectors are established.

The number of ZIKV infections in women of childbearing age, despite these women having received pretravel advice, also poses a significant public health concern, coupled with the possible risk for sexual transmission from a partner arriving from a risk area, even several months after travel (*36*). Although sexual transmission of DENV seems to be a rare route of transmission and the risk is considered extremely low, clinicians should consider this possibility for patients with DENV and no compatible travel history and should advise patients with DENV that the risk for transmission via this route may be minimized through safe sex practices.

These data highlight the challenges posed by these imported infections and the need for improvements, such as the establishment of specific protocols for screening potential blood and organ donors and the enhancement of laboratory capacities. The strengthening of information sources (community-based information and online resources) should be prioritized, not only for at-risk travelers, such as women of fertile age and immunosuppressed patients, but also for physicians in non–arbovirus-endemic areas faced with specific diagnostic and management challenges.

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Acknowledgment

We are grateful to Liliana Moreno Velásquez for her contribution to data entry and management.

+Redivi is supported by the Red de Investigación Colaborativa de Enfermedades Tropicales, grant no. 16/0027/0020.

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Decreased Susceptibility to Azithromycin in Clinical *Shigella* Isolates Associated with HIV and Sexually Transmitted Bacterial Diseases, Minnesota, USA, 2012–2015

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Shigellosis outbreaks caused by *Shigella* with decreased susceptibility to azithromycin (DSA-*Shigella*) among men who have sex with men (MSM) have been reported worldwide. We describe sexual health indicators and antimicrobial drug resistance for shigellosis cases in Minnesota, USA. We analyzed a sample of isolates received during 2012–2015 and cross-referenced cases with the Minnesota Department of Health Sexually Transmitted Disease Database to ascertain patients' HIV status and recent chlamydia, gonorrhea, and syphilis infections. Of 691 *Shigella* isolates, 46 (7%) were DSA-*Shigella*; 91% of DSA-*Shigella* patients were men, of whom 60% were living with HIV. Among men, those with DSA-*Shigella* infection had greater odds of living with HIV, identifying as MSM, or having a recent diagnosis of a sexually transmitted disease. DSA-*Shigella* was associated with MSM, HIV infection, and recent sexually transmitted disease. To decrease spread of DSA-*Shigella*, interventions targeted at communities at high risk are needed.

Shigella is an enteric bacterial pathogen that causes diarrhea (sometimes bloody), fever, and cramps (*1*). An estimated 500,000 *Shigella* infections occur annually in the United States (*2*). *Shigella* transmission is fecal–oral; it is easily spread person-to-person because of a low infectious dose. Outbreaks are most frequently documented in childcare settings but have also been reported among men who have sex with men (MSM) (*3*–*7*). *Shigella* infections are typically self-limiting, but treatment is recommended for patients with severe illness or underlying immunocompromising conditions (*1*,*8*). Antimicrobial drug treatment might shorten illness duration and is often used in childcare-associated outbreaks to prevent secondary transmission (*1*,*8*). When *Shigella* antimicrobial drug susceptibilities are unknown (e.g., when empiric therapy is started before culture and sensitivity results are available) or if the isolate is resistant to ampicillin and trimethoprim/sulfamethoxazole, oral treatment options include ciprofloxacin or azithromycin (*1*). However, fluoroquinolones, including ciprofloxacin, are generally avoided for treatment in children because of the risk for musculoskeletal damage (*9*).

The emergence of *Shigella* with decreased susceptibility to azithromycin (DSA) has been reported in Asia, Europe, North America, and Oceania (*10*–*17*). Local outbreaks and intercontinental sexual transmission of DSA-*Shigella* have been observed among MSM (*10*,*15*,*18*–*20*). Co-infection with other enteric pathogens and sexually transmitted diseases (STDs) have also been reported among MSM (*20*–*24*).

In 2016, the Clinical and Laboratory Standards Institute (CLSI) defined epidemiologic cutoff values for azithromycin resistance in *S. sonnei* or *S. flexneri* for the first time (*25*). Previously, the National Antimicrobial Resistance Monitoring System (NARMS) had documented DSA (azithromycin MIC >16 μg/mL) among *Shigella* isolates; during 2011–2015, DSA prevalence increased from 0.9% to 6.1% among *S. sonnei* isolates and from 12.1% to 32.9% among *S. flexneri*

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DOI: https://doi.org/10.3201/eid2604.191031

isolates submitted to NARMS (*26*). The Minnesota Department of Health (MDH) Public Health Laboratory (PHL) began testing 10% of *Shigella* isolates for DSA in 2010 and found such an isolate in 2013. As a result, we began more extensive testing of isolates from 2012 onward to characterize the emergence of DSA-*Shigella*. The purpose of this study was to describe the relationship between the emergence of DSA-*Shigella* in Minnesota and HIV and bacterial STDs.

Materials and Methods

In Minnesota, all *Shigella* infections must be reported to MDH. In addition, clinical laboratories must submit isolates or clinical specimens to the MDH PHL. Phone interviews with patients are attempted by using a standard questionnaire.

We analyzed all shigellosis cases in Minnesota residents who had a sample collected during January 1, 2012–December 31, 2015, that was culture-confirmed by the MDH PHL. Patients with >1 isolate were considered to have a new infection if specimens were collected ≥90 days apart or were different *Shigella* species. We collected information by telephone interview on demographics, travel history, children in the household, contact with a childcare facility, ill contacts, symptom history, and antimicrobial drug treatment. During 2012–2014, sexual activity in the week before onset was noted on the case report form when reported by the patient, but it was not routinely collected.

In 2015, sexual exposure questions ("in the 7 days before your onset of illness did you have any sexual contact with a male; did you have any sexual contact with a female?") were added to the *Shigella* questionnaire for adult patients. Case name and date of birth were linked with the MDH STD, HIV, and Tuberculosis Section database to obtain HIV, chlamydia, gonorrhea, and syphilis test results, and antimicrobial drug treatment for such infections occurring during the 12 months before each *Shigella* isolation. Risk factors for HIV infection, such as identifying as an MSM during an HIV surveillance interview, were obtained for persons living with HIV (PLWH). Children were defined as persons <18 years of age. Recent STD was defined as chlamydia, gonorrhea, or syphilis infection in the 12 months before *Shigella* isolation.

We performed antimicrobial susceptibility testing by using the modified Kirby-Bauer disk diffusion method on all *Shigella* isolates collected during 2013 and 2014. We also performed antimicrobial susceptibility testing on the following isolates collected during 2012 and 2015: all *S. flexneri* isolates, all isolates from international travelers and adults, 75% of pediatric outbreak isolates, and 30% of the remaining sporadic pediatric isolates. The antimicrobial susceptibility testing panel included 8 classes of antimicrobial drugs: aminoglycosides (gentamicin, streptomycin), cephems (cephalothin, ceftriaxone), folate-pathway inhibitors (sulfisoxazole, trimethoprim/sulfamethoxazole), macrolides (azithromycin), penicillins (ampicillin), phenicols (chloramphenicol), quinolones (ciprofloxacin, nalidixic acid), and tetracylines (tetracycline). Susceptibility was classified by using CLSI guidance, including classifying DSA in *S. flexneri* as a ≤15 mm zone of inhibition for a disk containing 15 μg of azithromycin (BD BBL Sensi-Disc; Becton Dickinson, https://www.bd.com) (*25*). Because CLSI guidelines do not include disk diffusion epidemiologic cutoff values for use with *S. sonnei*, we conservatively defined DSA as no zone of inhibition (6 mm) by disk diffusion using disks containing 15 μg of azithromycin. A sample of isolates with DSA was submitted to NARMS for further characterization by PCR for the macrolide resistance genes *mph*A and *erm*B. Multidrug resistance (MDR) was defined as resistance to ≥3 antimicrobial drug classes and clinical resistance as resistance to >1 of the major antimicrobial drug class (cephems, folate-pathway inhibitors, macrolides, penicillins, quinolones).

We compiled descriptive and summary statistics by using SAS version 9.4 (SAS Institute, https://www.sas.com). We analyzed categorical variables by using Fisher exact or χ^2 tests as appropriate and compared medians by using 2-sided Wilcoxon rank-sum tests. Statistical significance was set at $\alpha<0.05$.

Results

During 2012–2015, a total of 882 infections in 878 patients were confirmed as *Shigella* by the MDH PHL. Among the 882 infections, 750 (85%) were caused by *S. sonnei*, 125 (14%) by *S. flexneri*, 5 (0.6%) by *S. boydii*, and 2 (0.2%) by *S. dysenteriae*. Half (n = 442) of the infections were in children, 223 (25%) were in adult women, and 213 (24%) were in adult men (Figure 1).

Antimicrobial Drug Resistance

Antimicrobial susceptibility testing was performed for 691 (78%) isolates; 46 (7%) isolates had DSA (24 [4%] of 559 *S. sonnei* isolates, 21 [17%] of 125 *S. flexneri* isolates, and 1 [20%] of 5 *S. boydii* isolates) (Table 1; Figure 1). Two DSA-*Shigella* isolates each were collected during 2012 and 2013; the remaining 42 isolates were collected during 2014 (*S. sonnei*, 16; *S. flexneri*, 4) and 2015 (*S. sonnei*, 7; *S. flexneri*, 15) (Figure 2).

Isolates with resistance to ≥1 of the 8 classes of antimicrobial drugs tested were observed among all sex and age groups (Table 2). After we excluded international travelers, we found that similar proportions of

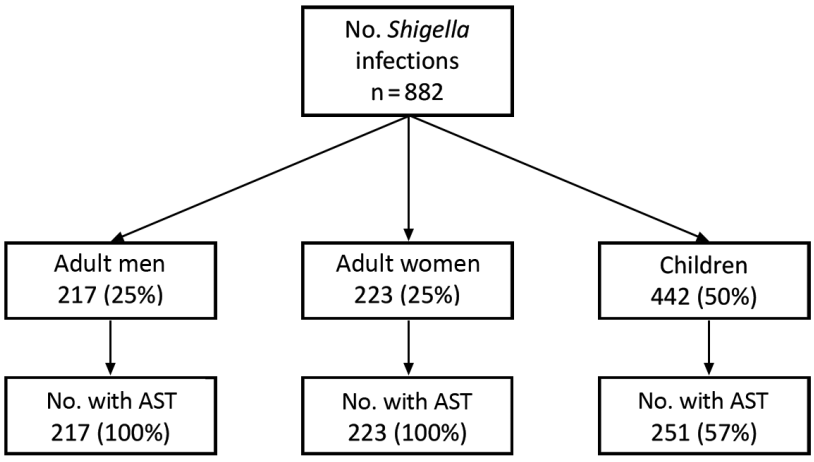

Figure 1. Number of *Shigella* isolates for which AST was conducted, by demographic group among isolates received at the Minnesota Department of Health, 2012–2015. AST, antimicrobial susceptibility testing.

isolates from children and women were resistant to each of the antimicrobial drug classes except for folate-pathway inhibitors (children 57% resistant vs. women 40% resistant; p<0.001) and phenicols (children 9% resistant vs. women 3% resistant; p = 0.01). Isolates from men had a higher prevalence of resistance to all drug classes except for cephems and aminoglycosides and were more likely to have clinical resistance (Table 2).

Forty-two (91%) of the 46 DSA-*Shigella* infections were in men; among the other 4 infections, 1 case in 2012 and 1 case in 2013 were in children and 2 cases in 2014 were in women (Figure 2). In comparison, only 175 (27%) azithromycin-susceptible infections were in men (odds ratio [OR] 28.2, 95% CI 10.0–79.8; p<0.001).

Three men had multiple *Shigella* infections. One patient had an azithromycin-susceptible *S. sonnei* infection in 2013 and a DSA *S. flexneri* 3b infection in 2015; 1 patient had a DSA *S. flexneri* 3b infection in 2015 and an azithromycin-susceptible *S. sonnei* infection that had a specimen collection date 66 days later; and 1 patient had an azithromycin-susceptible *S. sonnei* infection in 2012, an azithromycin-susceptible *S. flexneri* 3a infection in 2014, and a DSA *S. flexneri* 3b infection with a specimen collection date 104 days after the *S. flexneri* 3a infection.

Sixteen DSA isolates were further characterized by NARMS for macrolide resistance genes. All isolates had the *mph*A resistance gene, and 15 isolates had the *erm*B resistance gene.

Illness Severity and Treatment

We found no major differences in the proportion of patients with fever, bloody diarrhea, or hospitalization among patients with DSA-*Shigella* compared with patients who had azithromycin-susceptible *Shigella*. The median duration of illness was longer for patients with DSA-*Shigella* (11 days) than for patients with azithromycin-susceptible *Shigella* (9 days) (p = 0.004) (Table 3).

Information about antimicrobial drug treatment for shigellosis was available for 752 (85%) infections; 514 (68%) patients were treated with 1 antimicrobial drug and 56 (7%) with multiple antimicrobial drugs. For 182 (24%) infections, the patient either refused treatment (n = 178) or could not recall whether treatment had been given (n = 5). We found similar rates

Table 1. Frequency of azithromycin zone of inhibition for *Shigella* isolates by species, Minnesota, USA, 2012–2015*

	No. (%) isolates			
Inhibition zone, mm	*S. sonnei*, n = 559	*S. flexneri*, n = 125†	*S. boydii*, n = 5	*S. dysenteriae*, n = 2
6	24 (4)	18 (14)	1 (20)	0
8	0	1 (0.8)	0	0
12	0	1 (0.8)	0	0
14	0	1 (0.8)	0	0
15	1 (0.2)	0	0	0
17	2 (0.4)	0	0	0
18	3 (1)	0	0	0
19	5 (1)	2 (2)	0	0
20	23 (4)	4 (3)	0	0
21–25	442 (79)	48 (38)	2 (40)	1 (50)
26–30	58 (10)	44 (35)	2 (40)	0
>30	1 (0.2)	6 (5)	0	1 (50)

*Decreased susceptibility to azithromycin is defined as no zone of inhibition (6 mm) by disk diffusion using a disk containing 15 μg of azithromycin.
†Decreased susceptibility to azithromycin is defined as a ≤15 mm zone of inhibition with a disk containing 15 μg of azithromycin.

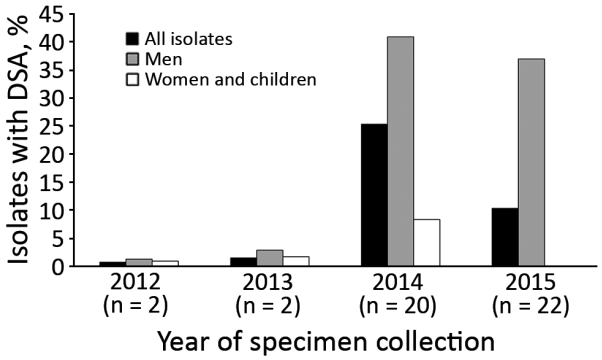

Figure 2. Percentage of 46 clinical *Shigella* isolates tested at the Minnesota Department of Health that had DSA, by year and demographic group, 2012–2015. DSA, decreased susceptibility to azithromycin.

of antimicrobial drug treatment infections with and without DSA (84% vs. 77%; p = 0.54). Patients who received antimicrobial drug treatment reported longer durations of illness (median 9 days vs. 7 days; p = 0.04). Of 103 patients who had antimicrobial susceptibility testing and reported treatment with azithromycin, 4 (4%) had DSA-*Shigella* isolates. Among patients given azithromycin, illness caused by DSA-*Shigella* tended to last longer than that caused by azithromycin-susceptible *Shigella*, but the difference was not significant (median 17 days vs. 7 days; p = 0.06).

Risk History

Patient interviews were completed for 610 (88%) of the 691 isolates with antimicrobial susceptibility testing results, including 38/46 (82%) with DSA-*Shigella* and 572/645 (88%) that were susceptible to azithromycin. No or few patients with DSA-*Shigella* were exposed to childcare settings (0% vs. 31%; OR undefined; p<0.001) or had children in their home (11% vs. 62%; OR 0.1, 95% CI 0.0–0.2; p<0.001). International travel was reported by the patient or patient's healthcare provider for 3 (8%) of 39 patients with DSA-*Shigella* and by 66 (12%) of 573 patients with azithromycin-susceptible *Shigella* (OR 0.6, 95% CI 0.2–2.1; p = 0.61).

Travel to Asia was reported by all 3 patients with DSA-*Shigella* infections who reported international travel; 2 were women and 1 was a child. Among those asked, sexual contact with a man in the week before illness onset was reported by 16 (62%) of 26 men with DSA-*Shigella* and 13 (33%) of 40 men with azithromycin-susceptible *Shigella* isolates (OR 3.3, 95% CI 1.2–9.3; p = 0.02). Of the 29 male patients reporting sexual contact with a man, 1 patient with an azithromycin-susceptible infection reported international travel; none reported contact with childcare or children in the household. Among the 66 patients who had sexual contact information on their shigellosis interview and after combining sexual history data from the HIV/STD surveillance database (n = 63), 88% of men with DSA-*Shigella* either identified themselves as an MSM during an HIV/STD interview or reported sexual contact with a man versus 33% of those with azithromycin-susceptible *Shigella*.

No recent STDs were reported among children. Among the 440 isolates collected from adults, 66 (15%) were from PLWH, and 41 (9%) were from patients with a recent STD. Seven (3%) of 223 women were either PLWH (n = 1) or had a recent STD (n = 6); none of these patients had DSA-*Shigella*. Eighty (37%)

Table 2. Antimicrobial drug class resistance for *Shigella* isolates, by patient sex, age, and international travel status, Minnesota, USA, 2012–2015*

Characteristic	Total, n = 691	Known international travel, n = 69	Men, n = 194†	Children and women, n = 428‡	OR (95% CI)§	p value
Antimicrobial drug class (drug)						
Aminoglycoside (GEN, STR)	660 (96)	61 (88)	191 (98)	408 (95)	3.1 (0.9–10.6)	0.07
Cephem (CRO, CEF)	13 (2)	2 (3)	3 (2)	8 (2)	0.8 (0.2–3.1)	1.0
Folate-pathway inhibitor (SUL, SXT)	416 (60)	56 (81)	148 (76)	212 (50)	3.3 (2.2–4.8)	<0.001
Macrolide (AZT)	46 (7)	3 (4)	42 (22)	1 (0)	118.0 (16.1–864.7)	<0.001
Penicillin (AMP)	162 (23)	27 (39)	74 (38)	61 (14)	3.7 (2.5–5.5)	<0.001
Phenicol (CHL)	76 (11)	22 (32)	26 (13)	28 (7)	2.2 (1.3–3.9)	0.01
Quinolone (CIP, NAL)	52 (8)	23 (33)	23 (12)	6 (1)	9.5 (3.8–23.6)	<0.001
Tetracycline (TET)	257 (37)	60 (87)	124 (64)	73 (17)	8.6 (5.9–12.7)	<0.001
Class resistance						
No resistance detected	6 (1)	2 (3)	0 (0)	4 (1)	NA	0.32
≥3 classes	266 (38)	60 (87)	130 (67)	76 (18)	9.4 (6.4–13.9)	<0.001
Clinical resistance¶	486 (70)	66 (96)	173 (89)	247 (58)	6.0 (3.7–9.9)	<0.001

*Values are no. (%) resistant except as indicated. Adults are persons ≥18 years of age; children are persons <18 years of age. AMP, ampicillin; AZT, azithromycin; CHL, chloramphenicol; CEF, cephalothin; CIP, ciprofloxacin; CRO, ceftriaxone; GEN gentamicin; NA, not applicable; NAL, nalidixic acid; OR, odds ratio; STR, streptomycin; SUL, sulfisoxazole; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline.
†Total excludes 23 known international travelers.
‡Total excludes 46 known international travelers.
§Comparison of men with children and women. Reference group is children and women.
¶Resistance to ≥1 of the following antimicrobial drug classes: cephem, folate-pathway inhibitor, macrolide, penicillin, quinolone.

Table 3. Reported symptoms and illness severity of patients with shigellosis, by azithromycin susceptibility status and CD4 count, Minnesota, USA, 2012–2015*

	DSA status, n = 691				CD4 count, cells/mm^3, n = 878†				
Characteristic	DSA, n = 46‡	Azithromycin susceptible, n = 64§	OR (95% CI)¶	p value	<200, n = 6	200–500, n = 21	>500, n = 35	Not known to be PLWH, n = 816	p value
Bloody diarrhea	13 (35)	247 (45)	0.7 (0.3–1.3)	0.31	2 (50)	6 (46)	9 (33)	319 (46)	0.65
Fever	23 (66)	406 (74)	0.7 (0.3–1.4)	0.32	3 (75)	9 (69)	18 (69)	525 (75)	0.89
Hospitalized	8 (17)	136 (21)	0.8 (0.4–1.7)	0.71	3 (50)	8 (38)	13 (37)	143 (18)	<0.001#
Death	0	1 (0)	NA	1.00	0	0	0	1 (0)	0.99
Median illness duration, d (range)	11 (3–91)	9 (1–125)	NA	0.004	7 (3–15)	11.5 (4–23)	11 (3–32)	8 (0–125)	0.03**

*Values are no. (%) unless otherwise indicated. DSA, decreased susceptibility to azithromycin; OR, odds ratio; NA, not applicable; PLWH, person living with HIV.
†Total number of infections was 882. However, CD4 count was not available for 4 PLWH.
‡Species distribution: *Shigella sonnei*, 52%; *S. flexneri*, 46%; *S. boydii*, 2%.
§Species distribution: *S. sonnei*, 83%; *S. flexneri*, 16%; *S. boydii*, 0.6%; *S. dysenteriae*, 0.3%.
¶Reference group is azithromycin-susceptible patients.
#Patients not known to be PLWH had lower hospitalization rates.
**Patients not known to be PLWH had shorter median duration of illness than PLWH with CD4 counts ≥200 cells/mm^3.

of 217 isolates collected from men were either from PLWH (n = 65, including 2 patients with 2 *Shigella* infections, and 1 patient with 3 *Shigella* infections) or had a recent STD (n = 36, including 21 patients who were also PLWH). Thirty-two (76%) of 42 men with DSA-*Shigella* were PLWH (n = 25) or had a recent STD (n = 20, including 13 who were also PLWH).

Men with DSA-*Shigella* had greater odds of recent infection with chlamydia (OR 8.3; p<0.001), gonorrhea (OR 5.2; p = 0.001), syphilis (OR 11.7; p = 0.003), any recent STD (OR 9.0; p<0.001), and multiple recent STDs (OR 9.3; p<0.001) compared with men who had susceptible *Shigella* infections (Table 4). In addition, HIV infection was more common among those with DSA-*Shigella* (60% vs. 23%; p<0.001).

We used a multivariate model that included only men and the variables HIV infection and recent STD. In this model, we found that HIV infection (adjusted OR 3.5, 95% CI 1.6–7.6; p = 0.001) and a recent STD (adjusted OR 6.7, 95% CI 2.8–15.5; p<0.001) were independently associated with DSA-*Shigella*.

Men who were PLWH were not ill longer (median 11 days vs. 9 days; p = 0.10) and did not have higher rates of hospitalization (38% vs. 28%; p = 0.14) than other men and did not have higher rates of hospitalization except for PLWH with CD4 counts <200 cells/mm^3. We found no difference in illness severity among PLWH based on CD4 count. However, patients who were not known to be PLWH had lower rates of hospitalization and shorter median duration of illness than patients who were PLWH with CD4 counts ≥200 cells/mm^3 (Table 3). Rates of antimicrobial drug treatment for shigellosis were not higher for PLWH among men (79% vs. 83%; OR 0.8, 95% CI 0.3–1.8; p = 0.66). Isolates from men who were PLWH were more likely than those from other adult males to be MDR (91% vs. 61%; OR 6.4, 95% CI 2.6–15.8; p<0.001), to have clinically relevant resistance (100% vs. 86%; OR undefined; p<0.001), or to be DSA (38% vs. 11%; OR 5.0, 95% CI 2.4–10.1; p<0.001).

Discussion

We identified DSA among multiple *Shigella* species in Minnesota during 2012–2015. In addition to DSA, we found ≥1 isolates with resistance to clinically relevant oral antimicrobial drugs and a parenteral agent (ceftriaxone). Increasing resistance to azithromycin has been reported through national antimicrobial drug resistance surveillance every year since 2011 (*26*). However, resistance profiles differed across groups at risk for shigellosis; 42 of the 43 domestically acquired DSA-*Shigella* cases in Minnesota occurred among men, 60% of whom were also infected with HIV. This trend has been observed in other jurisdictions and has implications for clinical testing, public health surveillance, case management, and prevention efforts (*27*–*29*).

DSA-*Shigella* was strongly independently associated with HIV infection and having a recent STD diagnosis. Identifying as an MSM or being a man reporting sexual contact with a man was also associated with DSA-*Shigella* despite the limited data available. Among men with shigellosis, 37% overall and 76% of those with DSA-*Shigella* were either PLWH or had a recent STD. These findings are consistent with other shigellosis outbreak reports among MSM but present more robust estimates of recent STD prevalence among shigellosis patients (*20*,*22*,*27*). Co-infection with STDs is of particular concern because azithromycin is recommended as a treatment for chlamydia, as well as for gonorrhea in conjunction with ceftriaxone (*30*). Nearly half of men with DSA-*Shigella* in this study

Table 4. Sexually transmitted diseases reported in the 12 months before *Shigella* specimen collection for 217 men with shigellosis, by azithromycin susceptibility status, Minnesota, USA, 2012–2015*

Disease	DSA, n = 42, no. (%)	Azithromycin susceptible, n = 175, no. (%)	OR (95% CI)†	p value
Chlamydia	13 (31)	9 (5)	8.3 (3.2–21.1)	<0.001
Gonorrhea	10 (24)	10 (6)	5.2 (2.0–13.4)	0.001
Syphilis	5 (12)	2 (1)	11.7 (2.2–62.6)	0.003
HIV‡	25 (60)	40 (23)	5.0 (2.4–10.1)	<0.001
Any bacterial STD§	20 (48)	16 (9)	9.0 (4.1–20.0)	<0.001
Multiple bacterial STDs§	9 (21)	5 (3)	9.3 (2.9–29.4)	<0.001

*All *Shigella* infections reported in men are included. Two men who were PLWH had 2 *Shigella* infections, and 1 man who was PLWH had 3 *Shigella* infections during the study period. DSA, decreased susceptibility to azithromycin; OR, odds ratio; PLWA, persons living with HIV; STD, sexually transmitted disease.
†Reference group is men with azithromycin-susceptible *Shigella*.
‡HIV positive at any time before *Shigella* isolation.
§Chlamydia, gonorrhea, or syphilis only.

were given a diagnosis of, and presumably treated for, an STD during the previous year. Treatment of STDs with azithromycin might select for drug-resistant *Shigella* strains circulating in this population. In addition, immunosuppression caused by HIV infection might increase shedding duration and thus the likelihood of *Shigella* transmission (*31*). In our study, severity and duration of illness were not related to CD4 count. However, the sample sizes were small.

Transmission of *Shigella* through sexual contact or activity among MSM was first noted during the 1970s, and outbreaks continue to be documented in the United States and worldwide (*14*,*19*,*32*). Similar to the findings presented in our study, MDR shigellosis has been documented among MSM populations worldwide (*18*,*20*,*33*). Overall, MSM are at greater risk for sexually transmitted *Shigella* infections, and HIV infection can increase the risk for contracting shigellosis (*34*). Efforts to prevent the spread of shigellosis should include outreach to MSM communities to encourage hand and body washing before and after sex, washing sex toys, waiting to have sex until convalescent-phase stool testing confirms that shedding has stopped (or a few weeks if convalescent-phase testing is not performed), asking sexual partners whether they have recently been ill with diarrhea, and use of barriers to prevent fecal–oral contact during sexual activity (*35*).

Additional data about shedding in the context of DSA or HIV infection would help refine this guidance. General shigellosis prevention messages, such as avoiding certain activities such as preparing food for others and swimming while ill, should also be included. In addition, because of the high prevalence of HIV and recent STDs among men with shigellosis, clinicians might use a diagnosis of shigellosis in a man as a reason for HIV and STD screening and use HIV or STD diagnoses to counsel about prevention of shigellosis and other sexually transmitted enteric infections (*29*). Because patients could seek care from clinicians working with MSM in the context of STDs or general practitioners who might not provide treatment for STD patients, cross-training is needed to educate providers for prevention of shigellosis and potential indicators for STD risk.

Shigellosis is typically self-limited and does not require antimicrobial drug treatment, even among PLWH, unless CD4 counts are <500 cells/mm^3 (*8*). If treatment is deemed necessary because of severity of illness or public health restrictions, antimicrobial susceptibility testing results should be used to guide antimicrobial drug treatment if possible. However, clinical breakpoints for azithromycin resistance in *Shigella* have not been established for clinical laboratories. CLSI recently established epidemiologic cutoff values (ECVs) for azithromycin nonsusceptibility by using MICs for *S. flexneri* and *S. sonnei* and disk diffusion for *S. flexneri* (*25*). In the absence of clinical breakpoints, ECVs are needed for disk diffusion for *S. sonnei*. Although ECVs are useful for epidemiologic purposes, they are not useful for clinical decision making. Azithromycin is a recommended treatment option for shigellosis; therefore, clinical breakpoints are urgently needed. Clinicians should consider antimicrobial drug–resistant shigellosis in MSM patients who have diarrhea, and should specifically request antimicrobial drug susceptibility testing for *Shigella*-positive specimens because such testing is not performed routinely in many clinical laboratories.

In our study, patients with DSA-*Shigella* had a longer duration of illness, but did not experience more severe illness by other measures (i.e., fever, bloody diarrhea, hospitalization). Appropriate antimicrobial drug treatment can shorten the duration of *Shigella* carriage and is often used in an attempt to reduce transmission during outbreaks. However, data about the effectiveness of this strategy are lacking (*1*). Increasing awareness among clinicians is key to increasing stool testing and subsequent antimicrobial susceptibility testing before treatment. Regardless of treatment strategy, adults with shigellosis should

be counseled about prevention, including waiting to engage in sexual activity while experiencing and convalescing from diarrhea and using barriers to prevent fecal–oral contact.

In addition to *Shigella* outbreak detection, whole-genome sequencing (WGS) can be used to determine genes that predict resistance to antimicrobial drugs. Most of the DSA isolates tested in our study contained *mph*A and *erm*B genes, both of which are known to confer macrolide resistance (*36*). The increasing use of culture-independent diagnostic testing (CIDT) at clinical laboratories could result in reduced availability of isolates for WGS and susceptibility testing if the capacity to culture CIDT-positive specimens is eliminated from clinical laboratories. To preserve the isolates necessary for determining antimicrobial drug susceptibility and outbreak detection, clinicians are encouraged to order reflex culture for CIDT-positive specimens. Ultimately, isolates should be submitted for antimicrobial susceptibility testing, or WGS if antimicrobial susceptibility testing is not available, to determine antimicrobial drug susceptibility.

Our study findings have limitations. These findings might not be generalizable beyond Minnesota. Case-patients who were given any antimicrobial drugs for shigellosis had longer duration of illness; however, these patients might have received antimicrobial drugs because they had been ill longer, had more severe illness, or had other concurrent conditions. Both data sources are likely incomplete. MSM are probably underreported because both data sources for sexual practices were consistently obtained only during 2015 for shigellosis patients, and sex of sexual partners is not always included on STD or HIV case reports. Also, the period of interest varied between the *Shigella* and HIV/STD case reports, which limited the conclusions that can be drawn from combining those data. Finally, the number of DSA-*Shigella* patients was small, which limited the power of analyses by *Shigella* species.

In conclusion, antimicrobial drug–resistant shigellosis is a growing threat to public health. Treatment recommendations have been modified from ampicillin or trimethoprim/sulfamethoxazole to azithromycin, ciprofloxacin, or ceftriaxone to account for increasing resistance to ampicillin and trimethoprim/sulfamethoxazole (*1*,*8*). However, in Minnesota, prevalence of DSA is increasing among *Shigella* isolates, primarily among adult men, PLWH, and those who have had a recent diagnosis of an STD. Men with *Shigella* infections appear to be at higher risk for MDR; almost 70% of isolates are not susceptible to >3 antimicrobial drug classes. Almost half of men with DSA-*Shigella* infections had a recent STD, indicating that further population-level interventions, such as educational campaigns, are needed to reduce enteric infections spread through sexual activity.

Acknowledgments

We thank the staff of the Minnesota Department of Health Public Health Laboratory, HIV/STD Epidemiology and Surveillance Unit, and Foodborne Diseases Unit; and Davina Campbell for assistance with this study.

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High Incidence of Active Tuberculosis in Asylum Seekers from Eritrea and Somalia in the First 5 Years after Arrival in the Netherlands

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Three quarters of tuberculosis (TB) patients in the Netherlands are foreign-born; 26% are from Eritrea or Somalia. We analyzed TB incidence rates in asylum seekers from Eritrea and Somalia in the first 5 years after arrival in the Netherlands (2013–2017) and performed survival analysis with Cox proportional hazards regression to analyze the effect of age and sex on the risk for TB. TB incidence remained high 5 years after arrival in asylum seekers from Eritrea (309 cases/100,000 person-years) and Somalia (81 cases/100,000 person-years). Age ≥18 years was associated with a higher risk for TB in asylum seekers from Eritrea (3.4 times higher) and Somalia (3.7 times higher), and male sex was associated with a 1.6 times higher risk for TB in asylum seekers from Eritrea. Screening and treating asylum seekers from high-incidence areas for latent TB infection upon arrival would further reduce TB incidence in the Netherlands.

The Netherlands is a low-incidence country for tuberculosis (TB). The TB notification rate was 4.6 cases/100,000 population in 2017, the lowest ever recorded in the country. Most TB patients in the Netherlands are immigrants and asylum seekers. In 2017, of the Netherlands' 787 TB patients, 74% were foreign-born; persons from Eritrea and Somalia together accounted for 26% of all foreign-born patients (*1*). In line with the World Health Organization (WHO) End TB Strategy and the related framework and plans toward TB elimination in low-incidence countries, the Netherlands is aiming to reduce TB incidence by 25% in the next 5 years (*2*–*5*). The ultimate aim is to reach the preelimination phase for TB (<1 TB patient/100,000 population/year) and subsequently elimination (<1 TB patient/1 million population/year). One of the priority actions for low-incidence countries to proceed toward this goal is to have screening programs in place for active TB and latent TB infection (LTBI) in selected high-risk groups, such as asylum seekers from high-incidence countries (*4*).

Nearly one fourth of the world population has LTBI, which is especially prevalent among those living in countries with high incidence of active TB (*6*). LTBI refers to a persistent host immune response to *Mycobacterium tuberculosis* antigens without evidence of clinically manifest active TB. Persons with LTBI generally have no symptoms of TB but are at risk for active TB. This risk is highest in the first 2 years after infection. Unlike active TB, which can usually be diagnosed on the basis of a combination of signs and symptoms, imaging (e.g., chest radiograph), and bacteriologic examination, LTBI is diagnosed by tuberculin skin test and interferon-γ release assays. Therefore, screening programs for active TB differ from those for LTBI. Treatment of LTBI, which typically requires fewer antibiotic drugs over a shorter period compared with active TB, can prevent future onset of active TB and transmission of the disease (*7*).

In the Netherlands, asylum seekers and immigrants from countries with a WHO-estimated TB incidence of ≥50 cases/100,000 population and who have an intended stay in the Netherlands of >3 months undergo mandatory screening for active TB. Asylum seekers are screened within the

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DOI: https://doi.org/10.3201/eid2604.190123

first 3 days after reporting to a reception center; immigrants are screened usually within the first 3 months of arrival in the country. Furthermore, asylum seekers and immigrants from countries with a WHO-estimated TB incidence of ≥200 cases/100,000 population (e.g., Somalia, which has an incidence of 270 cases/100,000 population) (*8*) or from countries with a high TB prevalence at entry screening in the Netherlands (e.g., Eritrea, which has a WHO-estimated incidence of 74 cases/100,000 population but a prevalence of >280 cases/100,000 population at entry screening in the Netherlands) (*8*,*9*) are offered biannual follow-up screening by chest radiograph during the first 2 years after arrival (*5*,*9*). The coverage of entry screening of asylum seekers is nearly 100%. However, the coverage of follow-up screening drops to 14% at 6 months after entry and 6% at 2 years after entry. A recent 5-year evaluation of the Netherlands' TB screening program for asylum seekers concluded that radiologic follow-up screening is not effective because of its low coverage. Only 36% of TB patients who were eligible for follow-up screening and did not have TB diagnosed at entry screening were found through follow-up screening (*10*).

Replacing radiologic follow-up screening with an LTBI screening and treatment program for asylum seekers from high-incidence countries upon arrival in the Netherlands is likely to be more effective in reaching the targets set by the Netherlands' TB Control Strategy. Such screening would identify persons at risk for active TB in the future and provide opportunities to prevent the disease. The Netherlands' Committee for Practical TB Control already recommended replacing radiologic screening with LTBI screening for immigrants and asylum seekers <18 years of age. As of the publication date of this article, this approach was being implemented for immigrants <18 years of age but not yet for asylum seekers (*5*,*9*). To inform policy makers and professionals on the potential benefit of an LTBI screening program for asylum seekers from high-incidence countries, we analyzed trends in TB incidence rates in asylum seekers from Eritrea and Somalia in the first 5 years after their arrival in the Netherlands.

Methods

We performed a retrospective cohort study in asylum seekers from Eritrea and Somalia who arrived in the Netherlands from January 1, 2013, through December 31, 2017. Because Eritrea was not yet an independent country before 1991, persons from Eritrea who were born before 1991 were actually born in Ethiopia. Because our data sources reported on country of birth (instead of nationality), we combined the groups of asylum seekers from Eritrea whose country of birth was listed as Eritrea or Ethiopia (3.4% persons in this combined group were born in Ethiopia).

We obtained data from the Netherlands' Immigration and Naturalization Service (IND) on asylum seekers from Eritrea and Somalia arriving in the Netherlands during the study period. IND provided data on numbers of asylum seekers and reason of request (e.g., first applications, repeated applications, family reunifications, and invited asylum seekers) by country of birth, month and year of arrival, sex, and age group. We excluded repeated applications because these are usually from asylum seekers who reapply after a failed first application without leaving the country in between applications. The IND does not provide information on duration of stay in the Netherlands of asylum seekers after registration (i.e., no linked data of asylum seekers who left the country or died are available). Because asylum seekers from Eritrea and Somalia are usually granted asylum in the Netherlands and therefore stay in the country (*11*), we assumed that the follow-up period of each person in the study population lasted either until the end of the study period (December 31, 2017) or until the event (TB diagnosis) occurred.

We obtained data from the Netherlands' National TB Register (NTR) on TB patients from Eritrea and Somalia who arrived in the Netherlands during the study period (i.e., persons diagnosed with active TB through December 31, 2017). NTR contains detailed information on patient demographics (including date of arrival in the Netherlands, age, sex, and country of birth), diagnostic and disease characteristics, and treatment outcome. NTR also has information on whether patients belong to specific risk groups, such as asylum seekers or immigrants. Because asylum seekers who have onset of TB after obtaining asylum are registered as immigrants in NTR, we included asylum seekers and immigrants from NTR as long as they had arrived on or after January 1, 2013. We excluded TB patients with unknown date of arrival in the Netherlands (n = 41).

We defined cases of prevalent TB as active TB in patients who were registered in NTR as being found through entry screening, independent of the time that had passed since arrival. We defined cases of incident TB as active TB in patients who were not found through entry screening, including patients who self-reported symptoms and patients

found through follow-up screening or contact tracing. Patients with pulmonary and extrapulmonary TB were included. Because entry screening is conducted by using chest radiograph, mainly pulmonary and some forms of intrathoracic TB (e.g., pleural TB) are detected by screening. TB diagnosis follows the criteria set by European Union member states (i.e., cases are confirmed if *M. tuberculosis* is found in patient specimens; cases are deemed probable or possible if bacteriologic or clinical criteria are met) (*12*).

We merged both datasets (the cohort of asylum seekers from Eritrea and Somalia who arrived during the study period and the TB patients registered in NTR) by month and year of arrival, country of birth, sex, and age. We calculated the total follow-up time (in person-months) for cases by subtracting the date of arrival from the date of diagnosis, and for noncases by subtracting the estimated date of arrival (set at the 15th day of the registered month of arrival) from December 31, 2017.

We analyzed data by using the statistics software package Stata/SE 15.1 (https://www.stata.com). We described and compared characteristics of (prevalent and incident) cases and noncases and calculated incidence rates by number of years after arrival. Because we were interested in the risk for TB over time in persons with different follow-up periods, we performed survival analysis with Cox proportional hazards regression. We calculated cumulative incidences and analyzed the effect of country of birth, calendar year of entry, age, and sex on the risk for active TB.

Results

Characteristics of the Study Population

The study population consisted of 26,057 persons (21,182 [81%] asylum seekers from Eritrea and 4,875 [19%] from Somalia (Table 1). The number of asylum seekers from Eritrea and Somalia arriving per calendar year varied. Whereas the peak of asylum seekers from Eritrea arriving in the Netherlands occurred in 2015, the arrival of asylum seekers from Somalia peaked in 2013 (and the years before), resulting in different median follow-up periods of the study population: 27 months (interquartile range 13–32 months) for those from Eritrea and 49 months (interquartile range 39–53 months) for those from Somalia. Asylum seekers from Eritrea were more often ≥18 years of age (65%) than those from Somalia (30%) (p<0.001). The proportion of men and boys in the study population from Eritrea was higher than that in the study population from Somalia (61% vs. 48%; p < 0.001).

A total of 546 TB patients were identified. Seventy-eight patients (61 from Eritrea and 17 from Somalia) had prevalent TB found through entry screening, indicating a TB prevalence at entry of 288 (95% CI 224–370) cases/100,000 population for asylum seekers from Eritrea and 349 (95% CI 217–560) cases/100,000 population for those from Somalia. The other 468 patients had incident TB (338 were from Eritrea and 130 from Somalia), corresponding to overall incidence rates of 747 (95% 672–831) cases/100,000 population for asylum seekers from Eritrea and 712 (95% CI 600–846) cases/100,000 population for those

Table 1. Characteristics of asylum seekers from Eritrea and Somalia in whom active TB was detected, the Netherlands, 2013–2017*

Characteristic	Total study population	Country of origin		p value†
		Eritrea	Somalia	
Population size, no.	26,057	21,182	4,875	NA
Arrivals per year				
2013	3,741 (14.4)	911 (4.3)	2,830 (58.1)	<0.001
2014	5,353 (20.5)	4,168 (19.7)	1,185 (24.3)	<0.001
2015	8,889 (34.1)	8,378 (39.6)	511 (10.5)	<0.001
2016	3,484 (13.4)	3,250 (15.3)	234 (4.8)	<0.001
2017	4.590 (17.6)	4,475 (21.1)	115 (2.4)	<0.001
Age group				
<18 y	10,750 (41.3)	7,320 (34.6)	3,430 (70.4)	<0.001
≥18 y	15,307 (58.7)	13,862 (65.4)	1,445 (29.6)	<0.001
Sex				
F	10,731 (41.2)	8,191 (38.7)	2,520 (51.7)	<0.001
M	15,326 (58.8)	12,991 (61.3)	2,355 (48.3)	<0.001
Persons with prevalent TB	78 (0.3)	61 (0.3)	17 (0.4)	0.48
Of whom had PTB	59/78 (75.6)	49/61 (80.3)	10/17 (62.5)	0.068
Persons with incident TB	468 (1.8)	338 (1.6)	130 (2.7)	<0.001‡
Of whom had PTB	238/468 (50.9)	181/338 (53.6)	57/130 (43.5)	0.060
Detected in follow-up screening	77/468 (16.5)	67/338 (19.8)	10/130 (7.6)	0.002

*Values are no. (%) unless indicated. NA, not applicable; PTB, pulmonary tuberculosis; TB, tuberculosis.
†Case-patients from Eritrea compared with those from Somalia.
‡Proportion of incident cases cannot truly be compared between case-patients from Eritrea and those from Somalia because follow-up time was different (median of 28 months for case-patients from Eritrea versus 49 months for those from Somalia).

from Somalia. Sixteen percent of incident cases were identified through follow-up screening. The proportion of pulmonary TB was higher among patients identified through entry screening than those identified after arrival (76% vs. 51%; p<0.001).

Incidence Rates over Time and Survival Analysis

We determined the trend in TB incidence rate over the first 5 years after arrival in the Netherlands, stratified by country of birth (Figure 1). Among asylum seekers from Eritrea, the incidence rate dropped from 925 (95% CI 796–1,073) cases/100,000 person-years in the first year after arrival to 150 (95% CI 62–360) cases/100,000 person-years in the fourth year after arrival and to 309 (95% CI 44–2,195) cases/100,000 person-years in the fifth year after arrival. For asylum seekers from Somalia, the incidence rate dropped from 1,086 (95% CI 828–1,425) cases/100,000 person-years in the first year after arrival to 260 (95% 135–500) cases/100,000 person-years in the fourth year after arrival and to 81 (95% CI 11–575) cases/100,000 person-years in the fifth year after arrival. The large 95% CI around the fifth year incidence rate in asylum seekers from Eritrea reflects the small number of this population that had been followed up in this study for 5 years.

Figure 2 depicts the Kaplan-Meier curve for onset of TB over time. The cumulative risk for TB was ≈3% over the first 5 years after arrival in both groups.

Effect of Age and Sex

When stratified by country of birth, age ≥18 years was associated with a higher risk for TB in the study population (hazard ratio 3.4 [95% CI 2.4–4.9] in asylum seekers from Eritrea and 3.7 [95% CI 2.6–5.3] in those from Somalia) (Table 2). In asylum seekers from Eritrea, male sex was also associated with a higher risk for TB (hazard ratio 1.6 [1.3–2.1]); in those from Somalia, no association with sex was found. Because of strong (graphic) evidence against the proportional hazards assumption for the variable calendar year of arrival, we further stratified by this variable (data not shown). The effect of age and sex in a model with stratification by country of birth and calendar year of arrival appeared similar to the model with stratification by country of birth only but was not statistically significant because of small numbers in each stratum.

Discussion

Our study showed that asylum seekers in the Netherlands from Eritrea and Somalia have a high risk for TB: 0.3% had TB upon arrival, and ≈3% had onset of TB in the first 5 years after arrival. Although incidence rates gradually declined, they were still 10–50 times higher than the overall TB incidence in the Netherlands. Furthermore, our study provides additional insight in specific risk groups for active TB: adult (mainly those 18–35 years old) asylum seekers from Eritrea and Somalia were at higher risk for TB compared with those <18 years of age, as were men and boys from Eritrea compared with women and girls from Eritrea.

These results are consistent with a study conducted in the Netherlands in 2004, which showed that the incidence of pulmonary TB remained high in immigrants from high-incidence countries at least a decade after arrival in the Netherlands (*13*). A study in Denmark showed that in the 1990s, the annual incidence of TB in immigrants from Somalia decreased only gradually during the first 7 years of residence, from 2,000 to 700 cases/100,000 population (*14*).

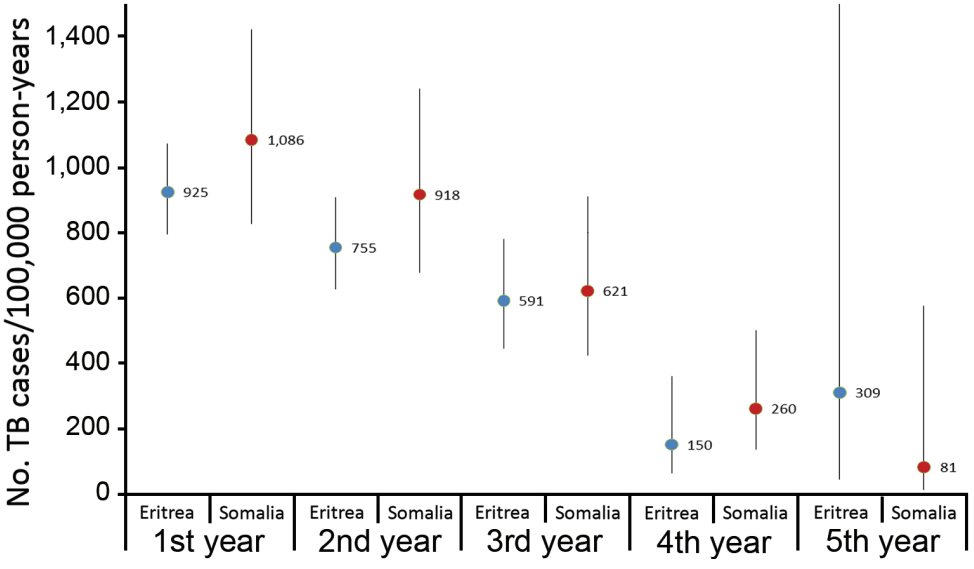

Figure 1. Trend of TB incidence rates (cases/100,000 person-years) of asylum seekers arriving from Eritrea and Somalia in the Netherlands, 2013–2017, by year after arrival. Error bars indicate 95% CIs; upper limit of the 95% CI for persons from Eritrea in the fifth year after arrival (2017) is 2,195. TB, tuberculosis.

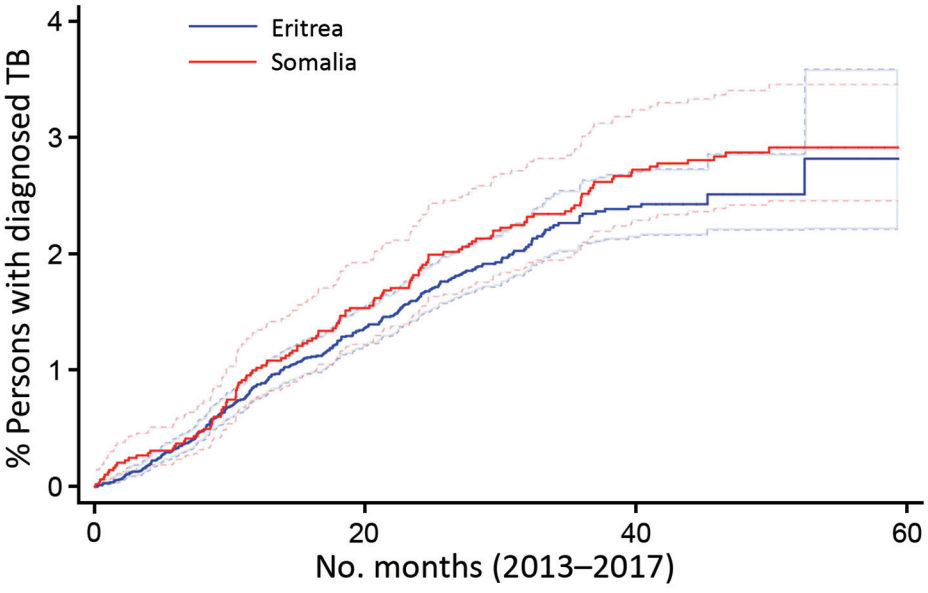

Figure 2. Kaplan-Meier curve indicating risk for TB among asylum seekers arriving from Eritrea and Somalia in the Netherlands, over a 60-month follow-up period (2013–2017). TB, tuberculosis.

Although these studies provide data on changes in incidence over time, we used survival analysis methods to analyze and depict the risks of a cohort of newly arrived asylum seekers, which enabled us to take into account the different follow-up periods for each person in the study.

The TB prevalence and incidence rates in asylum seekers from Eritrea and Somalia in our study were much higher than the WHO-estimated TB incidence in Eritrea (74 cases/100,000 population) and in Somalia (270 cases/100,000 population) (*8*). A plausible explanation for this finding is the additional risk for infection while traveling to Europe, where overcrowding and unsanitary conditions are common along travel routes, on top of the baseline infection risk in the country of birth (*15*,*16*). Walker et al. (*17*) found molecular and epidemiologic evidence for this in their study of a cluster of multidrug-resistant *M. tuberculosis* infections among patients arriving in Europe from the Horn of Africa. A second explanation could be an increased risk for TB because of vitamin D deficiency, malnutrition, and stress (*15*). These conditions are common in asylum seekers during the often stressful asylum application procedures and during the first years of settlement in the new country. Third, transmission within ethnic groups in the new country of residence can also contribute to the higher TB rates found in asylum seekers. Occasional outbreaks have been reported in ethnic groups, including recently arrived asylum seekers (*18*). Finally, the WHO figures could be an underestimation of actual TB rates (*18*–*20*).

Our findings support the recommendations for LTBI screening of asylum seekers from high TB-incidence countries (*4*). In our study, most TB cases in asylum seekers from Eritrea and Somalia occurred after initial radiologic screening for active

Table 2. Results of Cox proportional hazards regression analysis indicating hazard ratios for age and sex, stratified by country of birth, among asylum seekers from Eritrea and Somalia with incident tuberculosis cases, the Netherlands, 2013–2017

Country of origin and characteristic	Total	No. cases (%)	Hazard ratio (95% CI)
Eritrea			
Age group			
<18 y	7,301	35 (0.5)	Referent
≥18 y	13,820	303 (2.2)	3.4 (2.4–4.9)
Sex			
F	8,185	86 (1.1)	Referent
M	12,936	252 (2.0)	1.6 (1.3–2.1)
Somalia			
Age group			
<18 y	3,419	54 (1.6)	Referent
≥18 y	1,439	76 (5.3)	3.7 (2.6–5.3)
Sex			
F	2,513	74 (2.9)	Referent
M	2,345	56 (2.4)	1.0 (0.7–1.4)

TB, and only a few cases were identified by radiologic follow-up screening (partly because of the low coverage of follow-up screening). LTBI screening and treatment can prevent active TB, including extrapulmonary forms. Furthermore, because LTBI might reactivate to active disease many years after infection, LTBI screening also has the potential to prevent TB in asylum seekers many years and even decades after arrival in the new country. Implementing an LTBI screening program in asylum seekers is not easy. A survey conducted by the WHO Regional Office for Europe and the European Respiratory Society showed that 53% of countries in Europe performed systematic LTBI screening in refugee populations (*21*). A study conducted in 11 selected countries of Europe indicated that these countries had very different methods and policies for migrant TB or LTBI screening (*22*). Systematic reviews have demonstrated that limited information is available on the yield and effectiveness of migrant LTBI screening (*22*,*23*). Furthermore, cost-effectiveness of LTBI screening as predicted in mathematical models is highly setting-specific, with best results achieved if restricted to migrants from high-incidence countries (*24*).

Our study had several strengths. We were able to combine national comprehensive data on immigration and TB notification; thus, we could calculate incidence rates for specific groups of asylum seekers and show trends over time after arrival. Moreover, by using a Cox proportional hazards regression model, we were able to assess the effect of risk factors such as sex and age on the risk for TB.

Our study also had some limitations. First, no data were available on the actual follow-up period of persons in the study population who did not have TB diagnosed. Although most asylum applicants from Eritrea and Somalia are granted asylum and will therefore stay in the Netherlands (*11*), some might have moved to another country (or died) before the end of our study period, meaning that we possibly underestimated the TB incidence rates in asylum seekers from Eritrea and Somalia. On the other hand, whereas we included only asylum seekers (not immigrants) from the IND database, we did not differentiate between asylum seekers and immigrants among the TB patients included in our study. The reason for this was that asylum seekers who obtained a residence permit before having their TB diagnosed are registered in NTR as immigrants, even though most arrived in the Netherlands as asylum seekers. Very few asylum seekers from Eritrea and Somalia come to the Netherlands as immigrants (*11*,*25*), so we expect that this limitation has not led to a substantial overestimation of TB incidences in our study. Second, no information on travel routes was available for our study population, and no distinction could be made between asylum seekers who had undertaken the often long and stressful journey by land and water and those who came directly by airplane, for example, for the purpose of family reunification. Future studies should take these differences into account. Moreover, we only analyzed data on asylum seekers from 2 countries with high TB incidence countries (Eritrea and Somalia), so the results might not reflect trends in onset of active TB in asylum seekers from other high-incidence countries. However, because asylum seekers from other countries in Africa often share the same hazardous journey, their risk for TB is probably similarly elevated. An investigation into whether the risks for active TB after arrival in the Netherlands differ between asylum seekers and other migrants from high-incidence countries (e.g., persons migrating to the Netherlands because of work or study) would be warranted. We recommend additional studies of longer follow-up periods to enable a more extensive analysis of trends in TB incidence rates, molecular studies differentiating disease caused by in-country transmission or reactivation of premigration acquired infection, and studies evaluating the effectiveness and impact of LTBI screening programs in asylum seekers.

In conclusion, our study results clearly show that asylum seekers from Eritrea and Somalia remain at high risk for active TB for at least the first 5 years after arrival in the Netherlands. This finding underscores the need for an LTBI screening and treatment program for high-risk groups. LTBI screening and preventive treatment will also accelerate TB control and contribute toward the elimination of TB.

Acknowledgment

We gratefully acknowledge Alina Nicolaie for her contributions to the statistical analysis.

About the Author

Dr. van den Boogaard is a medical doctor in infectious disease control who conducted this study as part of her fellowship in the European Programme for Intervention Epidemiology Training of the European Centre for Disease Prevention and Control, for which she worked at the National Institute of Public Health and the Environment in the Netherlands.

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Severe Dengue Epidemic, Sri Lanka, 2017

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In 2017, a dengue epidemic of unexpected magnitude occurred in Sri Lanka. A total of 186,101 suspected cases and 440 dengue-related deaths occurred. We conducted a comprehensive analysis of this epidemic by comparing national surveillance data for 2017 with data from the preceding 5 years. In all Sri Lanka districts, dengue incidence in 2017 increased significantly over incidence during the previous 5 years. Older schoolchildren and young adults were more clinically symptomatic than those at extremes of age. Limited virologic surveillance showed the dominant circulating variant was dengue virus type 2 cosmopolitan genotype in the most affected district. One quarter of total annual cases were reported 5 weeks after the southwest monsoon started. Changes in vector abundance were not predictive of the increased incidence. Direct government expenditures on dengue control activities in 2017 were US $12.7 million. The lessons learned from this outbreak are useful for other tropical nations facing increasing dengue incidence.

Global incidence of dengue has increased 7-fold, from 8.3 million cases in 1990 to >58.4 million in 2013 (*1*). Currently, ≈390 million new infections occur annually in 128 dengue-endemic countries (*2*). Worldwide, ≈14,000–20,000 dengue-related deaths occur each year (*1*,*2*). In dengue-endemic countries, *Aedes* (*Stegomyia*) *aegypti* and *Ae.* (*Stegomyia*) *albopictus* mosquito vectors transmit the disease.

Sri Lanka, a tropical island in the Indian Ocean (population 21 million) (*3*), has reported dengue cases since the 1960s; seasonal epidemics predominantly affect areas that have annual rainfall >2,500 mm (*4*). However, until 1988, the more severe form of dengue virus (DENV) infection, dengue hemorrhagic fever, was reported only sporadically (*5*,*6*). During 1991–2008, dengue epidemics occurred once every few years on the background of endemic transmission (*6*). A disproportionate epidemic occurred in 2009 comprising 35,008 suspected cases (incidence 170 cases/100,000 population) and 346 deaths (case-fatality rate 1%) (*7*). During 2010–2016, dengue became a major public health problem; cases increased steadily (from 28,473 in 2011 to 55,150 in 2016) throughout the country but disproportionately affected the most populated Western province (*7*). In 2017, a total of 186,101 suspected cases and 440 dengue-related deaths were reported to the Central Epidemiology Unit of the Ministry of Health, Sri Lanka (*7*). This number is the highest number of suspected cases reported in a single calendar year in Sri Lanka since dengue was designated a notifiable disease in 1996. We compared the temporal, epidemiologic, virologic, entomologic, and climatic characteristics of the 2017 dengue epidemic with those of the epidemics during the preceding 5 years (2012–2016).

Methods

Data Sources

Epidemiology

We obtained epidemiologic data from the integrated national communicable disease surveillance system, which captures symptomatic dengue patients classified according to a standard case definition based on the 1997–2011 World Health Organization classification (*8*). Etiologic screening was conducted with NS1 antigen testing or dengue antibody assays. However, given the limited diagnostic test availability, especially in remote areas of the country, many cases were clinically diagnosed using the surveillance case

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DOI: https://doi.org/10.3201/eid2604.190435

definition (*9*). We based the population data we used on the last national census before the outbreak (2012) and projections for the following years (*10*).

Virology

The Central Epidemiologic Unit has an established ongoing fever surveillance cohort of 500 families in the catchment areas of the National Institute of Infectious Diseases, the main referral hospital for dengue patients in the most affected Colombo district. Viruses isolated from blood samples of patients suspected to have dengue collected during December 2016–December 2017 were sequenced (Sanger technique) at the University of North Carolina (Chapel Hill, NC, USA; Appendix, https://wwwnc.cdc.gov/EID/article/26/4/19-0435-App1.pdf). The sequences were aligned using Sequencher Software version 5.4.6 (https://www.genecodes.com) and phylogenetics analysis was performed on MEGA X software (*11*).

Entomology

Systematic entomologic surveillance is conducted in all districts of the country according to the guidelines issued by the National Dengue Control Unit of Sri Lanka's Ministry of Health. This surveillance comprises routine cluster surveys (systematic sampling of 100 premises per block) conducted monthly by designated entomologists. The presence of *Ae. aegypti* and *Ae. albopictus* larvae and pupa are reported as Breteau index, Container index, and Premise index/House index (*12*). A premise or container was considered positive if a single larva or pupa was found in a receptacle within its boundaries, both indoors and outdoors.

Meteorology

We extracted daily rainfall and temperature data during 2012–2017 from the Department of Meteorology for each district. The weekly cumulative rainfall in millimeters and mean temperature (°C) were calculated using daily observations. We obtained Oceanic Niño Index data as a measure of El Niño Southern Oscillation activity from the US National Oceanic and Atmospheric Administration (https://www.noaa.gov). The Oceanic Niño Index tracks the 3-month average sea surface temperatures in the east-central tropical Pacific from 120° to 170° West (Niño 3.4 region) (*13*). We correlated meteorologic parameters with the dengue incidence in 2017 and of the preceding 5 years for Colombo district (Western province, wet zone; annual rainfall >2,500 mm) and Jaffna district (Northern province, dry zone; annual rainfall 1,250–2,500 mm). We selected these 2 districts because both maintained a high dengue incidence during the 6 years examined and are representative of Sri Lanka's 2 contrasting climatic zones. Colombo district, in the wet zone, receives most of its rainfall from the southwest monsoon during May–September. Jaffna district, in the dry/semiarid zone, receives most of its annual rainfall from the northeast monsoons during December–February.

Cost Estimates

We estimated the direct costs of dengue control and outbreak response during epidemic year 2017 from the Ministry of Health perspective. Direct costs were costs of routine dengue control activities and costs of outbreak response activities. More specifically, routine dengue control activities included standard measures of premise inspections and removal of vector breeding sites, larval control, entomologic surveillance, development of electronic databases, health education, advertising through media, purchases for integrated vector control (fogging machines and chemicals), and operating costs of administrative offices. Outbreak response activities (after the epidemic) were establishment of extra high-dependency units in public hospitals, door-to-door premise inspection and source reduction programs in high-risk districts through civil–military cooperation, deployment of vector control brigades in high-risk districts, and extra-duty allowance for public health staff during outbreak months (May–August). We extracted the financial costs from the National Dengue Control Unit annual budget and financial expenditure records. For public health staff not exclusively engaged in dengue-related activities, we calculated the amounts on a pro rata basis according to the approximate number of hours spent on dengue-related work. This cost evaluation did not include in-kind costs, such as uncompensated work hours by community members or other stakeholders, because of the unavailability of records. We extracted all costs in local currency and converted to US dollars using the annual official exchange rate of the Sri Lanka rupee (LKR) 152.5 to US $1 in 2017 (World Bank, https://data.worldbank.org/indicator/PA.NUS.FCRF?end=2016&locations=LK&start=1960&view=chart).

Statistical Analyses

We analyzed the data using SPSS Statistics 21.0 (IBM, https://www.ibm.com). Descriptive statistics are shown as frequencies, means (± SD), and proportions. We examined the statistical significance of population proportions (count data) and mean differences (continuous data) by z-score test and used χ^2 test for dichotomous data with statistical significance

set at 0.05. The effect size is expressed as odds ratios or relative risk with 95% CIs. We used a distributed lag nonlinear model (*14*) for the analysis of exposure-lag response association between weather variables and dengue incidence. We conducted distributed lag nonlinear model construction and lagged correlation analysis in the R statistical environment (*15*).

Sequence Availability and Ethics Clearance

We deposited the DENV-2 sequences used in phylogenetic analysis in GenBank (accession nos. MK579857–61. The Ethics Review Committee of the Faculty of Medicine, University of Colombo (EC-18-004) provided ethics clearance for this study.

Results

In 2017, a total of 186,101 suspected dengue cases (866 cases/100,000 population) and 440 dengue-related deaths (case-fatality rate 0.24%) were reported to the national surveillance system. The weekly average of reported dengue cases was significantly higher than the average for the preceding 5 years (3,570 vs. 792 cases/week, p<0.0001, greater than mean for 2012–2016 + 2 SD) throughout the year. The national surveillance picked up an unusual increase in cases in the northern and eastern parts of the country from the latter half of 2016. However, a much larger increase in incidence was notable from week 16 of 2017 and peaked at week 29 when 10,699 cases were reported. Approximately one quarter of total dengue cases in 2017 were reported during weeks 27–31 (Figure 1).

Spatial Distribution of Dengue Incidence

Sri Lanka's 9 provinces are divided into 25 administrative districts (Table 1; Figure 2). In 2017, the most dengue cases were reported from the 2 most populous Colombo and Gampaha districts within the Western province (Colombo, 1,419/100,000; Gampaha, 1,323/100,000). Three other districts also had an incidence rate >1,000 cases/100,000 population, and 10 districts reported rates above the national average (Table 1). The lowest incidence rate (118/100,000 population) was reported from the Nuwara-Eliya district, which is in the Central province at 1,800 m above sea level. In all districts, dengue incidence in 2017 was significantly higher than the average incidence in the preceding 5 years (Table 1). When we repeated the comparison against each of the preceding 5 years (instead of the average), the difference remained significant for all districts.

Demographic Profile

The sex distribution of dengue patients in 2017 was similar (99,464 [55.2%] male, 80,724 [44.8%] female; p>0.05). The mean age of dengue patients nationwide was 29.7 years, but age varied widely by province (Table 2). Mean age was significantly lower than the national average (p<0.001) in Eastern (22.3 years), Northern (27.6 years), and Western (28.7 years) provinces and significantly higher in Sabaragamuwa (36.5 years), North Central (34.1 years), and Southern (33.2 years) provinces (Table 2). Incidence was highest for persons 20–29 years of age (1,225 cases/100,000 population), followed by persons 10–19 years (1,057/100,000 population). Persons >50 years of age were least affected (580/100,000 population) (Figure 3). Age-specific incidence rates among provinces differed noticeably. For example, young children (5–9 years) were the most affected group in the Eastern province (Appendix Table 3).

Phylogenetic Analysis

From blood samples collected during December 2016–December 2017 from 140 persons with acute fever, a total of 44 (31%) persons were positive for DENV RNA (DENV-2, 39; DENV-1, 4; DENV-2/3, 1). Of these, 5 DENV-2 isolates were cultured and sequenced (PrM-E region). A neighbor-joining

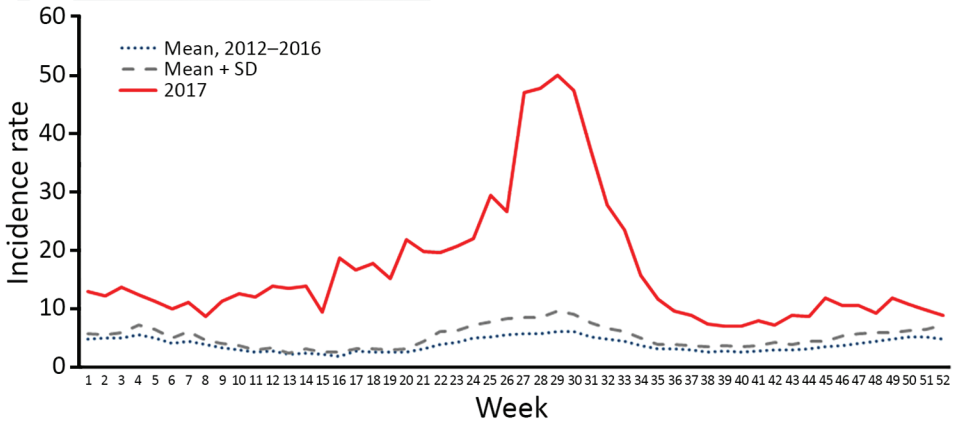

Figure 1. Comparison of the weekly mean attack rates of dengue reported in 2017 with the 5-year historical mean (2012–2016), Sri Lanka. Rates are cases per 100,000 population. Source: (*7*).

phylogenetic tree (Figure 4) made with a mix of other DENV-2 genotype references placed the new sequences within the cosmopolitan genotype (*17*,*19*). However, this strain differed from other DENV-2 cosmopolitan strains reported previously in Sri Lanka (*18*,*20*) and was more closely related to variants isolated from Singapore and China during 2014–2017.

Entomologic Surveillance

The mean national Breteau index for 2017 was similar to that for 2013–2016 (11.67% vs. 12.9%; p = 0.833). Provincial-level differences were not significant. Types and proportions of water-retaining containers positive for *Ae. aegypti* mosquitoes varied by province (Table 3). In Western, Sabaragamuwa, and North-Western provinces, discarded water-holding containers were the most common receptacles positive for larvae. In contrast, in Central and Northern provinces, which had an extended drought in 2017, vector breeding was observed mainly in water storage containers and tanks. However, entomologic parameters did not correlate with the disproportionate increase in dengue cases in 2017.

Climatic Factors

We assessed whether dengue incidence correlated with climatic parameters (Figure 5). The mean annual rainfall in Colombo district was lower in 2017 than in 2012–2016 (1,908 mm vs. 2,447 mm). In the Jaffna district, cumulative rainfall during 2017 (1,342 mm) was similar to the average annual rainfall for 2012–2016 (1,297 mm). El Niño conditions (Oceanic Niño Index >0.5) were observed in 2015 and 2016 but not in 2017. In addition, temperature patterns in both districts during 2017 and during the preceding 5 years did not change notably. Therefore, weather conditions were stable in both districts throughout 2012–2017, despite the disproportionate increase in dengue incidence in 2017.

Cost Analysis

We estimated the direct cost of dengue control and outbreak response activities during the epidemic year of 2017 to be US $12.7 million (LKR 1.938 billion), corresponding to a per capita cost of US $0.64 (Table 4). Of this total, US $4.4 million (35%; US $0.22 per capita) was spent on outbreak response activities.

Discussion

In 2017, a dengue epidemic in Sri Lanka resulted in an unprecedented number of cases and dengue-related deaths and a considerable strain on resources in terms of direct expenditures. Incidence increased substantially throughout the country; even the district with the lowest incidence for 2017 had a 3-fold increase over the average of the preceding 5 years. The epidemic primarily affected older schoolchildren and young adults in the workforce, with some marked disparities in age-specific incidence rates across provinces. Entomologic and climatic factors (except rainfall) did not explain the increased incidence. Limited virologic surveillance during 2017 in the district most affected (Colombo) showed DENV-2 dominance.

Temporal trends in dengue incidence are closely linked to the demographic characteristics of Sri Lanka. Historically, seasonal dengue outbreaks have heavily affected the country's urban areas and, more conspicuously, the Colombo district (which comprises 11% of the country's population and has the highest population density) (*10*). In entomologic surveys,

Table 1. Incidence of dengue, Sri Lanka*

Province, administrative district	Mean incidence†		
	2017	2012–2016	Fold increase in 2017‡
Western			
Colombo	1,419	503	2.8
Gampaha	1,323	252	5.2
Kalutara	861	190	4.5
Eastern			
Trincomalee	1,214	107	11.4
Batticaloa	1,001	157	6.4
Kalmunai	698	131	5.3
Sabaragamuwa			
Kegalle	1,090	174	6.3
Ratnapura	978	214	4.6
Northern			
Jaffna	996	251	4.0
Vavuniya	583	87	6.7
Mannar	500	175	2.9
Kilinochchi	418	70	6.0
Mulativu	402	130	3.1
Central			
Kandy	990	162	6.1
Matale	616	125	4.9
Nuwara Eliya	118	40	2.9
Southern			
Matara	976	89	10.9
Galle	553	138	4.0
Hambantota	419	107	3.9
North-Western			
Puttalam	965	119	8.1
Kurunegala	665	135	4.9
Uva			
Moneragala	668	64	10.4
Badulla	430	88	4.9
North-Central			
Polonnaruwa	325	96	3.4
Anuradhapura	317	60	5.3
National average	866	189	4.6

*Source: (*7*).
†Cases/100,000 population.
‡Increases were significant in all districts (p<0.05) compared with the average for 2012–2016.

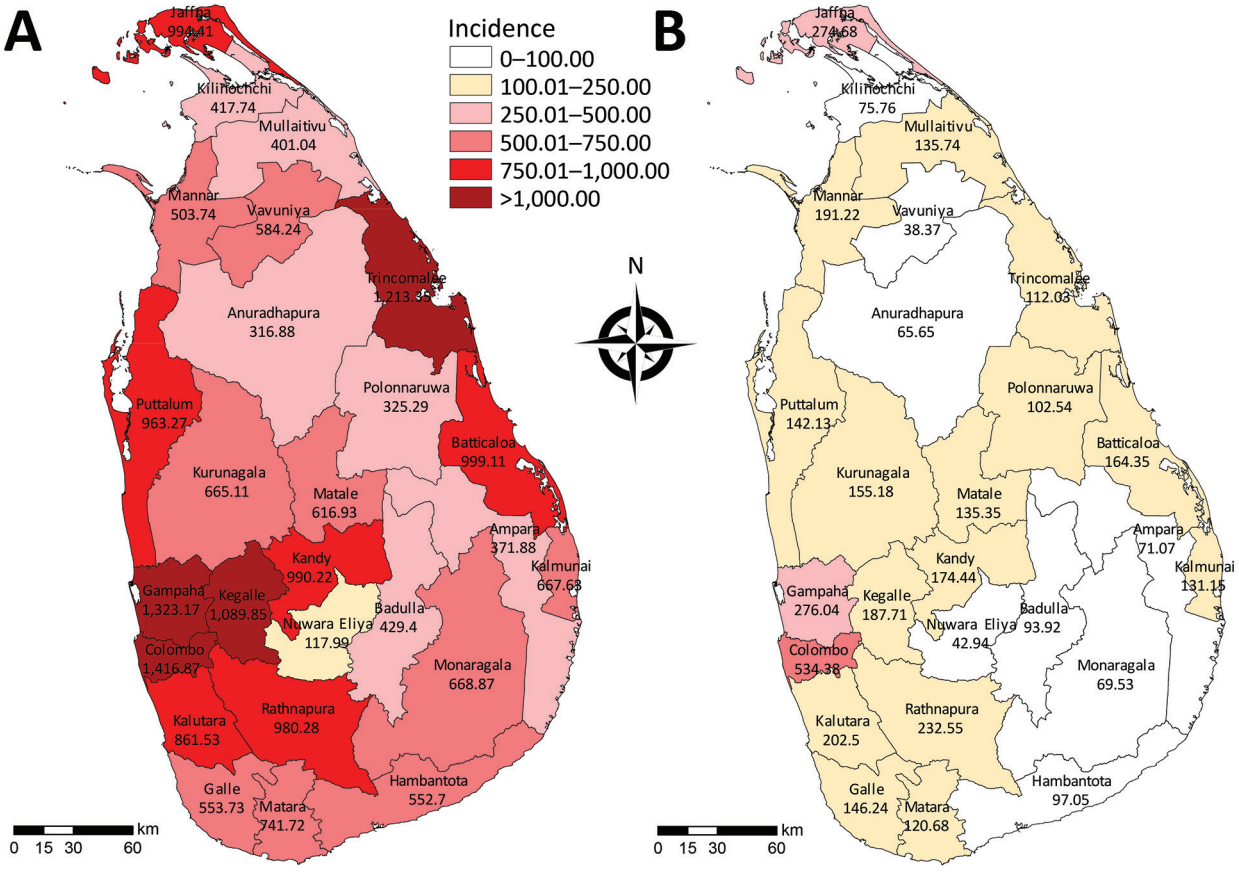

Figure 2. Comparison of dengue incidence rates per district in 2017 with the 5-year (2012–2016) average, Sri Lanka. A) Incidence rate in 2017. B) Historical mean incidence rate during 2012–2016. Incidence is cases per 100,000 population. Source: (*7*).

more than half of *Ae. aegypti* vector breeding occurred in discarded items (e.g., containers, tires) in the Western province, signifying the effect of poor container waste management, which is conducive for vector breeding. On a global scale, the high vector-to-host ratio profoundly affects dengue epidemiology (*21*–*25*). In Vietnam, a study of 2 outbreaks during 2005–2008 showed that dengue incidence increased in a critical window of population density (3,000–7,000/km^2) and declined thereafter (*26*).

Table 2. Age distribution of dengue patients, Sri Lanka, 2017*

Province	Mean age, y	+ SD	25th percentile	75th percentile
Western	28.7†	18.5	14.0	40.0
Eastern	22.3†	16.4	9.0	31.0
Sabaragamuwa	35.7‡	18.1	22.0	49.0
Northern	27.6†	16.5	17.0	36.0
Central	29.5	17.6	16.0	41.0
Southern	33.2‡	17.3	21.0	45.0
North-Western	31.1	18.3	17.0	44.0
Uva	31.1	16.9	19.0	42.0
North-Central	34.1‡	14.3	23.0	43.0
National average	29.7	18.2	16.0	42.0

*Source: (*7*).
†Significantly lower mean age than the national average, p<0.05.
‡Significantly higher mean age than the national average, p<0.05.

In most Sri Lanka provinces, including the Western province, the highest dengue incidence rate occurred among persons 20–29 years of age (without any sex difference), followed by persons 10–19 years of age. These age groups comprise children attending secondary schools (up to 18 years of age), students of tertiary education institutions, and young working adults. The same trend has been observed in the

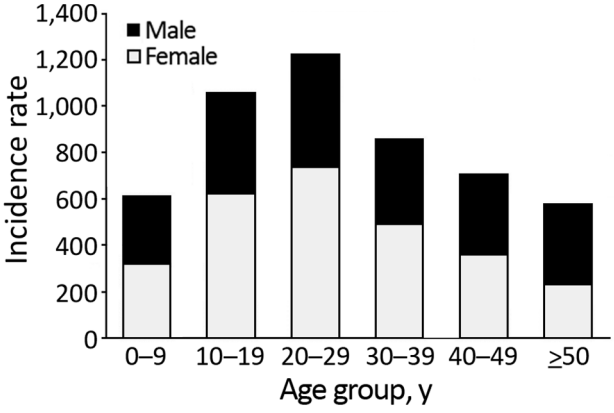

Figure 3. Dengue incidence rates by age group and sex, Sri Lanka, 2017. Incidence is cases per 100,000 population. Source: (*7*).

Table 3. Type and proportion of breeding habitats positive for *Aedes aegypti* mosquitoes, Sri Lanka, 2017*

Province	Discarded items, %	Water storage containers and tanks, %	Ponds and ornamental items, %	Wells and tube wells, %	Natural water collections, %	Other miscellaneous items, %†
Western	50.9	10.0	3.7	1.6	2.2	31.5
Eastern	33.9	22.9	3.0	11.5	1.6	27.0
Sabaragamuwa	55.6	5.1	5.1	9.5	0.8	24.0
Northern	18.8	55.0	2.9	0.2	0.1	23.0
Central	21.9	42.9	1.8	0.0	0.2	33.2
Southern	41.6	23.7	4.8	0.0	4.1	25.9
North-Western	46.4	21.7	1.1	5.6	0.2	24.9
Uva	41.2	39.2	1.2	0.0	0.5	17.9
North-Central	19.1	30.1	8.1	0.0	0.0	42.8
National average	38.7	23.9	3.4	5.8	1.3	26.8

*Total number of premises inspected: 279,728; total *Ae. aegypti*–positive containers: 9,699. Source: (*7*).
†Including refrigerator trays, nonfunctional cisterns, pet feeding cups, gutters, concrete slabs, and any other water-collecting containers.

Southeast Asia region, where older schoolchildren have a higher risk for clinical illness (*27*). However, the dengue incidence in younger age groups (0–9 years) might be underestimated because of asymptomatic or mild primary infection. Approximately half of primary dengue infections in children are inapparent (*28*,*29*), whereas primary infections in adults more often result in overt disease (*30*). In Sri Lanka, incidence also decreased among elderly persons, which cannot be explained by asymptomatic infections. According to Sri Lanka's 2012 census, 31.3% of the population was 10–29 years of age, and only 12.4% was >60 years of age (*10*). Comparable data from other countries in Asia give conflicting results about age-specific dengue incidence. For example, in Taiwan and Singapore during 2010–2015, the prevalence of dengue was higher in persons >60 years of age than in younger persons, a plausible reflection of

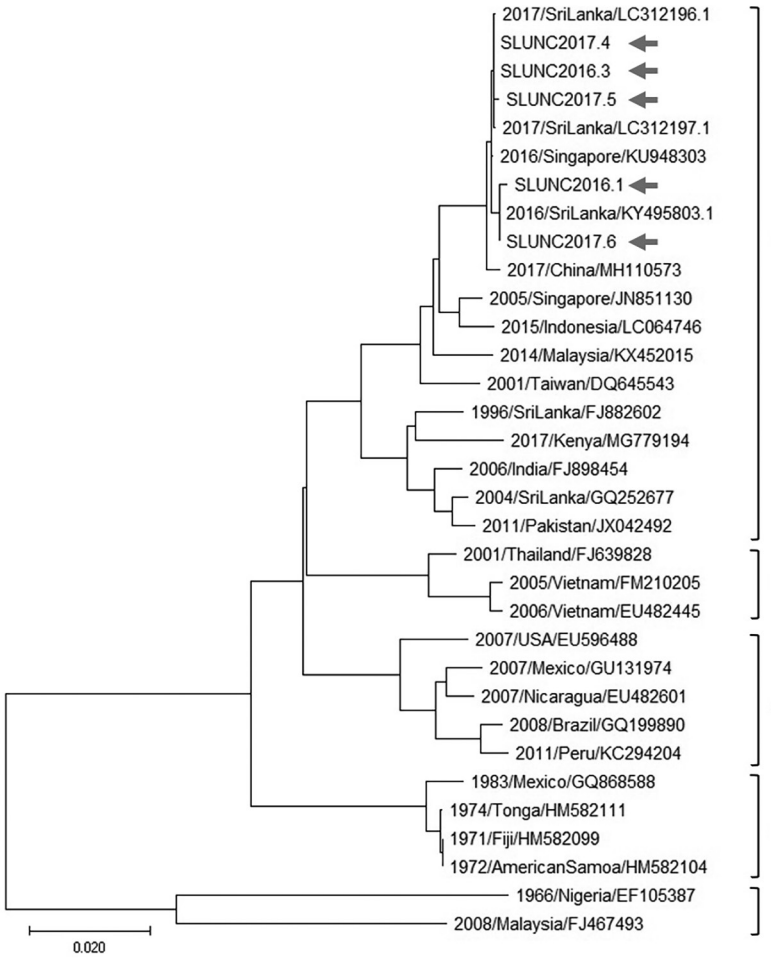

Figure 4. Phylogenetic tree for 5 dengue virus 2 (DENV-2) isolates from late 2016 and 2017 dengue epidemic (arrows), Sri Lanka, and reference DENV-2 strains. The tree is based on a 1,485-nt fragment that encodes the envelope protein. Classification and naming of DENV-2 genotypes are based on (*16*). The evolutionary history was inferred using the neighbor-joining method (*17*). The optimal tree with the sum of branch length = 0.44012906 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (*18*) and are in the units of the number of base substitutions per site. This analysis involved 33-nt sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The final dataset comprised 1,485 positions. Evolutionary analyses were conducted in MEGA X (*12*). Scale bar indicates nucleotide substitutions per site.

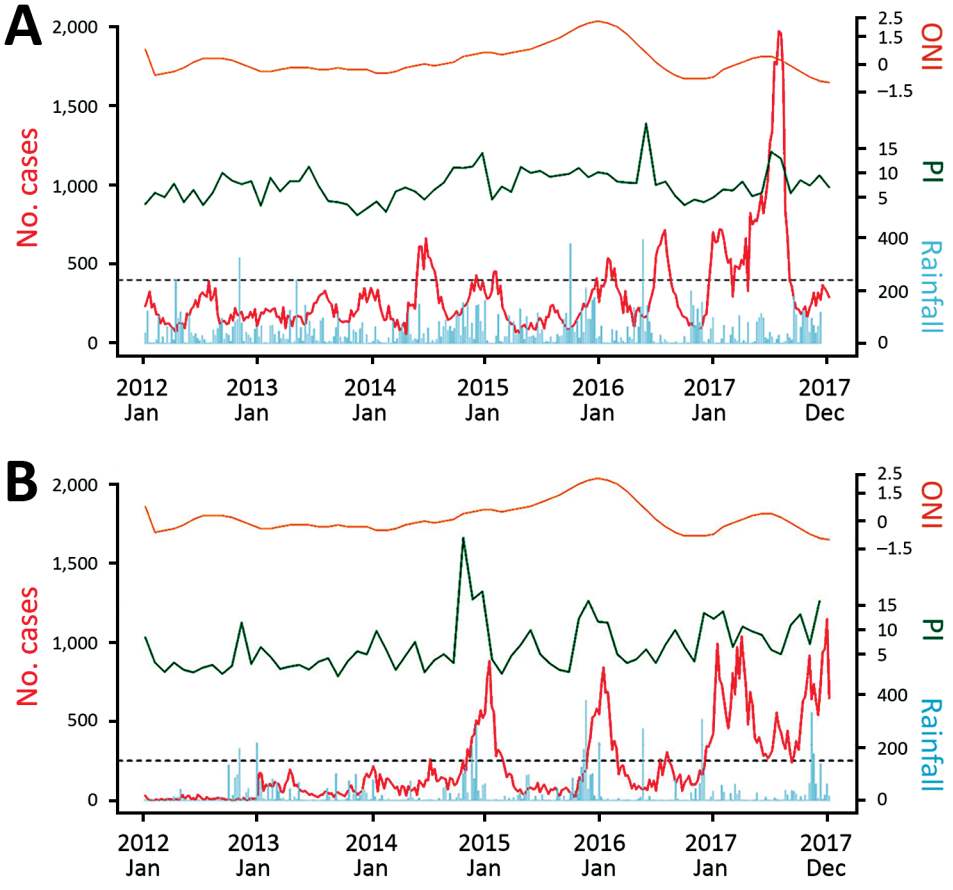

Figure 5. Time series plot showing weekly number of reported dengue cases (red line), ONI (orange line), PI (green line), and weekly cumulative rainfall, mm (blue line), Sri Lanka, 2012–2017. A) Colombo district. B) Jaffna district. ONI, Oceanic Niño Index; PI, Premise Index.

an aging but active elderly population (*31*–*33*). On the other hand, in countries such as India, Vietnam, and Brazil, dengue incidence is increasing in a higher proportion of persons in younger age groups (*27*,*34*,*35*).

In many countries, serotype switches of circulating dengue viruses have been associated with severe epidemics when the population is exposed to a new serotype (*36*,*37*). Host immunity against 1 dengue serotype only partially protects against other serotypes. In fact, heterotypic antibodies may exaggerate the inflammatory response during infection with a different serotype (*38*). Circulating serotypes in Sri Lanka were monitored in the past by a handful of research studies in the absence of a national virologic surveillance program. A study conducted during 2003–2006 (*39*) and another during a large epidemic in 2009 (*40*) concluded that DENV-1 was the dominant circulating serotype in all these periodic epidemics. This observation remained unchanged in 2 subsequent epidemics in 2010 and 2012 (*41*). However, during 2003–2006, DENV-2 and DENV-3 serotypes caused between-epidemic background dengue transmission (*39*). After 2010, the number of DENV-1 cases progressively increased, taking over the background transmission from DENV-2 infections, which might have led to a loss of immunity to DENV-2 in the population. Limited virologic surveillance from the Colombo district demonstrated DENV-2 dominance in the 2017 samples, but this finding cannot be extrapolated to the entire country. The viruses isolated from the 5 patients had almost identical sequences and belonged to the cosmopolitan genotype of DENV-2. The phylogenetic analysis demonstrates this strain to be a new strain (for Sri Lanka) that most likely came from Southeast Asia or China (*18*).

Of all the climatic factors studied, only monsoon rainfall had a clear relationship over the years with the dengue incidence. Historically, the incidence of dengue in Sri Lanka shows 2 peaks per year, each of which occurs a few weeks after onset of the 2 monsoons affecting the country. The southwest monsoon brings in heavy rainfall midyear (May–September) to Western, Sabaragamuwa, Southern, and Central provinces, and the northeast monsoon brings a relatively heavy rainfall during December–February for the Northern, North Central, Uva, and Eastern provinces. The midyear peak in dengue incidence in 2017

Table 4. Direct costs of dengue control and outbreak response activities, Sri Lanka, 2017*

Type of activity	Sri Lanka rupee	US $
Routine dengue control activities		
Personnel: public health staff salaries	1,060,340,000	6,955,512
Development of health education material and advertising	5,000,000	32,798
Implementation of dengue awareness campaign through electronic and print media	10,000,000	65,597
Integrated vector management; insecticides and fogging machines	156,000,000	1,023,313
Recurrent costs at National Dengue Control Unit	7,000,000	45,918
Routine vector control and recurrent costs in periphery	25,000,000	163,992
Subtotal	1,263,340,000	8,287,131
Outbreak response activities		
Personnel: public health staff extra duty pay for outbreak response, at national and regional/district levels	176,723,333	1,159,252
Outbreak response brigades: brigade staff salaries and other expenses during outbreak response	114,000,000	747,806
Purchase of mini cabs for vector control (50 cabs)	75,000,000	491,977
Establishment of high-dependency units in public hospitals	181,000,000	1,187,306
Intensified door-to-door premise inspection campaign in high-risk districts	128,000,000	839,642
Subtotal	674,723,333	4,425,983
Total	1,938,063,333	12,713,114
Cost per capita	96.90	0.64

*Source: (7).

was exceptionally high in all provinces affected by the southwest monsoon (weeks 25–35).

The increase in dengue incidence in 2017 could not be explained by entomologic parameters because they did not differ significantly between preceding years and 2017. The types of breeding sites reported in each of the provinces varied but also did not have any relationship with the reported case incidence.

Sri Lanka's government-led dengue control activities mainly focus on vector control. These methods include routine measures of integrated vector control that are in place periodically throughout the year and outbreak control measures during epidemics: health education, source reduction of vector breeding sites, chemical fogging, and punitive measures (e.g., fines, warnings for harboring mosquito breeding sites). Integrating a biological control method into this framework by introducing the larvicidal *Bacillus thuringiensis* bacterium did not yield promising results in a recently concluded field trial in the city of Colombo and its suburbs in the Western province (*42*). Outbreak control measures usually are conducted as civilian–military partnerships focusing on breeding site removal. These methods come into effect only when an epidemic is ongoing and are not helpful in preventing one. Government expenditure on dengue control activities (excluding costs of curative services and indirect costs) is substantial, and 35% of the cost was on outbreak control, which could have been averted by better outbreak prevention. During the epidemic year of 2012, per capita spending for dengue in Colombo district was US $0.42 (US $0.48 when adjusted for depreciation of the LKR during 2012–2017) (*43*). In 2017, per capita expenditure was US $0.64 in a countrywide cost assessment.

Our findings are subject to several limitations. A large volume of the national surveillance system data originates from the public health services and in-ward facilities of private hospitals. Data from outpatient departments of private hospitals and general practitioners are underrepresented. In addition, facilities for laboratory diagnosis are limited. Although most cases were reported as suspected cases diagnosed with syndromic definition using clinical criteria and serial blood counts, on-demand rapid antigen detection tests are becoming increasingly available. The number of dengue infections in Sri Lanka still can be underestimated because clinically inapparent infections are not counted. Because of the lack of national virologic surveillance, genomic data on infecting viruses are available only from the Western province, which might not be representative for the rest of the country.

In retrospect, the key early indicators of the disproportionate epidemic in 2017 were the increase in cases in the latter half of 2016 and the gradual transition of the circulating strain (limited evidence from 1 district). An increase in the number of cases every year after monsoon rains is clear in all years studied. The direct costs of dengue control are huge once an outbreak occurs and could have been better spent on outbreak prevention rather than on outbreak control if prevention activities were sustained throughout the intermonsoon period and intensified during the monsoon rains. Epidemiologic surveillance based on suspected cases (vs. confirmed cases) is not ideal but is the best many dengue-endemic countries can do with limited access to point-of-care diagnostic testing. Capturing data from currently underreporting healthcare providers (e.g., general practitioners) is a priority to get an accurate picture of dengue in

Sri Lanka. Establishing a virologic surveillance system covering the entire country is important and is becoming feasible as new third-generation sequencing technologies become portable and less expensive. However, health education that results in behavior change of communities remains the most effective way of reducing vector breeding sites to prevent outbreaks. The government should facilitate health education as well as a good waste management policy backed by periodic audits.

Acknowledgments

We sincerely appreciate the support extended by Nimalka Pannila Hetti, Anoja Dheerasinghe, Mizaya Cader, Sunith Kumarapperuma, Iroshini Abeysekara, Thanuja Rathnayaka, and Indika Weerasinghe. We gratefully acknowledge the technical and nontechnical staff of the National Dengue Control Unit. We appreciate the work of Sampath Weerakoon and Dinusha Perera.

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Severe Fever with Thrombocytopenia Syndrome, Japan, 2013–2017

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We conducted an epidemiologic study of severe fever with thrombocytopenia syndrome (SFTS) in Japan during 2013–2017. Of 303 cases reported during that period, 133 (44%) were included in this study. The median time between onset of illness and diagnosis of SFTS shortened, from 11.5 to 3.0 days, but the case-fatality rate remained high, at 27%. In 64 patients (48%), a close contact with companion animals was reported within 2 weeks of disease onset. Of these 64 patients, 40 were surveyed further, and we confirmed that 3 had direct contact with body fluids of ill companion animals; 2 had direct contact with the saliva of an ill feral cat or pet dog. These patients reported no history of tick bite, suggesting that ill companion animals might be a source of SFTS virus transmission. Direct contact with the body fluids of ill companion animals should be avoided.

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tickborne infectious disease, identified in 2009 in the rural areas of Hubei and Henan provinces in China (*1*,*2*); a total of 7,419 cases were reported from 23 provinces during 2010–2016 (*3*). SFTS is endemic not only to China but also to South Korea and Japan (*4*,*5*). Huaiyangshan banyangvirus (formerly SFTS virus [SFTSV]), the causative agent of SFTS, belongs to the genus *Banyangvirus* in the family *Phenuiviridae*. Although the name of the virus has recently been changed from SFTSV to Huaiyangshan banyangvirus by the International Committee on Taxonomy of Viruses (*6*), the term SFTSV is still used. SFTSV is found in tick species such as *Haemaphysalis longicornis*, *Amblyomma testudinarium*, and *Ixodes nipponesis* in China, South Korea, and Japan (*7*–*9*). Antibodies to SFTSV were detected in wild and domestic animals, such as goats, deer, cattle, dogs, and cats, in SFTS-endemic areas of these 3 countries (*10*–*15*). SFTSV is thought to circulate in an enzootic environment and to have a tick–vertebrate–tick cycle (*12*). In addition, human-to-human transmission through blood and respiratory secretions has been reported from China and South Korea (*16*–*18*).

Since the first case of SFTS was reported in Japan in 2013 (*5*), ad hoc retrospective and prospective surveillance has been conducted by Japan's Ministry of Health, Labor, and Welfare. A total of 23 suspected SFTS cases were retrospectively reported from 2005, of which 11 were confirmed through this surveillance (*5*,*19*). SFTS was included in Japan's Infectious Diseases Control Law as a category IV notifiable disease on March 4, 2013. Our previous report describing the nationwide epidemiology of SFTS during April 2013–September 2014 demonstrated that the case-fatality rate (CFR) in 49 patients was as high as 31% (*20*). As of October 2017, a total of 303 confirmed cases had been reported to Japan's National Epidemiologic Surveillance of Infectious Disease (NESID) (*21*).

The annual numbers of SFTS patients reported each year during 2013–2017 were 40, 61, 60, 59, and 83, respectively. These patients were reported from 23 of the 47 prefectures in Japan, and the geographic distribution of the SFTS cases expanded gradually each year from western to central Japan (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/26/4/19-1011-App1.pdf). Most patients had SFTS onset during April–October (Appendix Figure 2), and the annual national notification rates of SFTS ranged from 0.03 to 0.06 cases/100,000 person-years over the study period. These findings were similar to those previously reported for 2013 and 2014 (*20*).

Studies conducted in China, South Korea, and Japan reported that several clinical and laboratory

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DOI: https://doi.org/10.3201/eid2604.191011

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parameters were associated with a fatal outcome of SFTS (*20*,*22*–*25*). Because no effective therapeutic agents for SFTS are currently available (*22*,*25*,*26*), effective and specific treatments for SFTS must be developed. A recent case report demonstrated that a veterinarian who cared for 3 symptomatic cats, 2 of which were pets, was infected with SFTSV (*27*). On the basis of such information, we conducted questionnaire surveys of SFTS patients to collect information regarding their direct contact with ill companion animals 2 weeks before illness onset. We conducted a retrospective observational study to identify the changes in the epidemiologic findings of SFTS patients over the study period of March 2013–October 2017 to determine the prognostic factors for SFTS and to evaluate the possible risk of direct exposure to ill companion animals possibly infected with SFTSV.

Methods

National Surveillance of SFTS in Japan

NESID defines a case of SFTS as illness in a patient with fever or gastrointestinal symptoms, any laboratory findings, including thrombocytopenia (<10.0 × 10^4/μL), leukopenia (<4,000 cells/μL), or elevated liver enzymes, plus laboratory confirmation of SFTSV infection. The confirmatory examinations include detection of the SFTSV genome using reverse transcription PCR (RT-PCR), which was performed at the local public health institute, the National Institute of Infectious Diseases (Tokyo, Japan), or both, and SFTSV-specific antibody testing using an immunofluorescence assay or an indirect immunoperoxidase assay (*13*,*28*,*29*).

Study Design and Data Collection

We performed a retrospective observational study of SFTS cases reported to NESID during March 2013–October 2017. Physicians were asked to participate in this study by completing a questionnaire sent by mail. Demographic data, social history of outdoor activity for 2 weeks before illness onset, clinical symptoms, and laboratory data for SFTS patients were collected through the first questionnaire. The physicians who agreed to participate in this study collected the information about the patients through their medical charts or by telephone interviews with patients or their family members, after obtaining informed consent. We collected clinical information and laboratory data for the acute phase (within 2 weeks after illness onset). We obtained data for patients whose cases were reported during March–September 2014 from a previous study (*18*); our study added data collected during October 2014–October 2017. We extracted basic demographic data for each SFTS patient reported during the study period from the NESID database.

We sent a second questionnaire to the physicians who responded to the first questionnaire; the second questionnaire requested information about whether the confirmed SFTS patients had contact with companion animals (such as cats and dogs). The information requested included whether the SFTS patient had close contact with an ill companion animal within 2 weeks before illness onset or direct contact with body fluids of an ill companion animal and the outcome for the ill companion animal.

Statistical Analyses

We used SPSS Statistics 21.0 for Windows (IBM, https://www.ibm.com) for all statistical analyses. We performed trend analysis for different years by using the Spearman rank correlation test and the Jonckheere–Terpstra test. We used the Pearson χ^2 test or the Wilcoxon rank-sum test to compare the characteristics of survivors and nonsurvivors. We used logistic regression modeling for multivariable analysis. All p values were 2-sided, and $p<0.05$ was considered statistically significant.

Ethics Approval

This study was conducted under approval by the Medical Research Ethics Committee of the National Institute of Infectious Diseases (approval no. 706). All aspects of the study complied with the Helsinki Declaration. Each patient or their proxy provided written informed consent.

Results

Epidemiologic and Clinical Characteristics of Study Cases

Of 303 patients reported to NESID, we included 133 (43.9%) whose demographic and clinical data were collected through the first questionnaire in this study (Figure 1). Of these 133 patients, 130 (98%) had SFTS diagnosed by detection of an SFTSV-specific genome using RT-PCR and 3 (2%) by detection of an SFTSV-specific antibody. We observed no significant difference in sex and age distribution between these 133 patients and the 170 patients for whom no questionnaire was collected. The median ages of the 2 groups were 73 years (interquartile range [IQR] 65–82 years) for the patients with questionnaires and 75 years (IQR 67–82 years) for those without.

Although the median time from illness onset to initial hospital visit was 3 days (IQR 2–5 days) and

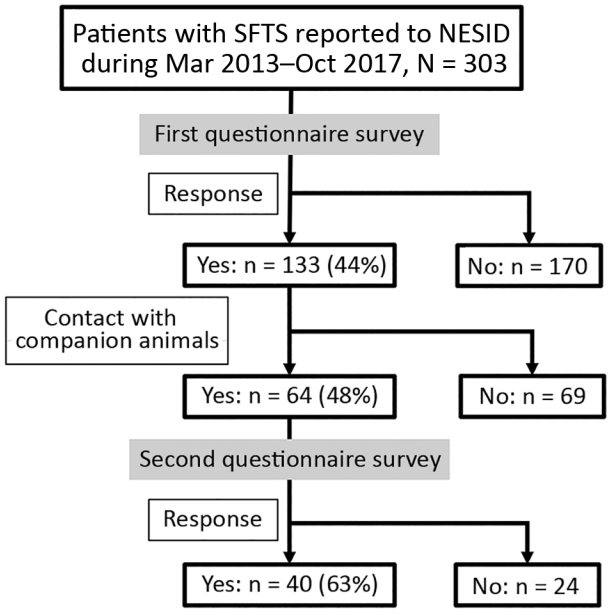

Figure 1. Flow diagram of epidemiologic study of 133 patients with severe fever with thrombocytopenia syndrome, Japan, March 2013–October 2017. NESID, National Epidemiologic Surveillance of Infectious Disease; SFTS, severe fever with thrombocytopenia syndrome.

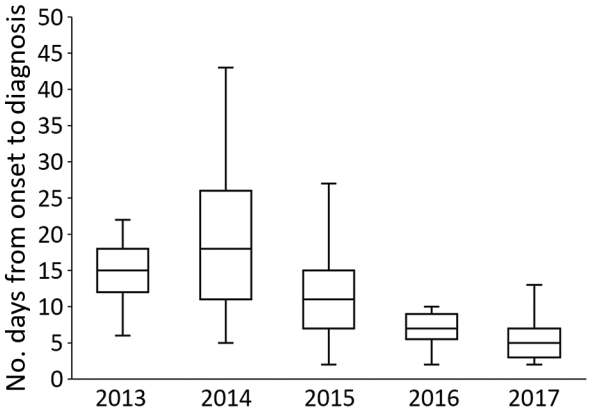

Figure 2. Comparison of time between illness onset and confirmed diagnosis in 133 patients with severe fever with thrombocytopenia syndrome, Japan, March 2013–October 2017. We conducted a trend analysis of time from initial visit to diagnosis over the study period by using the Jonkheere–Trapstra test (p<0.01). In the box plots, the bottom boundary of the box indicates the 25th percentile, the line within the box marks the median, and the top boundary of the box indicates the 75th percentile. Whiskers above and below the box indicate the 10th and 90th percentiles.

did not differ significantly between years, the median time between illness onset and a confirmed diagnosis for 133 SFTS cases shortened significantly over the study period (p<0.01) (Figure 2). The median time between illness onset and a confirmed diagnosis also significantly shortened over the study period for 97 survivors and 36 nonsurvivors (p<0.01). Thirty-six patients died, corresponding to a CFR of 27% (Figure 3, panel A). We observed no significant difference in age or sex distribution by year between the 97 survivors and 36 nonsurvivors. The Kaplan–Meier survival curve of 133 SFTS patients demonstrated that most deaths (94%) occurred within 2 weeks after illness onset (Figure 3, panel B).

Underlying illnesses among these 133 patients included hypertension (n = 47), diabetes mellitus (n = 27), and dyslipidemia (n = 15), as well as a few instances of malignant disease (n = 9) (Table 1). The

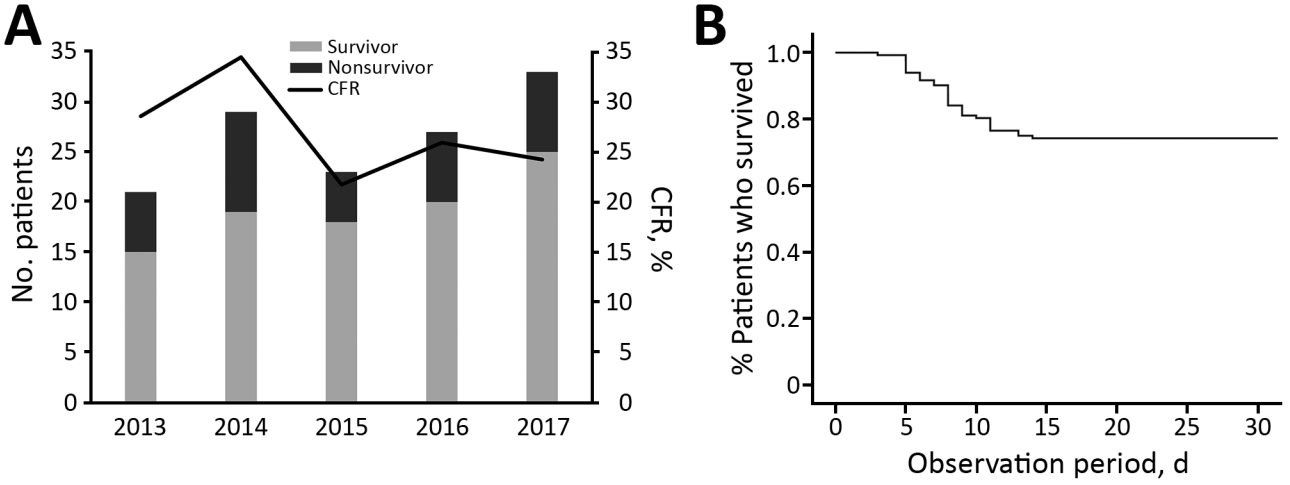

Figure 3. Annual number and CFRs and Kaplan–Meier survival curve of 133 patients with severe fever with thrombocytopenia syndrome, Japan, March 2013–October 2017. A) Trend analysis of CFRs over the study period by using Spearman rank correlation test (p = 0.285). B) Kaplan-Meier curve of 133 patients with severe fever with thrombocytopenia syndrome within 30 days after illness onset. CFR, case-fatality rate.

Table 1. Demographic characteristics of 133 patients with severe fever with thrombocytopenia syndrome, Japan, March 2013–October 2017*

Characteristic	No. (%) All case-patients, n = 133	Nonsurvivors, n = 36	Survivors, n = 97	p value†
Sex				
M	63 (47)	17 (47)	46 (47)	0.984
F	70 (53)	19 (53)	51 (53)	
Median age, y (IQR)	73 (65–82)	78 (68.25–84.75)	72 (63.5–80)	0.015‡
Underlying conditions				
Malignant tumor	9 (7)	6 (17)	3 (3)	0.006
Diabetes mellitus	27 (20)	7 (19)	12 (12)	0.860
Hypertension	47 (35)	14 (39)	33 (34)	0.707
Dyslipidemia	15 (11)	3 (8)	12 (12)	0.381
None	36 (27)	8 (22)	28 (29)	0.444

*Values are no. (%) unless indicated. IQR, interquartile range.
†Pearson χ^2 test used for all variables except age.
‡Wilcoxon rank-sum test.

proportion of patients with malignant disease was significantly higher among the nonsurvivors (17%) than among the survivors (3%) ($p<0.05$). Sixty-four (48%) patients were reported to have been bitten by a tick, and 55 (41%) had traces of a tick bite; 109 (82%) had participated in outdoor activities on hills and in fields. Sixty-four (48%) had close contact with companion animals, such as dogs and cats, within 2 weeks before illness onset.

We reviewed clinical symptoms and laboratory data at initial hospital visit of the 133 patients with available information (Appendix Tables 1, 2). We found no significant difference in neurologic symptoms, except for tremor, between survivors and nonsurvivors. The serum levels of aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, lactic acid dehydrogenase, and potassium were significantly higher, and activated partial thromboplastin time significantly longer, in nonsurvivors than in survivors.

Prognostic Factors

To investigate possible prognostic factors at the initial hospital visit, we performed multivariable analysis by selecting factors on the basis of the univariate analysis results, past reports, and clinical importance (Table 2). The multivariable logistic regression analysis indicated that a low platelet count was associated with a fatal outcome (odds ratio [OR] 1.38, 95% CI 1.07–1.78). The complications of malignant disease (OR 20.83, 95% CI 1.32–327.70) and presence of tremor at initial hospital visit (OR 17.37, 95% CI 1.26–239.39) also were associated with an increased risk for death.

Contact with Ill Companion Animals

Of 64 patients who responded to the second questionnaire, 40 (62.5%) reported contact with a companion animal within the 2 weeks before illness onset (Figure 1). For 5 of these patients, their companion animal appeared to be ill during this period, and 3 (patients 1, 2, and 3) had direct contact with the body fluids of the ill companion animals (Table 3). These three patients had no history of a tick bite. No information was available about the type of body fluid to which patient 1 was exposed. Patient 2 had direct contact with the saliva of a symptomatic feral cat, and she was also bitten by this cat, which subsequently died. No virologic examination of these 2 cats was attempted. Patient 3 had direct contact with the saliva of an ill dog that he owned. RT-PCR detected the presence of SFTSV genome in blood from this dog.

Table 2. Multivariable analysis of prognostic factors for 133 patients with severe fever with thrombocytopenia syndrome, Japan, March 2013–October 2017*

Variable	Univariable OR (95% CI)	Univariable p value	Multivariable OR (95% CI)	Multivariable p value
Age	NA	0.015	1.07 (0.98–1.16)	0.115
Malignant tumor	6.13 (1.45–26.04)	0.006	20.83 (1.32–327.70)	0.031
Disorientation	1.58 (0.71–3.52)	0.259	1.37 (0.30–6.35)	0.687
Tremor	8.60 (1.57–47.04)	0.004	17.37 (1.26–239.39)	0.033
Platelet	NA	0.661	1.38 (1.07–1.78)	0.014
Albumin	NA	0.791	0.99 (0.15–6.62)	0.994
ALT	NA	0.015	1.01 (1.00–1.02)	0.371
LDH	NA	0.012	1.00 (1.00–1.00)	0.378
CK	NA	0.591	1.00 (1.00–1.00)	0.842
APTT	NA	0.007	1.06 (0.97–1.16)	0.215

*ALT, alanine aminotransferase; APTT, activated partial thromboplastin time; CK, creatine kinase; LDH, lactate dehydrogenase; NA, not applicable; OR, odds ratio.

Table 3. Characteristics of 3 patients with severe fever with thrombocytopenia syndrome who had contact with body fluid of an ill companion animal before illness onset, Japan, March 2013–October 2017*

Patient no.	Onset year	Age, y/sex	Outcome	Tick bite	Species of ill companion animal	Direct exposure to ill animal's body fluid	Outcome of ill companion animals	SFTSV detection from animals
1	2014	46/F	Recovered	No	Cat	Yes	Recovered	Not tested
2	2016	57/F	Died	No	Cat	Yes (bite)	Died	Not tested†
3	2017	42/M	Recovered	No	Dog	Yes	Recovered	Yes

*SFTSV, severe fever with thrombocytopenia virus (Huaiyangshan banyangvirus).
†Clinical and laboratory findings were similar to those of human case.

Discussion

In this study, we investigated the epidemiologic and clinical features of 133 SFTS patients identified in Japan during 2013–2017. The overall CFR was 27%, which did not significantly change over the study period. We found a significant reduction in the interval between illness onset and the SFTS diagnosis and identified that underlying malignant disease, low platelet count, and appearance of tremor at hospital visit were significantly associated with increased risk for death after adjusting for age.

On the basis of national surveillance data, as of October 31, 2017, the proportions of fatal cases during the study period were 35% in 2013, 26% in 2014, 18% in 2015, 14% in 2016, and 13% in 2017 (https://www.niid.go.jp/niid/ja/sfts/sfts-idwrs/7415-sfts-nesid.html). Therefore, a discrepancy was noted between the CFRs observed in this study and the proportions of fatal cases in the national surveillance database. We found that the time between illness onset and hospital visit remained unchanged but that the time between illness onset and diagnosis shortened significantly, indicating an increased awareness of this disease among physicians during more recent years in the SFTS-endemic areas of Japan. Because physicians who diagnose a case of SFTS are requested to report the case to NESID immediately after diagnosis, the time between illness onset and reporting had also been shortened (from 14 days [IQR 11–24 days] during 2013–2014 to 6 days [IQR 4–8 days] in 2017).

Our findings demonstrate that deaths commonly occurred within 2 weeks after illness onset (Figure 3, panel B). During the study period, 11 patients (1 patient each in 2013 and 2015, 2 patients in 2016, and 7 patients in 2017) died after their diagnosis was reported to NESID. This fact might explain, in part, the discrepancy between the CFRs in this study and the proportions of fatal cases in national SFTS surveillance data.

Our finding that a low platelet count at initial hospital visit was a risk factor for a fatal outcome is in agreement with the results of previous studies (*20*,*22*,*23*). Although age-adjusted underlying malignant disease also was significantly associated with fatal outcome in our study, whether it has a direct effect on the survival of SFTS patients remains uncertain because of the limited number of such cases (n = 9). A previous study based on univariable analysis showed that the proportion of patients with tremor at admission was not significantly higher in fatal cases (20%) than in nonfatal cases (3%) (*20*), but our multivariable analysis demonstrated that appearance of tremor at initial hospital visit was significantly associated with a fatal outcome.

Three patients had direct contact with the body fluid of ill companion animals (1 dog and 2 cats) before illness onset, and these patients reported no history of tick bite. Importantly, 2 of the 3 patients had direct contact with the saliva of an ill feral cat or an ill pet dog. In addition, the cat that bit patient no. 2 subsequently died (Table 3). These findings suggested that the ill companion animals could be the direct source of SFTSV infection.

Since 2017, a total of 24 pet cats living in western Japan have been diagnosed with SFTSV infection (*30*). All cats infected with SFTSV showed acute onset of clinical signs. High fever (>39.5°C) was noted in 15/22 cats (68.2%) and vomiting in 10/24 cats (41.7). Animal experiments by Park et al. (*31*) confirmed that cats are susceptible to SFTSV infection; the authors reported that 4/6 cats infected with SFTSV died, and all cats showed signs that were similar to or more severe than those signs observed in humans infected with SFTSV. Those authors also found high viral loads in serum, saliva, and eye swabs taken 7 days postinfection from cats that subsequently died from infection. Collectively, these findings suggest that SFTSV transmission in patient no. 2 (Table 3) very possibly occurred from an ill cat through the patient's direct contact with the saliva of the cat.

Our study has several limitations. First, only 133/303 (43.9%) patients reported to NESID were included in the study. Although we found no significant difference in sex and age distribution between the 133 patients who participated in this study and the 170 patients who did not, the 133 study participants might not represent the population of interest for research purposes. Second, although 3 patients

who had contact with a body fluid of ill companion animals reported no history of a tick bite, whether these patients actually had a tick bite that was not reported because of recall bias is unknown. Therefore, we cannot exclude the possibility that the animals carried ticks and that a tick bite, rather than exposure to body fluid, might have been the actual mode of transmission. Third, no information is available on whether the 3 SFTS patients possibly infected from their ill companion animals were infected with the same strain of SFTSV as the animals. For any patients possibly infected from their companion animals, testing should be performed to determine whether the viruses isolated from the patient and from their animal are identical. In our study, we conducted the questionnaire surveys to evaluate the SFTSV infections from companion animals but were unable to determine the risk for SFTSV infection from these companion animals. Therefore, further case–control studies are required to determine the risk of exposure to the body fluids of companion animals.

In conclusion, we demonstrated that underlying malignant disease, low platelet count, and appearance of tremor at the first hospital visit were significantly associated with a fatal outcome among SFTS patients. The CFR of SFTS patients in Japan remained high at 27%. Three (2%) of 133 SFTS patients had direct contact with the body fluids of ill companion animals but no reported history of tick bite within the 2 weeks before illness onset, suggesting that ill companion animals might be a source of SFTSV transmission to humans. Although further studies on the epidemiologic and virologic analyses are needed, the owners of companion animals and veterinarians in the SFTS-endemic area should be fully aware of the risk of direct contact with body fluids of ill companion animals. The owners should avoid direct contact with body fluids, such as saliva, of their ill companion animals and should take care not to be bitten by them.

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Acknowledgments

We thank the members of the SFTS Epidemiological Research Group–Japan and staff at local public health centers and public health institutes.

This study was supported by the Agency for Japan Medical Research and Development (grant no. 17fk0108202).

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Comprehensive Profiling of Zika Virus Risk with Natural and Artificial Mitigating Strategies, United States

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Zika virus is transitioning to become a long-term public health challenge, and countries should remain informed of the risk for emergence. We developed a stochastic epidemiologic model to profile risk for Zika virus emergence, including trimester-specific fetal risk across time, in all 3,208 counties in the United States, including Puerto Rico. Validation against known transmission in North America demonstrated accuracy to predict epidemic dynamics and absolute case counts across scales (R^2 = 0.98). We found that, although sporadic single transmission events could occur in most US counties, outbreaks will likely be restricted to the Gulf Coast region and to late spring through autumn. Seasonal fluctuations in birth rates will confer natural population-level protection against early-trimester infections. Overall, outbreak control will be more effective and efficient than prevention, and vaccination will be most effective at >70% coverage. Our county-level risk profiles should serve as a critical resource for resource allocation.

Zika virus is a flavivirus spread by *Aedes* mosquitoes that for >60 years remained only an esoteric threat to human health (*1*). However, the recent Zika epidemic, which erupted in South America in 2015 and became the largest in history, brought the virus to prominence, particularly because infection has been linked to fetal microcephaly and other neurodevelopmental and neurologic sequelae (*2*).

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DOI: https://doi.org/10.3201/eid2604.181739

Although no longer classified a global emergency by the World Health Organization (WHO), Zika virus emergence and transmission continues globally, and WHO warns that Zika virus is set to remain as a long-term public health challenge (*3*). Given the critical importance of preventing Zika virus infections, especially during pregnancy, transmission anywhere requires that nations remain vigilant and informed at local, state, and national levels to prevent and control introduction and onward transmission (*4*,*5*). This imperative is especially important for countries such as the United States that simultaneously harbor the *Aedes* vectors and maintain essentially entirely susceptible populations.

Numerous models for the potential emergence of Zika virus in the United States focus largely on the ecologic niche of *Aedes* mosquitoes (*6*–*10*). Projections that simultaneously consider vector dynamics and human demographics, including birth seasonality, to resolve both relative and absolute epidemic risk and potential control measures across space and time throughout the year are more limited.

Here we present a stochastic Zika virus compartment model that considers the overlap of vector dynamics and human demographics at the county level in the United States, including Puerto Rico. The model was used to profile the risk for Zika virus transmission, assuming an initial introduction into each county, including trimester-specific fetal exposures for each of the 3,208 counties and municipalities within the United States including Puerto Rico over time and under varying control measures. We tested 3 approaches to controlling Zika virus transmission and assessed their utility in preventing or abrogating Zika virus transmission. These approaches include reducing human–vector contact (i.e.,

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behavior modification and ubiquitous technologies such as air conditioning, screens, and long clothing); depleting adult vectors (i.e., mosquito fumigation programs); and vaccination, which, should a successful candidate vaccine come to market, might reduce individual and community risk for infection once herd-immunity thresholds are achieved (*11*).

Methods

We modeled county-level Zika virus transmission using a coupled 2-system stochastic human–mosquito differential equation compartment model (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/26/4/18-1739-App1.pdf). The human system was a susceptible-exposed-infected-recovered model and the mosquito system a susceptible-exposed-infected compartment model that incorporates vector and viral life-stage dynamics as functions of temperature throughout the year, as well as climate (temperature) and demographic data, including county- and municipality-level seasonality of births. More specifically, we coupled high-resolution *Aedes* vector risk maps (*12*) describing the ecologic extent of the major vectors of Zika virus, *Ae. aegypti* and *Ae. albopictus* mosquitoes, with dynamic temperature-dependent Zika virus and *Aedes* life-stage models, local climate data, and county-level demographic information, including population and monthly birth cohort data. We simulated Zika virus transmission given a single importation (index case) into each county across time and under varying control measures. We simulated stochastic trajectories by using an adaptation of the fundamental Gillespie stochastic simulation algorithm, an adaptive tau-leaping procedure (*13*) for continuous-time Markov processes, which we implemented by using the AdaptiveTau R package (*13*).

For each county and each scenario, we conducted ≥500 simulations and derived probability of initiation of a transmission chain from the index case, expected outbreak size when transmission occurs, and, by fitting nonlinear models to county-level monthly birth data, trimester-specific fetal Zika virus exposures. To remain relevant to local, state, and national entities, all 3,208 counties and municipalities were investigated independently, assuming only that an index case-patient arrives in the county, regardless of origin (i.e., spread from a neighboring county or an international import).

Model Parameters

We selected parameters from ecologic and epidemiologic literature (Appendix Table 1; Appendix Figure 1). Given the novelty of the Zika virus as a major human pathogen, relatively limited information on its dynamic life-stage properties is available. Thus, properties relating to transmission and extrinsic incubation period were borrowed from the large body of literature on dengue virus dynamics, because dengue virus is a closely related but more completely studied mosquitoborne flavivirus that shares the same mosquito vector host system as Zika virus. Such a strategy is commonly used in modeling for emerging pathogens, including other Zika virus transmission models (*7*,*14*).

Trimester-Specific Pregnancies and Exposure Calculations

Infection with Zika virus is most concerning during pregnancy, where maternal infection has been linked to congenital birth defects, most notably microcephaly (*15*). These defects appear to be most strongly associated with Zika virus infection during the first and second trimesters (*16*,*17*). Therefore, we derived trimester-specific maternal–fetal exposures from county-level demographic data, including birth seasonality.

Throughout the year, the proportion of a population's births fluctuate in a predictable manner across calendar months. To estimate the numbers of children born per month, and thus calculate expected numbers of first-, second-, and third-trimester pregnancies per month, we used the number of births per month for each county over an 8-year period (2007–2014) based on US Census data. For each county, we fit generalized additive models to the monthly data to estimate the fraction of annual births per month for each county. We then coupled the county-level generalized additive model output, indicating the expected proportion of annual births in each calendar month, to annual birth numbers for each individual county to calculate monthly county-specific expected pregnancies.

From the monthly birth data for each county, we back-calculated the time of conception, assuming a 40-week gestation and a constant rate within a given month. On the basis of this calculation, we derived pregnancy cohorts, defined as the number of women becoming pregnant per month of the simulation, which allowed us to follow each cohort throughout their pregnancies and evaluate the number of pregnancies in their first, second, or third trimester during each month for each county or municipality. To calculate infections during pregnancy during the simulations, we derived the number of fetal exposures per week per trimester by drawing simultaneously from 3 binomial distributions each week, each

with the size equal to the number of first-, second-, or third-trimester pregnancies in the county during the week of interest, and with a probability equal to the proportion of the population infected during that week (Appendix). By drawing from a binomial distribution, we incorporated stochastic effects that influence the number of infections among gravid women, relative to the proportion infected across the population as a whole.

Model Validation

We compared models by comparing reported or published estimated incidence and case counts for known Zika that have arisen from local transmission in the United States including Puerto Rico against the respective simulated data. In addition, given similarities between Zika virus and dengue virus, such as common transmission vectors and dynamics (*18*), we compared reported or published estimated incidence and case counts for known dengue outbreaks that have arisen from local transmission in the contiguous United States against the respective simulated data. Validation data for Brownsville County, Texas, and Miami-Dade County, Florida, came from the Texas and Florida departments of health, respectively (*19*,*20*). Validation data for Monroe and Martin counties in Florida came from serosurvey data collected by the Florida department of health and the Centers for Disease Control and Prevention (CDC) (*21*,*22*).

Model validation for Puerto Rico used 2 resources to derive estimates of monthly and cumulative Zika virus incidence across Puerto Rico's 8 health regions. Official reported data from the Puerto Rico Ministry of Health (http://www.salud.gov.pr/Estadisticas-Registros-y-Publicaciones/Pages/VigilanciadeZika) provided information on the monthly dynamics (fractional abundance) of the epidemic for each health region, namely the proportion of cases per month in each of the 8 health regions. Separately, to obtain best estimates for total cumulative incidence, we used a recent and thorough report from CDC by Chevalier et al. (*23*), which analyzed blood donor screening data from April 3 through August 12, 2016, from the 2 largest blood banks in Puerto Rico to estimate overall epidemic size. The use of both resources used the strengths of both types of reports (accurate fractional abundance over time and in each health region, and accurate estimates of cumulative incidence) to derive best estimates for model validation (Appendix).

Results

Across the United States, when an infectious Zika virus–infected person was introduced during peak *Aedes* abundance for each county (Appendix Figure 2), the model predicted at least minimal transmission (defined as ≥1 transmission event in ≥0.05% of simulations) in 86% of US counties (Figure 1, panel A), essentially reflecting the limit of *Aedes* mosquito distribution (Appendix Figure 3). However, the probability of any transmission varied widely and was focused in the Southeast United States, Puerto Rico, and portions of Texas (Figure 1, panel A; Appendix Figures 4–6).

Once initiated, transmission chains were very limited. Of counties where the model indicated at least minimal transmission from index case-patients during peak vector abundance, 93% of transmission chains (interquartile range [IQR] 88%–98%) had median incidence (among simulations with transmission) of <1% of the population (Figure 1, panel B), and 63% (IQR 48%–78%) of chains had final outbreak sizes of <10 total cases (Figure 1, panel C; Appendix Figure 6). Where *Ae. aegypti* mosquitoes are scarce compared with *Ae. albopictus* mosquitoes (Appendix Figure 7, panel A), 95% of counties had median outbreaks of <10 total cases (Appendix Figure 7, panel B), demonstrating that onward transmission is driven primarily by *Ae. aegypti* mosquitoes.

Along the Gulf Coast, outbreaks were more sustained. In Harris County, Texas, home to the Houston metropolitan area (population ≈4.8 million), the model predicted the largest epidemics in the 50 states, with a median epidemic size of 6,538 infections (IQR 1,846–17,440 infections) from an import during peak vector abundance. Although the entire Gulf Coast region is at risk for outbreaks, only 3 states contributed 97% of the top 100 counties with the largest simulated outbreaks: Florida (40%), Texas (35%), and Louisiana (22%). Mississippi contributed the other 3%.

According to our model, no counties within the 50 states sustained transmission beyond the first winter (Figure 1, panel D; Appendix Figure 8), although Miami-Dade and Broward counties in Florida sustained transmission as late as February in a fraction of simulations. Only Puerto Rico, Hawaii, and select counties, primarily in Florida and Texas, supported any transmission from the index cases occurring outside of the late spring through early autumn months (Appendix Figure 9, 10). Within the 50 states, only Miami-Dade County had evidence of transmission as early as February, and outbreaks there were limited in size (median 2 cases).

Our model showed final epidemic size was particularly sensitive to time of introduction (Appendix Figure 11), especially among counties most susceptible to transmission. Among the top 10% of counties

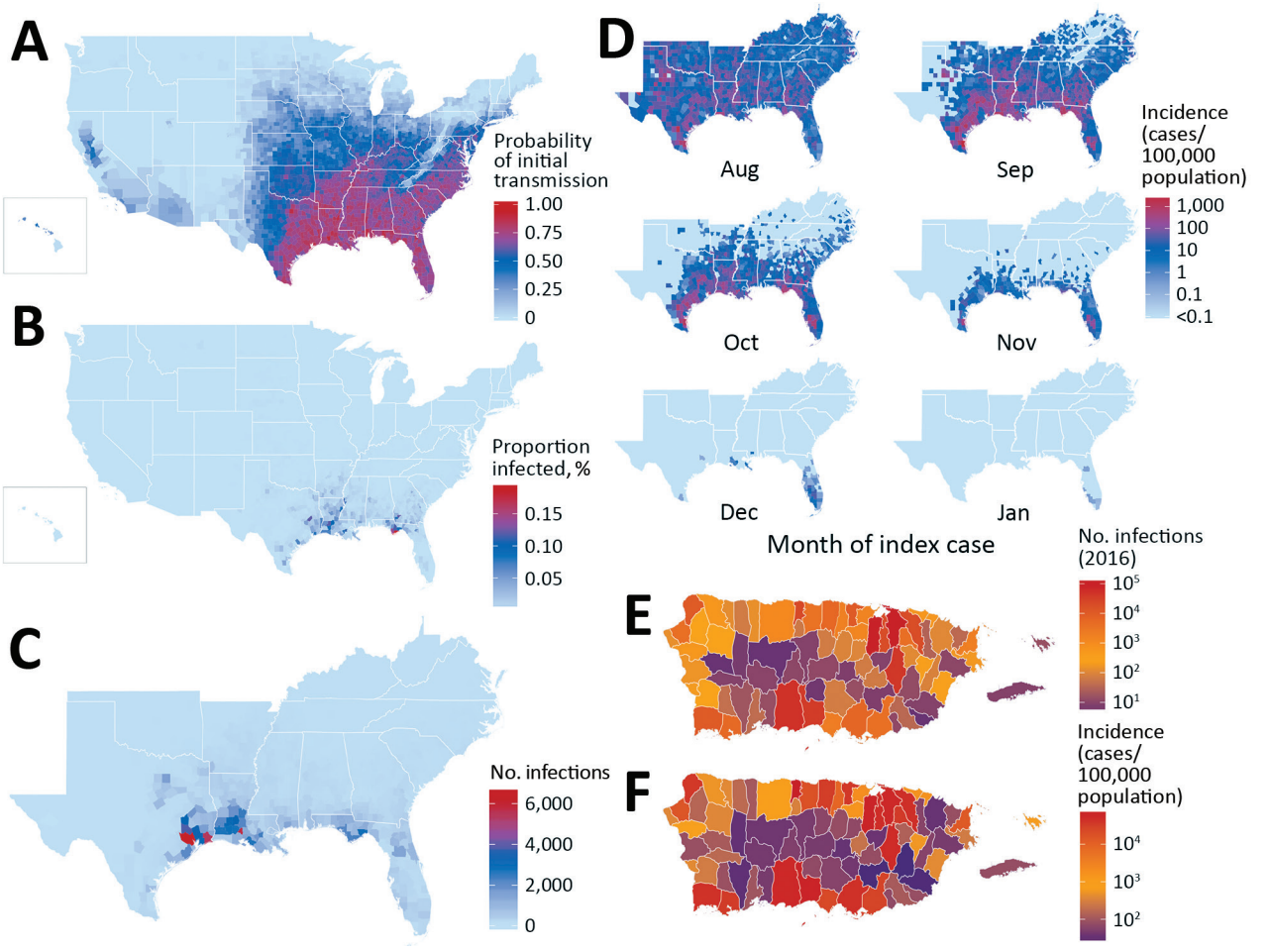

Figure 1. County-level Zika virus risk profiling, United States including Puerto Rico. A) Probability of initial transmission from an index case introduced during peak vector abundance, calculated as the proportion of simulations with ≥1 transmission event, for every county. B) Proportion of population infected. C) Total case counts for the southeastern United States (nationwide data depicted in Appendix Figure 6, https://wwwnc.cdc.gov/EID/article/26/4/18-1739-App1.pdf) when transmission does occur after index cases during peak abundance (median calculated among simulation with ≥1 transmission event). D) Monthly incidence and duration of outbreaks. Shown is the median monthly incidence of Zika virus infections from August index cases. E) Total number of simulated exposures in Puerto Rico ending December 31, 2016. F) Final epidemic size (incidence) at the end of simulations. For panels E and F, imports into each municipality corresponded temporally with initial cases reported in 2015 and 2016. All simulations assess counties and municipalities independently.

by predicted final epidemic size, the time of import that maximized incidence was ≈2 months earlier than that which maximized initial transmission (May vs. July; p<0.001) (Appendix Figure 12, panels A, C), and final incidence was as much as 10-fold greater. This difference disappeared among the 80% of counties with the smallest predicted final epidemic size (Appendix Figure 12, panels B, D), where both metrics were maximized by imports during peak vector abundance, reflecting very limited transmission chains in most counties.

In Puerto Rico, simulated epidemics were more sustained and greater in magnitude. When index case-patients were introduced into each municipality to match timing of first reported cases in the 2016 epidemic (*6*), through 2016 our model detected 479,025 (IQR 310,365–662,257) total infections (Figure 1, panel E), representing a median incidence of 14% (IQR 9%–19%) of the population. The model also showed that San Juan (population 365,576) had the largest epidemics, which usually persisted for up to 3 years and infected 58% (IQR 52%– 74%) of the population (Figure 1, panel F; Appendix Figure 13). These findings are consistent with previous Zika epidemics among island populations, where seropositivity reached 50%–70% (*24*,*25*). Across simulations, the total incidence on the island of Puerto Rico was 24% (IQR 19%–30%), suggesting that most infections had already occurred in 2016, when the index case was introduced. In addition, only 19%

(IQR 13%–23%) of the municipalities in the model sustained transmission through the first winter, whereas 14% and 3% sustained through the second and third winters, respectively.

Zika Virus Infections during Pregnancy

We found that natural seasonality in births results in waves of first- and second-trimester pregnancies that are out-of-phase with peak Zika virus infections in the model (Figure 2, panel A) and thus confers significant population-level protection against early-trimester exposures (Figures 2, panels B, C). Our model indicates that birth seasonality alone reduced risk for Zika virus exposure during first- (versus second- and third-) trimester exposures by 11% (relative risk 0.89 [95% CI 0.80–0.99]; p = 0.012).

Although counties in our model with the greatest numbers of fetal exposures generally tracked with epidemic size, distinct demographics led to deviations. In particular, Florida contributed 12% fewer counties to the top 100 counties, when ordered by rates of fetal exposures versus rates of total infections. Within the continental United States, Harris County, Texas, had the highest number of exposures during pregnancy (78 [IQR 20–183] exposures) after introduction of index cases during peak vector abundance (Figure 2, panel D; Appendix Figure 14). In Miami-Dade County, when simulated with a July index case introduction to match the 2016 outbreak (see also model validation below), we detected only 1 (IQR 0–3) fetal exposure from locally transmitted infections.

In Puerto Rico, when index cases were introduced into each municipality to match timing of initial cases reported in the current outbreak, through 2016 we detected 4,187 [IQR 2,733–5,760] infections during pregnancy (Figure 2, panel E), representing 10% (IQR 6%–13%) of all births. Throughout the entire simulated epidemic in Puerto Rico, the IQR for exposures in pregnancy was 5,800–9,100 (Figure 2, panel F).

Control Strategies

When human–vector contact rates were reduced from baseline in the model, the probability of initial transmission remained relatively insensitive, until contact was reduced by ≥70%, at which point initial transmission fell sharply (Figure 3, panels A, B). Incidence was exquisitely sensitive to reductions in contact, and fell log-linearly, with the magnitude of the slope proportional to the baseline incidence (Figure 3, panel C).

Depletion of adult *Aedes* mosquitoes through reductions in *Aedes* mosquito average lifespan in the model was effective at decreasing likelihood of initial transmission and epidemic size across all levels of intervention, again with incidence more sensitive than initial transmission from the index case (Figure 3, panels D–F).

Vaccination was relatively more effective at preventing initiation of transmission than reducing incidence, particularly once vaccination coverage exceeded 70% (Figure 3, panels G–I). This finding is consistent with an R_0 for Zika virus of 3–4, based on the simple but robust formula for the vaccination coverage V) required to achieve herd immunity: $V = 1–1 / R_0$, where R_0 is the basic reproductive number), in agreement with previous estimates (*26*,*27*).

Model Validation

We validated the model against known Zika outbreaks in the United States including Puerto Rico since late 2015, including Miami-Dade County (Florida), Brownsville County (Texas), and Puerto Rico, with separate tests across each of Puerto Rico's 8 health regions. Overall, the model accurately predicted the dynamics and absolute case counts for each site tested ($R^2 = 0.980$; $p<0.001$; Figure 4, panel A). In Miami-Dade County, where Zika virus transmitted locally in 2016, the model estimated a total epidemic size of 185 (IQR 45–467) cases (Figure 4, panel B), in strong agreement with the 225 locally transmitted cases reported by the Florida Department of Health and the 214 reported by CDC (*20*,*28*,*29*). In Brownsville County, Texas, where local Zika virus transmission was detected during October–December 2016 and infected 6 persons (*19*), the model estimated a median of 4 cases (IQR 1–8).

Because much of the model is parameterized on the basis of existing biological data measured for dengue viruses, we also validated the model against known dengue outbreaks in Florida. In Monroe County, Florida, local dengue transmission was detected in September 2009. A serosurvey conducted in the surrounding areas of the locally acquired cases estimated an infection rate of 3%–5% among residents during July–September 2009, where 5% includes presumptive infections in addition to acute and recent infections. The model estimated a median proportion infected of 1.4% (IQR 0.07%–3.38%) (*21*,*22*). In Martin County, Florida, local dengue transmission was detected in August 2013 and resulted in 22 cases. By late September 2013, a serosurvey in the surrounding area of the reported cases estimated a total of 29 cases. Given an import in early August, the model estimates a median of 69 (IQR 11–236) cases; for September import, the estimate is 14 (IQR 3–33) cases (*22*,*30*). When index cases were introduced into each municipality in Puerto

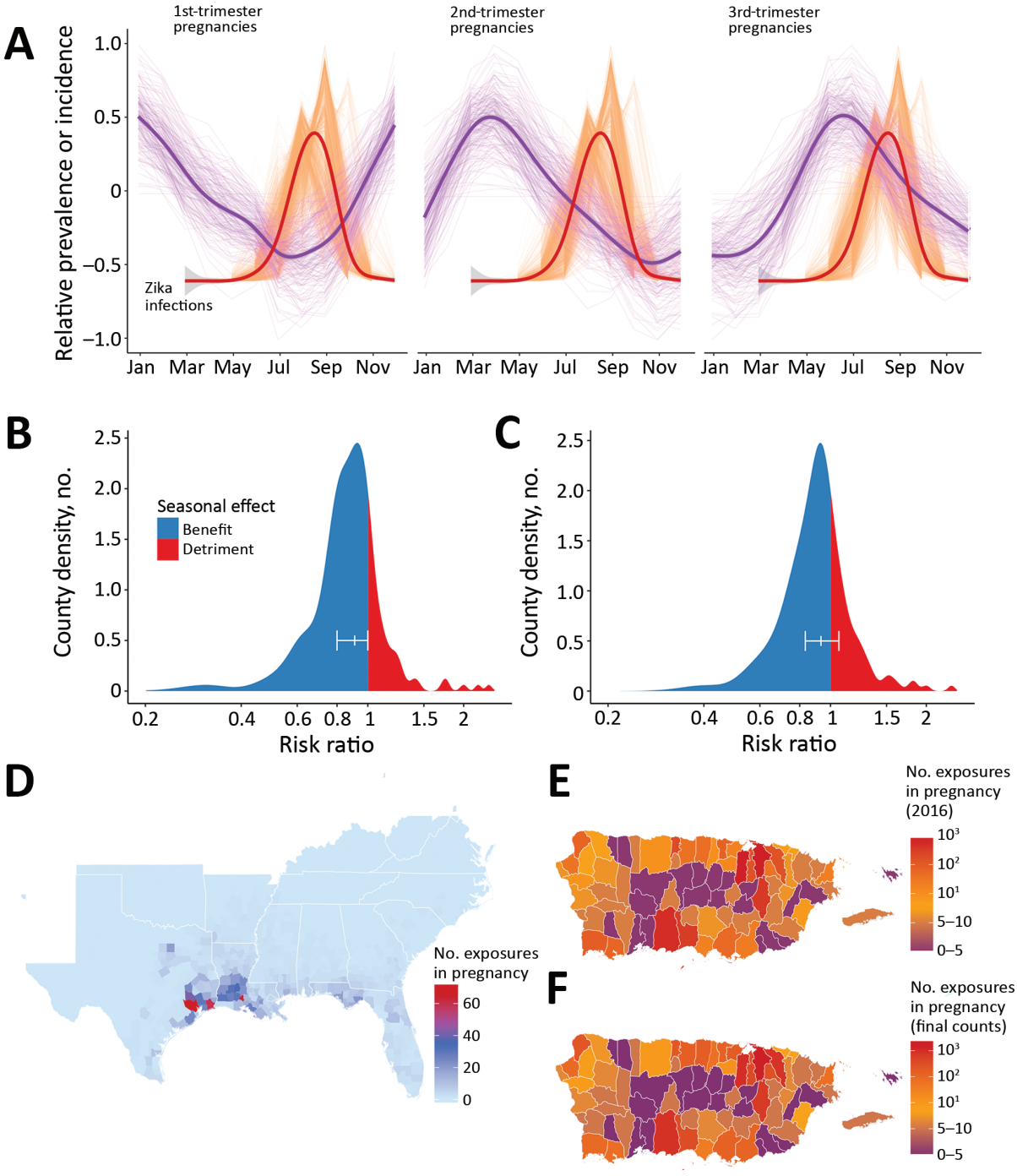

Figure 2. Zika virus infections during pregnancy and effects of natural birth dynamics, United States including Puerto Rico. A) Standardized prevalence of first-, second-, and third-trimester pregnancies throughout a year in the southeastern United States and Texas are plotted against the simulated and standardized Zika epidemic curves for each county and for every month of import. Thin purple lines indicate county-specific prevalence of pregnancy in each respective trimester, and thick purple lines show a generalized additive model fit. Thin orange lines indicate median outbreak per county, including distinct lines for each month of import during March–November. Thick orange line is a generalized additive model fit to the county-level data. B, C) Zika virus exposure risk ratio and 95% CI during (B) first (versus third) and (C) second (versus third) trimester of pregnancy, driven by the dynamics depicted in panel A (Appendix Table 2, https://wwwnc.cdc.gov/EID/article/26/4/18-1739-App1.pdf). D) Median number of infections (simulated) during pregnancy when index cases are imported during peak vector abundance. E, F) Median number of infections (simulated) during pregnancy for each municipality (E) in Puerto Rico in 2016 and (F) over the entire epidemic. Data in panels E and F include index cases that were introduced into each municipality to correspond with initial introductions reported in the current epidemic

Rico to correspond temporally with initial cases per health region reported by the Puerto Rico Ministry of Health (*6*), the model accurately predicted the monthly and cumulative case counts in Puerto Rico (325,000 infections vs. 314,209 simulated infections; Figure 4, panel C) and performed nearly as well for each of Puerto Rico's 8 health regions (Figure 4, panels D, E; Appendix Figure 15), each representing an independent validation set. Across the 10 independent sites that we were able to validate the model against, the actual (realized) incidence was within the IQR of our simulations, and usually within a single-fold difference from the median simulation.

Discussion

Overall, our model predicts interventions would be more effective at preventing additional transmission initiated by the index case than reducing the probability of an outbreak taking place. Therefore, given limited resources, a reactive approach focused on infection control rather than complete prevention might prove most beneficial. However,

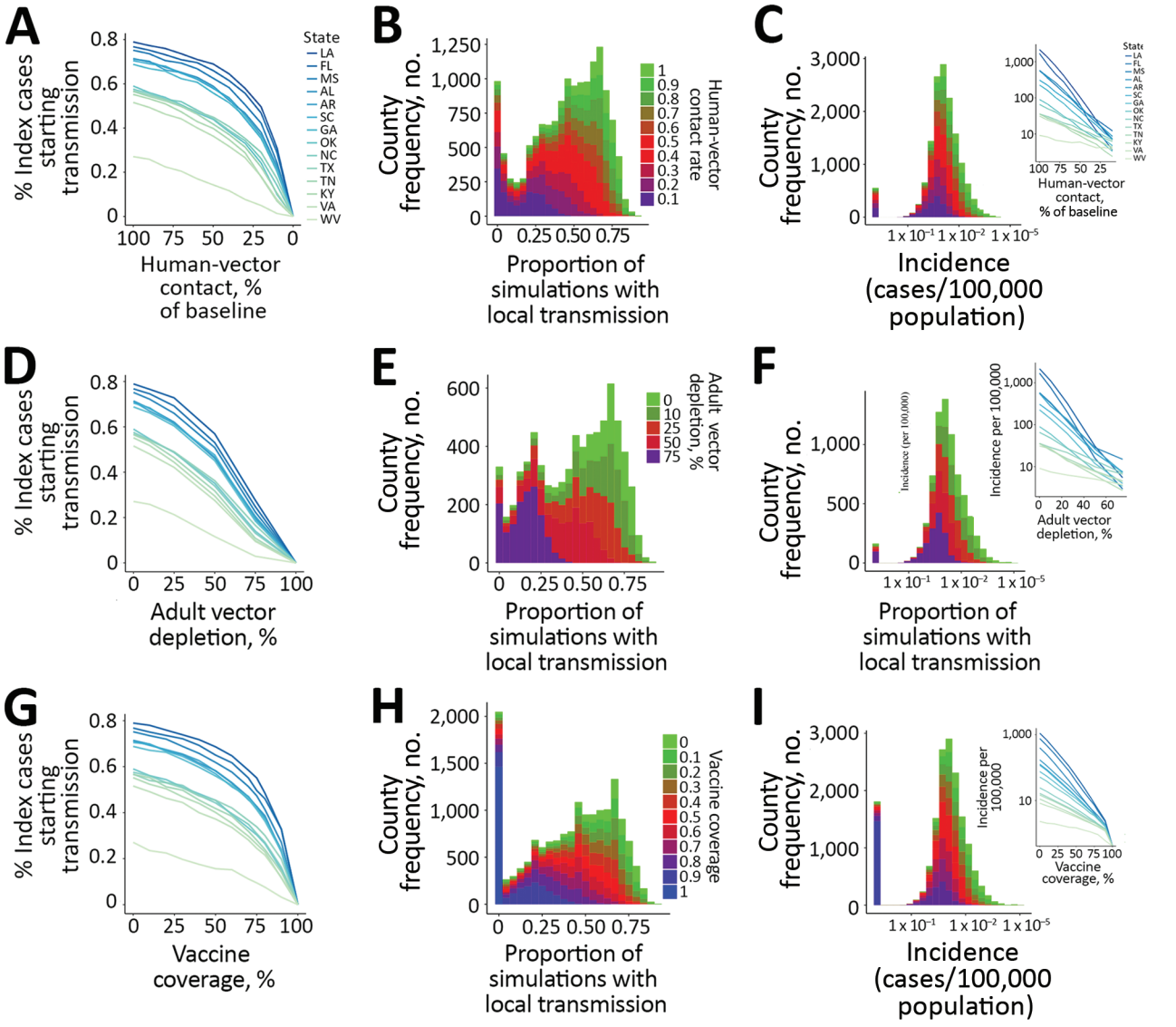

Figure 3. Zika prevention and control strategies, United States. For each county in the United States including Puerto Rico, classes of prevention or control strategies were assessed, including (A–C) reductions in human–vector contact, (D–F) adult vector depletion, and (G–I) vaccination. A, D, G) Proportion of index cases initiating ≥1 transmission event versus extent of each intervention. Each line represents the statewide average across each of the constituent county's median simulations. B, E, H) Histograms depicting number of counties versus probability of permitting ≥1 transmission event from the index case, color coded by the level of each respective intervention. C, F, I) Histogram showing the number of counties versus incidence across levels of respective intervention (color coding as in panels B, E, and H). Insets in panels C, F, and I show incidence (on a log-linear scale) versus extent of each respective interventions.

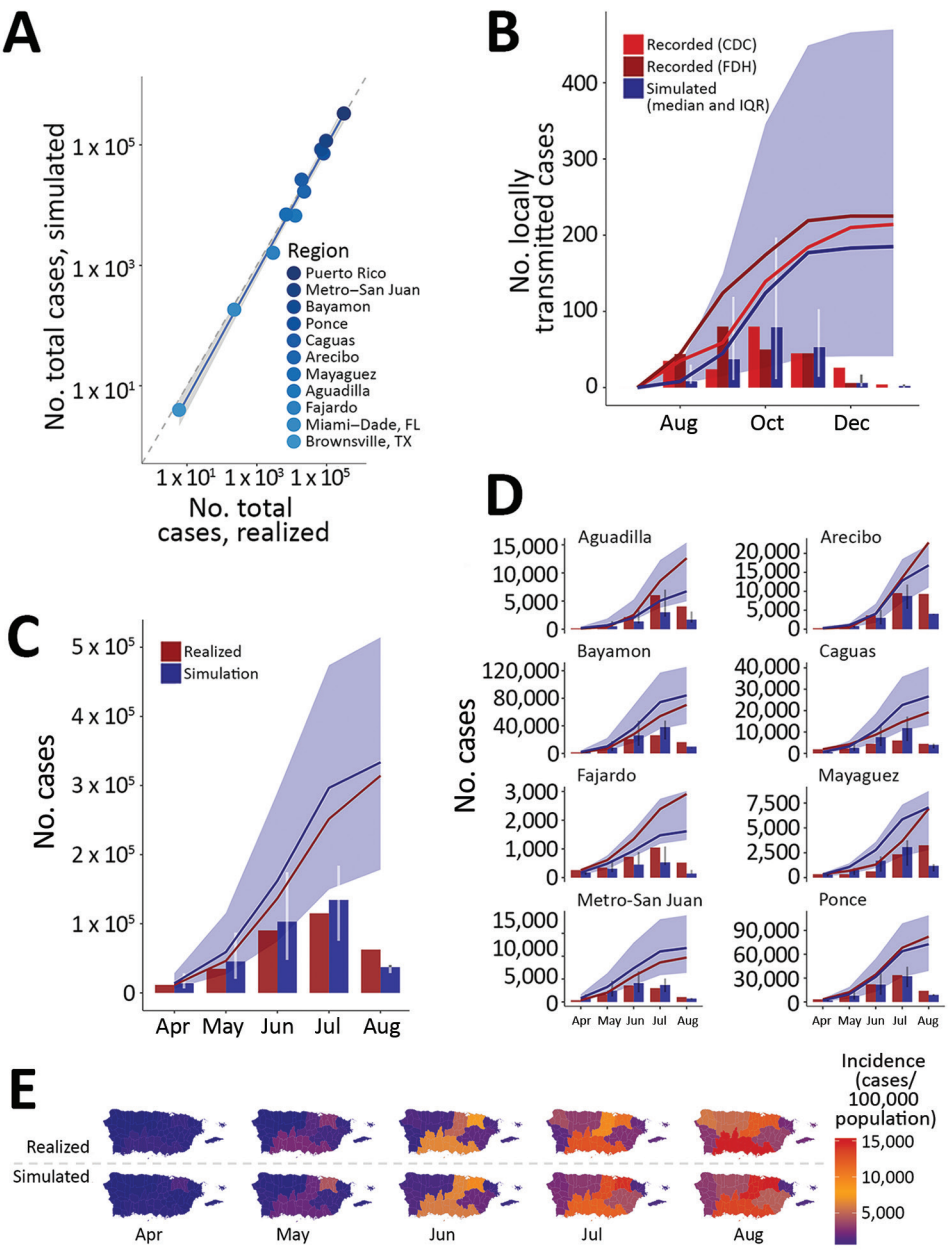

Figure 4. Model validation for Zika virus infection cases reported or estimated and simulated for outbreaks, United States including Puerto Rico, 2016. A) Total cases (median simulated) versus total cases reported or estimated (realized) for each of the regions are plotted as a scatter plot. Dotted line indicates 1:1 relationship. B–D) Monthly and cumulative simulated cases are plotted against reported or estimated cases for (B) Miami–Dade County, Florida, (C) Puerto Rico, and (D) each of the 8 health regions of Puerto Rico. Dark blue columns and line in panel B show monthly and cumulative case counts for the median simulated outbreak (among simulations with ≥1 transmission event) and shaded region shows the interquartile range. Red columns and red solid lines indicate the respective monthly and cumulative cases recorded or estimated, as noted. Data in panels C and D are as in B, but summed over the constituent municipalities (i.e., the dark blue line in panel C shows the sum of the cumulative case counts for each municipality in Puerto Rico). For panels C and D, validation data were available only for April 3–August 12, and thus realized and simulated case counts represent only cases measured or predicted within this period. E) Cumulative cases realized (upper panel) and simulated (lower panel) for each health region of Puerto Rico during April–August 2016. Validation data were available only for April 3–August 12, and thus realized and simulated case counts represent only cases measured or predicted within this period. CDC, Centers for Disease Control and Prevention; FDH, Florida Department of Health; IQR, interquartile range.

for those counties with highest overall invasion potential (Appendix Figure 16), early strategies aimed at preventing any transmission might be warranted, especially areas with high rates of potential imported cases (e.g., southeastern US cities with international airports). A vaccination coverage >70% would be most effective in preventing future outbreaks in these high-risk areas. However, pockets of unvaccinated persons are associated with elevated risk for infectious disease outbreaks (*31*). Therefore, this threshold might vary given a nonhomogeneous spatial distribution of vaccination coverage. In addition, these counties might serve as optimal US settings for Zika vaccine efficacy trials.

Zika outbreaks are likely to be highly restricted by both time and space, limited within the 50 US states almost exclusively to the summer months and the Gulf Coast region, where the *Ae. aegypti*

mosquito vector is most abundant. Although our model predicts many counties within the United States could support ≥1 transmission event from an index case, nearly all transmission outside of this region was extremely limited. Therefore, outside of the southeastern United States, a detected transmission event from an imported case will most likely represent only a sporadic occurrence, with minimal onward transmission even in the absence of local control efforts. These transmission patterns are consistent with previous local transmission patterns of other mosquitoborne flaviviruses in the United States, such as chikungunya and dengue, and with other model estimates (*9*,*32*).

Our model also shows that few municipalities in Puerto Rico sustained transmission through the first winter, with even less transmission sustained through the second and third winters. This finding suggests that sustained transmission throughout Puerto Rico requires continued case exports from municipalities with uninterrupted transmission. These findings are also consistent with CDC's reported Zika virus disease trends among travelers for 2016 and 2017, which show a decrease in the number of reported cases, from 4,205 cases in 2016 to only 331 cases in 2017 (*32*).

We also found that the natural seasonality in human births will likely serve to reduce population risk for early-trimester infections, which alone should serve to abrogate the number of fetal exposures resulting in neurologic complications. In addition, planned seasonal conception (based on birth seasonality and local Zika virus transmission data) is a viable intervention to pursue while maternal Zika virus vaccine and risk profiles, as they relate to gestational age, are being developed (*33*,*34*). Our model showed that in Puerto Rico, most fetal exposures occurred within the first year of the epidemic, suggesting that most fetal exposures have already occurred in 2016. Previous estimates by Ellington et al. (*29*) using Zika case data projected that ≈7,800 exposures in pregnancy would occur through 2016. Of note, that study anticipated an overall population incidence of 25% through 2016, whereas more recent estimates (*23*) place the actual incidence closer to 15%–20%. Thus, Ellington et al. might have overestimated actual fetal exposures by 20%–40%, which, when corrected for the updated incidence, places our model estimate well in line with theirs.

Some limitations to the proposed model might influence county level risk profiles for Zika virus transmission. For areas with a high level of travel-associated imports of Zika virus cases, such as cities with cruise ship ports and large airports, the associated risk might be an underestimate because our model does not consider multiple imports of infectious persons (*35*). In addition, certain parameters (e.g., incubation period and period of infectiousness of Zika virus infection) and transmission pathways (e.g., sexual transmission of Zika virus) are not fully understood and might contribute to elevated risk for Zika epidemics (*36*,*37*).

Zika virus transmission is expected to persist as a long-term public health challenge, and the United States remains an entirely susceptible population, with risk for transmission. As long as Zika virus circulates anywhere, the continued importation into the United States remains a potential risk. Our comprehensive profiling efforts should serve a critical need for decision making across all levels of government regarding efficient use of local, state, and national resources aimed at preventing and controlling Zika virus transmission and should provide critical information to inform future vaccination efforts.

Acknowledgments

We thank C. Jessica Metcalf and Bryan Grenfell for their critical review, comments, and suggestions for this manuscript.

Author contributions: All authors fulfill the contribution requirements as per the International Committee of Medical Journal Editors' role of authors and contributors' guidelines. All authors conceptualized and designed the study and critically reviewed and revised the manuscript. M.J.M. carried out the analyses and wrote the initial manuscript. L.B.G. extracted data and carried out analyses, K.E.A. extracted data, and M.J.M. and S.B.O. oversaw manuscript development.

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Genomic Insight into the Spread of Meropenem-Resistant *Streptococcus pneumoniae* Spain^{23F}-ST81, Taiwan

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Incidence of invasive pneumococcal disease caused by antimicrobial-resistant *Streptococcus pneumoniae* types not included in pneumococcal conjugate vaccines has increased, including a penicillin- and meropenem-resistant serotype 15A-ST63 clone in Japan. During 2013–2017, we collected 206 invasive pneumococcal isolates in Taiwan for penicillin and meropenem susceptibility testing. We found serotypes 15B/C-ST83 and 15A-ST63 were the most prevalent penicillin- and meropenem-resistant clones. A transformation study confirmed that penicillin-binding protein (PBP) 2b was the primary meropenem resistance determinant, and PBP1a was essential for high-level resistance. The rate of serotype 15B/C-ST83 increased during the study. All 15B/C-ST83 isolates showed an *ermB* macrolide resistance genotype. Prediction analysis of recombination sites revealed 12 recombination regions in 15B/C-ST83 compared with the *S. pneumoniae* Spain^{23F}-ST81 genome. Pneumococcal clones rapidly recombine to acquire survival advantages and undergo local expansion under the selective pressure exerted by vaccines and antimicrobial drugs. The spread of 15B/C-ST83 is alarming for countries with high antimicrobial pressure.

Streptococcus pneumoniae is a major human pathogen that causes bacteremia, meningitis, pneumonia, and sepsis (*1*). Although the use of pneumococcal conjugate vaccines (PCVs) has dramatically decreased incidence rates of diseases caused by vaccine-targeted serotypes in children (*2*), the rates of pneumococcal disease caused by non-PCV serotypes have risen, including as 35B in the United States; 8, 12F, and 9N in the United Kingdom; 24F in France; 12F in Israel; and 15A in Japan (*3*,*4*). Of note, 35B and 15A have high-level β-lactam resistance (*3*,*4*).

Taiwan launched a national vaccination catch-up program in 2013, in which 1 dose of 13-valent PCV (PCV13) was administered to children 24–60 months of age. In 2014, the program was expanded to include 2 doses at 12–23 months of age, and in 2015, a 2+1 national infant immunization program was implemented (*5*). Since 2015, PCV13 coverage has been >90%, and the incidence of invasive pneumococcal disease (IPD) in children ≤5 years of age was reduced by 70%, from 18.9/100,000 children during 2010–2012 to 6.3/100,000 children during 2015–2017 (*6*). Surveillance data from the Taiwan Centers for Disease Control (Taiwan CDC) demonstrated that serogroup 15 isolates caused most IPD in children ≤5 years of age during 2015–2017, which increased from 0.67/100,000 children in 2010 to 2.61/100,000 children in 2017 (*6*). In Japan, serotype 15A sequence type (ST) 63 (15A-ST63) was highly associated with resistance to penicillin (MIC >2 mg/L) and meropenem (MIC ≥0.5 mg/L) (*3*).

Meropenem is a broad-spectrum carbapenem antimicrobial drug recommended for initial empirical therapy in some countries and is indicated for treating bacterial meningitis caused by *S. pneumoniae* in children ≥3 months of age (*7*,*8*). Meropenem resistance seen in the *S. pneumoniae* 15A-ST63 clone in Japan was thought to be due to acquisition of penicillin-binding protein (PBP) 1a (type 13) via recombination with a formerly predominant global serotype 19A-ST320 vaccine strain (*3*).

Before widespread use of PCVs in Taiwan, the Pneumococcal Molecular Epidemiology Network

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DOI: https://doi.org/10.3201/eid2604.190717

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(PMEN) standardized nomenclature of prevalent multidrug-resistant international clones causing most invasive diseases, including Spain^{23F}-ST81 (PMEN1), Taiwan^{19F}-ST236 (PMEN14), Taiwan^{23F}-ST242 (PMEN15), and 19A-ST320 (*9*,*10*). These clones usually harbored PBPs that are not susceptible to penicillins, third-generation cephalosporins, and meropenem (*3*,*11*). Because *S. pneumoniae* is highly recombinogenic, continued circulation of these highly β-lactam–resistant PBP determinants is of concern in the era of PCV13. A previous study in Taiwan demonstrated that the prevalence of non-PCV13 serotypes 15A-ST63; 15B-ST83; a single locus variant of Spain^{23F}-ST81; and 23A-ST338 has increased since 2012 (*12*).

We examined the in vitro activity of penicillin and meropenem against invasive pneumococcal strains isolated in Taiwan during 2013–2017, analyzed their genetic relatedness, and assessed the role of amino acid changes in PBP1a, 2b, and 2x in meropenem resistance. In addition, we used whole-genome sequencing (WGS) to characterize the prevalent clone and its relationship to historical strains.

Materials and Methods

Hospitals and Patients

During January 2013–December 2017, we collected invasive pneumococcal isolates at a 3,700-bed branch of Chang Gung Memorial Hospital (CGMH) in Linkou, northern Taiwan, and at a 2,700-bed CGMH branch in Kaohsiung, southern Taiwan. Since 2006, CGMH has archived all pneumococcal isolates from patients with IPD, which was defined as an illness in which *S. pneumoniae* was isolated from ≥1 normally sterile site. In addition, Taiwan has an active surveillance system that made IPD a nationally notifiable disease in 2007. Therefore, doctors must report clinical information and submit pneumococcal isolates to the Taiwan CDC (*6*). This study was approved by the research ethics committee of CGMH, Taiwan (approval no. 201801433B0).

Bacterial Strains

We grew 206 nonrepetitive *S. pneumoniae* isolates at 37°C in Todd Hewitt broth supplemented with 0.5% yeast extract or on blood agar with 5% defibrinated sheep blood (Becton Dickinson, https://www.bd.com) in an atmosphere of 5% CO_2. We used standard latex agglutination and quellung reactions for serotyping and the broth microdilution method to determine MICs of meropenem and penicillin for *S. pneumoniae* isolates (*13*). For penicillin, we defined the criteria for meningitis as MIC ≤0.06 mg/L, susceptible; and MIC ≥0.12 mg/L, resistant. We defined criteria for nonmeningitis as MIC ≤2 mg/L, susceptible; MIC = 4 mg/L, intermediate; and MIC ≥8 mg/L, resistant. For meropenem, we defined MIC ≤0.25 mg/L, susceptible; MIC = 0.5 mg/L, intermediate; and MIC ≥1.0 mg/L, resistant. We used the *S. pneumoniae* MLST database (https://pubmlst.org/spneumoniae) to perform multilocus sequence typing (MLST) by PCR for the 125 meropenem-nonsusceptible isolates identified from the 206 isolates (*14*).

PBP Profiles

We performed PCR to compare the sequences of the transpeptidase regions of the *pbp1a*, *pbp2b*, and *pbp2x* genes of all 125 meropenem-nonsusceptible isolates by using 12 primers (Appendix 1 Table 1, http://wwwnc.cdc.gov/EID/article/26/4/19-0717-App1.pdf). We separated the resulting PCR products by using 1% agarose gel electrophoresis and extracted DNA for Sanger sequencing. We used BLAST 2.10.0 (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare the nucleotide sequences of each PBP transpeptidase region to the PBP sequences in the US Centers for Disease Control and Prevention database (*15*) and in previous studies (*3*,*11*,*16*,*17*).

Transformation of *S. pneumoniae*

We used the nonencapsulated laboratory strain R6, a meropenem-susceptible pneumococcus (MIC 0.015 mg/L), as the recipient in transformation studies. We used PCR to amplify the entire *pbp1a*, *pbp2b*, and *pbp2x* genes of the 15A-ST63 clinical isolates (Appendix 1 Table 1). We cloned the products into pJET1.2/blunt (ThermoFisher, https://www.thermofisher.com) according to the manufacturer's instructions and transformed it to *Escherichia coli* DH5α. We extracted the *pbp* plasmids from DH5α cells with the Plasmid DNA Mini Kit (QIAGEN, https://www.qaigen.com) and then transformed it to *S. pneumoniae*, as described previously (*18*). We spread the transformation on Mueller-Hinton agar containing 5% sheep blood and different concentrations of meropenem. After a 24-h incubation, we selected transformants from the plates containing the highest meropenem concentration with colonies.

WGS

To investigate evolutionary relationships of *S. pneumoniae*, we performed WGS on 27 isolates, including 24 meropenem-nonsusceptible isolates of serotype 15B/C-ST83 collected during 2013–2017 and 3 clinical isolates of serotype 23F-ST81 identified in our

previous study (*10*). We used a QIAamp DNA Mini Kit (QIAGEN) to extract *S. pneumoniae* genomic DNA and prepared libraries for WGS sequencing by using the TruSeq Nano DNA High Throughput Library Prep Kit (Illumina, https://www.illumina.com). We multiplexed library samples and sequenced on an Illumina MiSeq with 2×300-bp paired-end reads. We used SPAdes version 3.13.0 (*19*) to quality trim raw reads before assembling by using k-mer values 21–77 in careful mode. We used QUAST (*20*) to evaluate this assembly, which produced an average of 140 contigs with an N50 length in 101,632 bp. Because *S. pneumoniae* PMEN1 ATCC-700669 (GenBank accession no. FM211187), a serotype of the 23F-ST81 strain, is genetically related to ST83, a single locus variant of ST81, we chose this strain as a reference sequence for mapping. We deposited WGS data, ≈1.4 million pair-end reads per sample, to the Sequence Read Archive database (SRA; http://www.ncbi.nlm.nih.gov/sra) under accession nos. SRR8867342–68 (Appendix 1 Table 2).

Predicting Recombination Sites by Phylogenetic Analysis

We aligned the trimmed reads for the 27 isolates against the reference genome of ATCC-700669 by using Snippy version 4.4.5 (*21*), which uses the Burrow-Wheeler Aligner (https://github.com/lh3/bwa) to map the reads to the reference and then calls the subsequent single-nucleotide polymorphisms (SNPs) and insertions/deletions with FreeBayes per Garrison et al. (https://arxiv.org/abs/1207.3907). We used the whole-genome core SNPs alignment output from Snippy for downstream phylogenetic analysis, assessed recombination sites by using Gubbins version 2.3.4 (*22*), and generated a maximum-likelihood tree by using RAxML version 8.2.10 (https://github.com/stamatak/standard-RAxML). We ran RAxML by using the GTRCAT model, no rate heterogeneity, no ascertainment bias, rapid hill climbing, and default parameters. After 4 iterations, Gubbins reached a stable tree topology and determined regions of genetic recombination. We visualized the resulting phylogenetic tree, isolate metadata, core genome SNPs, and recombination sites by using Phandango version 1.3.0 (*23*).

Detection of Antimicrobial Resistance Genes

To detect the presence of antimicrobial resistance genes, we used BLASTN to search the assembled contigs of 27 *S. pneumoniae* against the reference sequences reported in previous studies (*3*,*24*–*26*). Our search included *ermB*, *cat*, *mef*(*A/E*), *tetO*, *tetM*, *folA*, *folP*, *rpoB*, *gyrA*, *gyrB*, *parC*, *parE*, and other Tn916-like transposon typing genes, including *aphA3*, *int*-Tn916, *xis*-Tn916, *tnpR*-Tn916, and *tnpA*-Tn916. We validated antimicrobial resistance genes and Tn916-like transposon types by using PCR (Appendix 1 Table 1).

Statistical Analysis

We compared categorical variables by using χ^2 test or 2-tailed Fisher exact test, when appropriate. We considered $p<0.05$ statistically significant. We performed analyses by using SPSS Statistics 15.0 (IBM, https://www.ibm.com).

Results

Isolate Demographics and Penicillin and Meropenem Susceptibility

We recovered 206 nonrepetitive *S. pneumoniae* isolates collected during the study: 136 from Linkou CGMH and 70 from Kaohsiung CGMH. Isolates were collected from blood (n = 178, 86.4%), cerebrospinal fluid (n = 6, 2.9%), and pleural fluid (n = 10, 4.9%). We grouped isolates from patients in the following age groups: <5 years of age, 22.8%; 5–64 years of age, 49%; and ≥65 years of age, 28.2%. Most (64.1%) isolates were from male patients. Serotype 19A (17.5%) was most common, followed by 15B/C (14.1%), 15A (9.7%), 14 (8.7%), and 19F (8.7%) (Table 1). Most (84.5%) isolates had an MIC of >0.06 mg/L for penicillin, and 5.3% had an MIC of >2 mg/L for penicillin. The MIC_{50} (MIC for 50% of the strains) for penicillin was 1 mg/L and the MIC_{90} (MIC for 90% of the strains) was 2 mg/L. Serotypes 19A (n = 36), 15B/C (n = 27), and 15A (n = 18) accounted for 46.6% of the isolates with an MIC >0.06 mg/L for penicillin. Isolates with an MIC >2 mg/L for penicillin were from serotypes 19A (n = 5), 19F (n = 4), 15B (n = 1), and 15A (n = 1).

The rate of nonsusceptibility to meropenem was 60.7%; the MIC_{50} for meropenem was 0.5 mg/L and the MIC_{90} was 1 mg/L. PCV13 serotypes had high rates of meropenem nonsusceptibility, including 88.9% of 19A, 83.3% of 19F, and 77.8% of 14, as did non-PCV13 serotypes, including 94.1% of 15B, 83.3% of 15C, and 65% of 15A (Table 1). The prevalence of the 7-valent PCV (PCV7) and PCV13 serotypes decreased, but 23-valent pneumococcal polysaccharide vaccine (PPV23) serotypes increased from 4.8% of isolates in 2013 to 24.4% in 2017 (p = 0.009; Figure 1). The prevalence of non-PCV13 serotypes 15A and 15B/C increased from 16.7% of isolates in 2013 to 37.8% in 2017 (p = 0.08; Figure 1).

Table 1. Serotype distribution and penicillin and meropenem susceptibility rates of invasive *Streptococcus pneumoniae* isolates from 2 tertiary hospitals in Taiwan, 2013–2017*†

		No. (%) isolates								
		Meropenem			Penicillin, meningitis			Penicillin, nonmeningitis		
Serotype	Isolates, no.	S	I	R	S	I	R	S	I	R
PCV7										
4	4	3 (75)	1 (25)	0	3 (75)	NC	1 (25)	4 (100)	0	0
6B	8	6 (75)	2 (25)	0	1 (12.5)	NC	7 (87.5)	8 (100)	0	0
9V	2	2 (100)	0 (0)	0	2 (100)	NC	0	2 (100)	0	0
14	18	4 (22.2)	14 (77.8)	0	1 (5.6)	NC	17 (94.4)	18 (100)	0	0
18C	0	0	0	0	0	NC	0	0	0	0
19F	18	3 (16.7)	8 (44.4)	7 (38.9)	1 (5.6)	NC	17 (94.4)	14 (77.8)	4 (22.2)	0
23F	10	5 (50)	5 (50)	0	0	NC	10 (100)	10 (100)	0	0
Subtotal	60	23 (38.3)	30 (50)	7 (11.7)	8 (13.3)	NC	52 (86.7)	56 (93.3)	4 (6.7)	0
PCV13–non-PCV7										
1	1	0	1 (100)	0	0	NC	1 (100)	1 (100)	0	0
3	15	15 (100)	0	0	9 (60)	NC	6 (40)	15 (100)	0	0
5	0	0	0	0	0	NC	0	0	0	0
6A	7	3 (42.9)	4 (57.1)	0	1 (14.3)	NC	6 (85.7)	7 (100)	0	0
7F	0	0	0	0	0	NC	0	0	0	0
19A	36	4 (11.1)	13 (36.1)	19 (52.8)	0	NC	36 (100)	31 (86.1)	5 (13.9)	0
Subtotal	59	22 (37.3)	18 (30.5)	19 (32.2)	10 (16.9)	NC	49 (83.1)	54 (91.5)	5 (8.5)	0
PPV23–non-PCV13										
8	0	0	0	0	0	NC	0	0	0	0
10A	0	0	0	0	0	NC	0	0	0	0
11A	4	2 (50)	1 (25)	1 (25)	2 (50)	NC	2 (50)	4 (100)	0	0
15B	17	1 (5.9)	8 (47.1)	8 (47.1)	0	NC	17 (100)	16 (94.1)	1 (5.9)	0
20	0	0	0	0	0	NC	0	0	0	0
22F	3	3 (100)	0	0	3 (100)	NC	0	3 (100)	0	0
33F	0	0	0	0	0	NC	0	0	0	0
Subtotal	24	6 (25)	9 (37.5)	9 (34.6)	5 (19.2)	NC	19 (79.2)	23 (95.8)	1 (3.9)	0
NVT										
6C	1	1 (100)	0	0	0	NC	1 (100)	1 (100)	0	0
7B/C	2	2 (100)	0	0	1 (50)	NC	1 (50)	2 (100)	0	0
9A/L	2	2 (100)	0	0	1 (50)	NC	1 (50)	2 (100)	0	0
11B	1	1 (100)	0	0	1 (100)	NC	0	1 (100)	0	0
11C	1	1 (100)	0	0	1 (100)	NC	0	1 (100)	0	0
12	2	0	2 (100)	0	0	NC	2 (100)	2 (100)	0	0
15A	20	7 (35)	12 (60)	1 (5)	2 (10)	NC	18 (90)	19 (95)	1 (5)	0
15C	12	2 (16.7)	8 (66.7)	2 (16.7)	2 (16.7)	NC	10 (83.3)	10 (100)	0	0
15F	1	0	1(100)	0	0	NC	1 (100)	1 (100)	0	0
18F	1	0	1 (100)	0	0	NC	1 (100)	1 (100)	0	0
19B	1	1 (100)	0	0	0	NC	1 (100)	1 (100)	0	0
23A	14	10 (71.4)	3 (21.4)	1 (7.2)	0	NC	14 (100)	14 (100)	0	0
23B	2	1 (50)	1 (50)	0	0	NC	2 (100)	2 (100)	0	0
35A/C	2	1 (50)	1 (50)	0	1 (50)	NC	1 (50)	2 (100)	0	0
35B	1	1 (100)	0	0	0	NC	1 (100)	1 (100)	0	0
Subtotal	63	30 (47.6)	29 (46)	4 (6.3)	9 (14.3)	NC	54 (85.7)	58 (98.3)	1 (1.7)	0

*I, intermediate; NC, not calculated; NVT, nonvaccine type; PCV7, 7-valent pneumococcal conjugate vaccine; PCV13, 13-valent pneumococcal conjugate vaccine; PPV23, 23-valent pneumococcal polysaccharide vaccine; R, resistant; S, susceptible.
†MICs for meropenem: S, ≤0.25; I, 0.5; R, >1; for penicillin, meningitis: S, ≤0.06; R, >0.12; and for penicillin, nonmeningitis: S, ≤2; I, 4; R, >8.

MLST and PBP Allelic Profiles of Meropenem-Nonsusceptible Pneumococcal Isolates

The mechanisms of penicillin resistance in *S. pneumoniae* have been well studied, but few data have been published on the mechanisms of meropenem resistance. We aimed to evaluate the role of PBP in meropenem resistance mechanisms.

We used MLST to evaluate the clonal relatedness of 125 meropenem-nonsusceptible isolates. Most (n = 94) isolates were from 3 major clonal complexes (CCs): CC320, including ST320, ST236, ST271, ST1464, ST7122, ST12444, and ST14357; CC81, including ST81, ST83, and ST14359; and CC63, including ST63 and ST14313 (Appendix 1 Table 3). CC320 was associated with serogroup 19, but CC81 and CC63 were associated with more diverse serotypes. We noted that the prevalence of IPD caused by serotypes 15B/C-ST83 increased from 7.1% (3/42) of isolates in 2013 to 26.7% (12/45) in 2017 (p = 0.004). Clonal expansion of 15B/C-ST83 appeared to be associated with an increase in meropenem nonsusceptibility and in the number of cases of IPD caused by serotypes 15B/C.

Among meropenem-nonsusceptible isolates, we noted 3 PBP1a, 2b, 2x allelic profiles, 13:11:16, 15:12:18, and 13:new1:43. Isolates of 19A-ST320 harbored most 13:11:16 allele profiles (n = 27); 15B/C-ST83 harbored

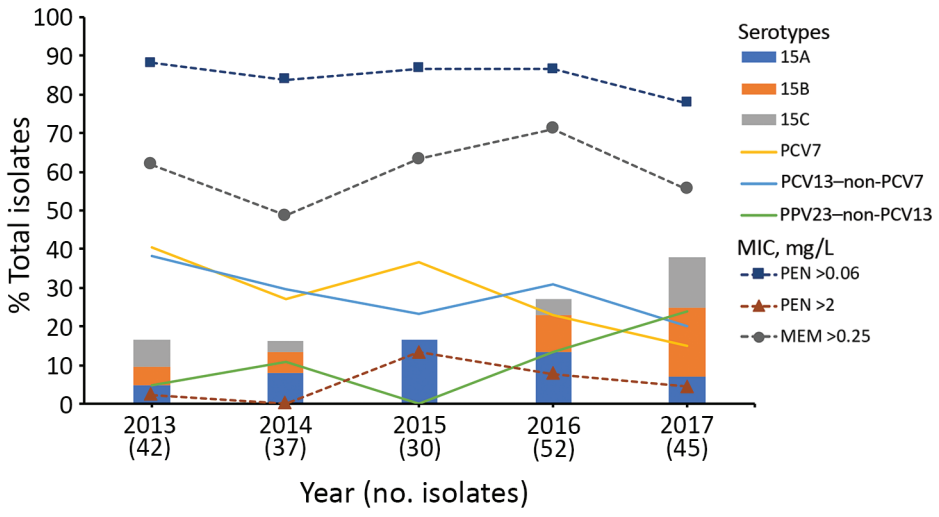

Figure 1. Serotype changes in *Streptococcus pneumoniae* strains isolated in Taiwan, 2013–2017. The prevalence of strains with an MIC >0.06 mg/L for penicillin was sustained during the study. PCV7 and PCV13–non-PCV7 serotypes decreased (p = 0.009) over time. In contrast, PPV23 serotypes and the non-PCV13 serotypes 15B/C increased (p = 0.002) and the rate of serotype 15A fluctuated. PCV7, 7-valent pneumococcal conjugate vaccine; PCV13, 13-valent pneumococcal conjugate vaccine; PPV23, 23-valent pneumococcal polysaccharide vaccine; PEN, penicillin; MEM, meropenem.

more 15:12:18 profiles (n = 23); and 15A-ST63 harbored more 13:new1:43 profiles (n = 10) (Appendix 1 Table 3).

We identified several new PBP1a, 2b, and 2x types in this study (Appendix 1 Tables 4–6) and we examined the relatedness of the amino acid (aa) sequences of PBP1a, 2b, and 2x among meropenem-nonsusceptible isolates (Appendix 1 Figure 1). The PBP2x sequences of all meropenem-nonsusceptible isolates had T338A, R384G, and D567N aa substitutions; all PBP2b sequences had the Q432A/L and T451A aa substitutions; and all PBP1a sequences had E388D, E397I/V, N405S/D, G414A/V, N443D, and D473N/S aa substitutions (Appendix 2, http://wwwnc.cdc.gov/EID/article/26/4/19-0717-App2.xlsx).

Effect of PBP Variants on the MIC of Meropenem for *S. pneumoniae* R6

The meropenem-susceptible pneumococcal strain R6 was transformed with individual entire *pbp* genes, as well as different combinations of the *pbp1a*, *pbp2b*, and *pbp2x* genes from a 15A-ST63 clinical isolate with a PBP profile of 13:new1:43 that had the highest MIC for penicillin and meropenem in our study (Table 2). No isolates of strain R6 that had *pbp2x* or *pbp1a* transformants had an increased MIC for meropenem, but when strain R6 was transformed with *pbp2b*, R6-2B transformants had an increased MIC of 0.06 mg/L for meropenem. DNA sequence analysis of the *pbp2b* gene from the R6-2B transformants confirmed that all the aa substitutions occurred in the PBP2b transpeptidase domain. Transformation of the R6-2B transformants with *pbp2x* generated R6-2B-2X transformants with an increased MIC of 0.25 mg/L for meropenem. DNA analysis of the *pbp2x* gene from the R6-2B-2X transformants revealed they possessed an additional 21 aa substitutions outside the transpeptidase domain of PBP2x. Transformation of the R6–2B-2X transformants with *pbp1a* produced R6-2B-2X-1A transformants and that had an increased MIC of 0.5 mg/L for meropenem. DNA sequence analysis of the *pbp1a* gene from the R6-2B-2X-1A transformants confirmed that the entire type 13 *pbp1a* was transformed. PBP2b and PBP2x appear to be involved in increased meropenem resistance, and PBP1a appears to be associated with breakthrough meropenem susceptibility.

Table 2. Pneumococcal transformation study results for study of invasive *Streptococcus pneumoniae* isolates from 2 tertiary hospitals in Taiwan, 2013–2017*

Recipient	Transforming DNA	Transformant name	Colony formation	MIC of transformants, mg/L		Integration of altered PBP in transformants	PBP profile 1A:2B:2X
				Meropenem	Penicillin		
R6	NA	R6-WT	ND	0.015	0.015	ND	2:0:2
15A-ST63	NA	15A-WT	ND	1	4	ND	13:new1:43
R6	*pbp1a*	R6–1A	–	ND	ND	ND	ND
R6	*pbp2b*	R6–2B	+	0.06	0.015	Yes, PBP2b	2:new1:2
R6	*pbp2x*	R6–2X	–	ND	ND	ND	ND
R6–2B transformants	*pbp2x*	R6–2B-2X	+	0.25	0.125	Yes, PBP2x	2:new1:43
R6–2B transformants	*pbp1a*	R6–2B-1A	–	ND	ND	ND	ND
R6–2B-2X transformants	*pbp1a*	R6–2B-2X-1A	+	0.5	1	Yes, PBP1a	13:new1:43

*NA, not applicable; ND, not done; PBP, penicillin binding protein; +, positive for colony formation on antimicrobial-containing agar plate; –, negative for colony formation on antimicrobial-containing agar plate.

Antimicrobial Resistance Genes and Phylogenomic Tree Analysis

All 15B/C-ST83 isolates in this study had *tetM*, *ermB*, *cat*, and *folA* mutations and *folP* insertions, but none had *ermTR*, *mef*(*A/E*), or *tetO* genes; none carried mutations in *rpoB*, *gyrA*, *gyrB*, or *parE*, but 2 had a mutation in the *parC* gene. Tn6002, carrying the *ermB*-mediated macrolide resistance gene, was detected in all 15B/C-ST83 isolates in our study (Table 3; Appendix 1 Table 7).

Using ATCC-700669 as an outgroup, we constructed a whole-genome phylogenic tree for the 24 15B/C-ST83 isolates and 3 23F-ST81 isolates (*10*). All 15B/C-ST83 isolates clustered in a clade, and the 23F-ST81 isolates clustered in another clade (Figure 2). We determined 12 recombination sites between all 15B/C-ST83 isolates, and we observed 7 of them in the 3 23F-ST81. We noted 5 specific recombination sites in all 15B/C-ST83 isolates: pneumococcal surface protein A (*pspA*) at positions 124732–124902; the capsule polysaccharide (*cps*) locus at positions 302151–329719; *spi* and *bacteriocin* at positions 362492–389805; putative DNA binding protein at positions 2094386–2095149; and choline binding protein A (*cbpA*) at positions 2171700–2171829 (Appendix 1 Table 8). We also constructed a recombination-free tree in Gubbins that demonstrates the same phylogenetic groups (Appendix 1 Figure 2).

Discussion

Widespread administration of PCVs decreases the incidence of antimicrobial drug–resistant *S. pneumoniae* because it targets serotypes that carry multiple genetic determinants of antimicrobial drug resistance that usually cause human disease (*27*). We found that previously successful clones changed their capsular types or served as a gene pool reservoir and donated genes associated with antimicrobial drug resistance,

Table 3. Antimicrobial resistance genes and Tn916-like transposon gene in 24 meropenem-nonsusceptible *Streptococcus pneumoniae* 15B/C-ST83 clinical isolates*

Isolate name, serotype	MIC, mg/L		PBP profile	Antimicrobial resistance genes								Mutations					
	PEN	MEM	1a:2b:2x	*tetM*	*tetO*	*ermB*	*ermTR*	*mef(A/E)*	Tn of Tn916-like	*cat*	CFT	*folA*†	*folP*‡	*gyrA*	*gyrB*	*parC*§	μ
2013																	
B1136, 15C	1	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
B999-49, 15B	1	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
B1300936, 15C	2	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
2014																	
1018-71,15B	2	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
1025-08, 15B	2	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
1054-32, 15C	2	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
2016																	
B1675, 15B	2	1	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
B0236, 15B	2	1	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
1148-64, 15C	2	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
1217-52, 15B	1	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
K54, 15B	2	1	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
K55, 15B	2	1	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
2017																	
B2581, 15B	2	1	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	+	
B5939, 15B	2	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	+	
B4505, 15C	2	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
B1757, 15C	2	1	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
B3127, 15C	2	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
B2404, 15B	2	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
B8812, 15B	2	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
1244-61, 15B	2	0.5	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
1267-33, 15B	2	1	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
1276-72, 15C	2	1	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
K72, 15B	2	1	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	
K110, 15B	4	1	15:12:18	+	–	+	–	–	Tn6002	+	R	+	+	–	–	–	

*CFT, ceftriaxone; MEM, meropenem; PEN, penicillin; R, resistant; +, positive; –, negative.
†I100L substitution.
‡Insertion of 1 codon between bases 168 and 201.
§S79Y substitution.

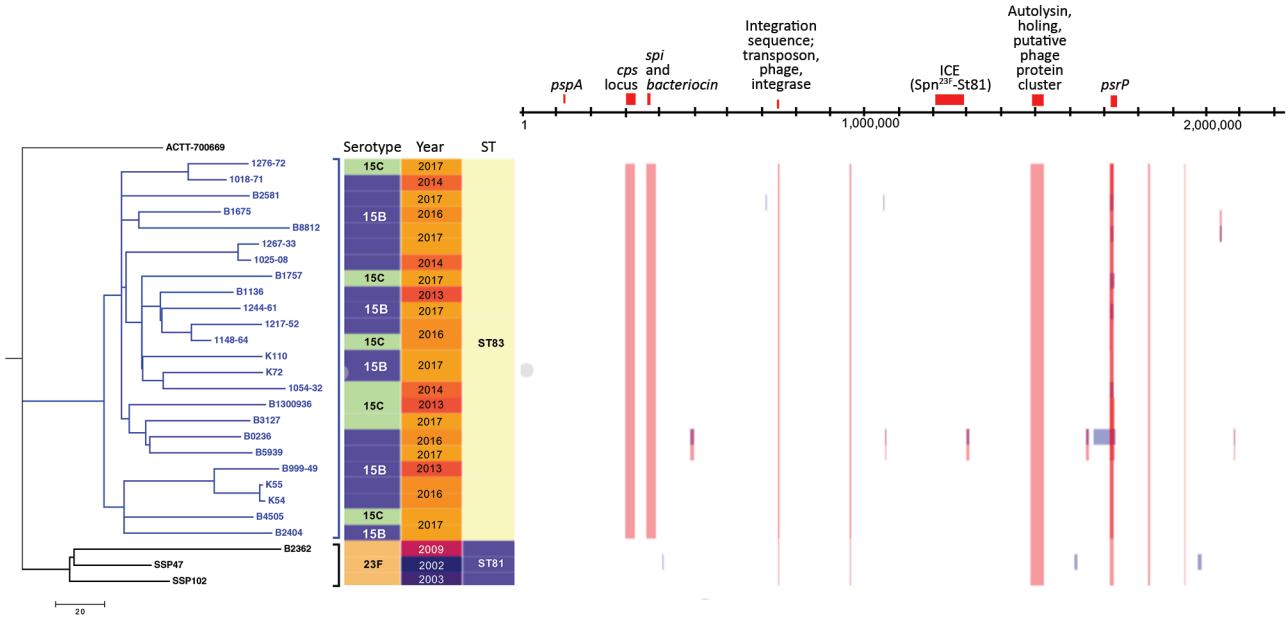

Figure 2. Phylogenic tree and recombination sites of 24 *Streptococcus pneumoniae* serotype 15B/C-ST83 isolates collected from Taiwan during 2013–2017 and 3 serotype 23F-ST81 isolates collected during an earlier study in Taiwan. Phylogenic tree compares these 27 isolates against the reference strain, *S. pneumoniae* Spain^{23F}-ST81 ATCC-700669 (GenBank accession no. FM211187). Blue text represents the 15B/C-ST83 serotype isolates from this study, which cluster together in the tree. Recombination positions are based on the 2,221,315-bp of the reference strain. Scale bar indicates nucleotide substitutions per site. ST, sequence type.

continuing their spread to other clones in the community. After universal vaccination with PCV13 in Taiwan, meropenem-nonsusceptibility was attributable to the emergence and spread of non-PCV13 serotype 15B/C-ST83 and 15A-ST63 clones, in addition to the PCV13 serotype 19A-ST320.

Of note, 15B/C-ST83 showed an increasing trend during 2013–2017. Rates of carriage and invasive disease caused by nonvaccine serotype 15A and 15B/C have increased with increased use of PCV13 (*28*). The serotype 15A-ST63 clone we detected in Taiwan was the same clone detected in Japan, which carries an identical whole PBP1a (type 13) and includes a new PBP2b associated with resistance to penicillin and meropenem. Alterations in PBP1a, PBP2b, and PBP2x are associated most often with β-lactam resistance (*29*). Limited data are available on the mechanism underlying meropenem resistance in pneumococcus. We demonstrated that PBP2b has a high affinity for meropenem and sets the susceptibility threshold of R6. Hence, transfer of the *pbp2b* gene to R6 decreased its susceptibility to meropenem, which resulted in an incremental increase in resistance after sequential introduction of the *pbp2x* and *pbp1a*. Acquisition of the PBP2b and PBP2x variants appears to be a prerequisite for creating the PBP1a variants that confer high-level resistance to meropenem, similar to the stepwise development of penicillin resistance (*30*). However, the MIC levels of resistance of the last R6-2B-2X-1A transformant for penicillin and meropenem were not as high as those of its parent strain, 15A-ST63. The results of this transformation experiment suggested that other PBP and non-PBP contributors are involved in the resistance mechanism.

In Hong Kong, ST8589 and ST199 were major clones in serotype 15B/C, and both were susceptible to β-lactam (*28*). In the United States, ST199 susceptible to β-lactam and ST3280 with low-level β-lactam resistance (MIC 0.12–1 mg/L) were major genotypes of serotype 15B/C (*11*). In Japan, most 15B/C isolates were ST199 with low-level β-lactam resistance (*31*). ST199 primarily circulated as either serotype 19A or 15B/C. After the use of PCV7 in the United States, ST199 with low-level β-lactam resistance was the most prevalent clone among 19A isolates before 2005, after which it was outcompeted by highly resistant ST320, presumably due to pressure from antimicrobial drugs (*32*).

In Taiwan, we did not find ST199 among any isolates (*12*,*33*), and serotype 15B/C-ST83 was the predominant clone. Serotype 15B/C-ST83 rarely was isolated in the United States, Japan, and Hong Kong (only 1–3 isolates among >100) (*11*,*28*,*31*), but is characterized exclusively with high-level β-lactam resistance (MIC ≥2 mg/L) and *ermB*- or *mef*(*A/E*)-mediated macrolide resistance. ST83 is a single-locus variant of

ST81 grouped into the PMEN1 lineage. PMEN1, predominantly circulating as the PCV7 vaccine serotype 23F, was one of the pandemic penicillin-resistant clones identified in Spain in the 1980s that subsequently spread worldwide (*34*). ST81 shows high rates of carriage and disease and possesses the ICE, MM1 phage, Na^+-dependent ATPase island, and TprA2/PhrA2 genomic regions, which are associated with increased colonization and virulence and led to their ecologic success and dominance (*35*,*36*). The Spain^{23F}-ST81 clone was resistant not only to tetracycline and chloramphenicol but also frequently developed resistance to fluoroquinolone, rifampin, and macrolides (*37*,*38*). In addition, members of the PMEN1 lineage frequently switched to alternative capsular types and rapid genomic evolution through recombination that occurred in response to the selective pressure exerted by vaccines and antimicrobial drugs (*39*).

We found a trend of 15B/C-ST83 clonal expansion among cases of IPD in Taiwan during 2013–2017. We also noted an increased prevalence of 15C-ST83. Reversible switching between serotypes 15B and 15C occurs during natural infection (*40*) and it is biologically plausible that the prevalence of 15C increased after 15B surged. 15B and 15C serotypes are distinguished by the presence or absence of an *O*-acetyl group attached to the capsular polysaccharide, which is ascribed to variation in the short tandem TA repeats in the *O*-acetyltransferase gene (*41*). We used WGS to confirm that the 15C isolates in our study had 7–13 tandem TA repeats and 15B isolates had 8 tandem TA repeats in the *O*-acetyltransferase gene. Of note, the functional antibodies generated against serotype 15B after administration of PPV23 have low cross-reactivity with serotype 15C (*42*).

Aside from the longest recombination fragment *cps* locus in 15B/C-ST83, we detected other recombination sites by WGS, including *pspA* and *cbpA*, which are reported to be recombination sites in the ST81 lineage (*39*,*43*), and the *spi* allele, which shifted from ST81 to ST83 and is the allele that most distinguishes the difference between ST83 and ST81. In addition, we detected the antibacterial toxin *bacteriocin*, which enables 1 strain to predominate over others and mediates intraspecies competition during nasopharyngeal colonization in human hosts (*44*). In addition, all 15B/C-ST83 isolates in our study were resistant to macrolide and harbored the Tn6002 element, via *ermB*, *tetM*, *int*-Tn916, and *xis*-Tn916. Tn6002 has been identified as the most common Tn916-like element among *ermB*-carrying strains (*45*).

Serotypes differ greatly in their carriage prevalence, mainly because of the biochemical structure of a specific capsular polysaccharide (*46*). Nonvaccine types with polysaccharides that have fewer carbons and low energy expenditure per repeat unit, such as 15A, 15B/C, and 35B, have become the next generation of colonizers after the vaccine types diminished (*46*). In this study, under the selective pressure exerted by vaccines and antimicrobial drugs, the 15A-ST63 clone from Taiwan also acquired PBP1a from 19A-ST320, similar to the 15A-ST63 clone from Japan (*4*). The meropenem-nonsusceptible 15A-ST63 clone identified in this study and a study from Japan might have been derived from the same ancestor or could have generated simultaneously by parallel evolution (*4*). The capsular switching recombination event in the ancestor, Spain^{23F}-ST81 clone, to 15B/C-ST83 continue the spread of PMEN1 lineage. Our data regarding the epidemiology of IPD aligns with carriage data (*47*) that showed that PCV reduced the overall carriage and disease, but antimicrobial resistance in pneumococcus was still high.

Although our study was performed at just 2 medical centers, the prevalent serotypes after implementation of PCV13 and the rates of β-lactam resistance we observed are consistent with national data for Taiwan (*6*). The PMEN1 lineage was not eliminated by PCV, and its spread is concerning. Rational antimicrobial drug use and continued surveillance are necessary to monitor the epidemiologic trends and protect public health.

This study was supported by the Ministry of Science and Technology (MOST), Taiwan (grant no. MOST-108-2314-B-182-020-MY3); the Chang Gung Memorial Hospital, Taiwan (grant nos. CMRPG3F1591-1592 and CMRPG3H1411-1412); and the Research Center for Emerging Viral Infections of the Featured Areas Research Center Program within the framework of the Higher Education Sprout Project supported by the Ministry of Education, Taiwan and MOST (grant no. MOST-108-3017-F-182-001).

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Isolation of Drug-Resistant *Gallibacterium anatis* from Calves with Unresponsive Bronchopneumonia, Belgium

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Gallibacterium anatis is an opportunistic pathogen, previously associated with deaths in poultry, domestic birds, and occasionally humans. We obtained *G. anatis* isolates from bronchoalveolar lavage samples of 10 calves with bronchopneumonia unresponsive to antimicrobial therapy. Collected isolates were multidrug-resistant to extensively drug-resistant, exhibiting resistance against 5–7 classes of antimicrobial drugs. Whole-genome sequencing revealed 24 different antimicrobial-resistance determinants, including genes not previously described in the *Gallibacterium* genus or even the *Pasteurellaceae* family, such as *aad*A23, bla_{CARB-8}, *tet*(Y), and *qnr*D1. Some resistance genes were closely linked in resistance gene cassettes with either transposases in close proximity or situated on putative mobile elements or predicted plasmids. Single-nucleotide polymorphism genotyping revealed large genetic variation between the *G. anatis* isolates, including isolates retrieved from the same farm. *G. anatis* might play a hitherto unrecognized role as a respiratory pathogen and resistance gene reservoir in cattle and has unknown zoonotic potential.

Infectious bronchopneumonia has a major economic impact, causing high morbidity and mortality rates in cattle production systems worldwide (*1*). Furthermore, it is the main indication for antimicrobial use in calves and youngstock (*2*), often resulting in acquired antimicrobial resistance (AMR) among bovine respiratory pathogens (*3*). Bacterial pathogens commonly involved in bronchopneumonia in cattle are *Histophilus somni*, *Mannheimia haemolytica*, *Mycoplasma bovis*, and *Pasteurella multocida* (*4*).

Gallibacterium anatis, a gram-negative coccobacillus within the family *Pasteurellaceae*, is historically considered an opportunistic pathogen of intensively reared poultry and domestic birds, where it is mainly isolated from the upper respiratory and lower genital tracts (*5*). *G. anatis* has emerged as a multidrug-resistant pathogen in poultry, mainly causing salpingitis (*6*), resulting in decreased egg production and increased mortality rates (*7*) but also peritonitis (*8*), epididymitis (*6*), and respiratory tract lesions (*9*). In humans, *G. anatis* has been occasionally associated with chronic bronchitis (*10*), lung abscesses (*11*), bacteremia, and death (*12*).

G. anatis has rarely been isolated in Belgium, from bovine feces (*13*) or from unknown sources (*13*,*14*), but has not, to the authors' knowledge, been reported from nasopharyngeal and tracheal bacterial communities of healthy cattle or cattle with bacterial bronchopneumonia (*15*). Therefore, whether *G. anatis* plays a role in the bovine respiratory disease complex as a facultative pathogenic bacterium remains unclear. Our study reports the detection of multiple independent *G. anatis* isolates from cattle with unresponsive infectious bronchopneumonia; our findings are supported by whole-genome sequencing (WGS) to characterize AMR and genetic relatedness.

Materials and Methods

Animal Sampling

We retrieved *G. anatis* isolates during a 2-year period (2017–2018) from 10 calves from 7 unrelated farms in Belgium; all 10 calves had a history of respiratory problems (≈5% of the total amount of samples). No poultry was present at these farms; however, at farm 2 (Table 1), raw eggs were occasionally fed to the calves. We obtained all isolates from animals 4–60 days old (Table 1) exhibiting signs of infectious bronchopneumonia, such as fever (>39.3°C), cough, nasal discharge, depression, and adventitious lung

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DOI: https://doi.org/10.3201/eid2604.190962

Table 1. Origin and characteristics of *Gallibacterium anatis* strains isolated from calves with unresponsive bronchopneumonia, Belgium, 2017–2018*

Isolate	Age of calf, d	Type (breed)	Farm	Culture	Other pathogens detected	MALDI-TOF MS log score†
GB2	36	Beef (BWB)	1	Pure culture	ND	2.40
GB3	20	Beef (BWB)	2	Dominant isolate	*Escherichia coli*	2.13
GB4	14	Beef (BWB)	2	Pure culture	ND	2.48
GB5	15	Beef (BWB)	2	Pure culture	ND	2.46
GB6	18	Beef (BWB)	2	Dominant isolate	*Histophilus somni*	2.47
GB7	60	Beef (BWB)	3	Dominant isolate	*Bibersteinia trehalosi*, *Mycoplasma bovis*	2.34
GB8	22	Beef (BWB)	4	Dominant isolate	*Trueperella pyogenes*	2.38
GB9	40	Beef (BWB)	5	Pure culture	ND	2.38
GB10	23	Beef (Blonde d'Aquitaine)	6	Dominant isolate	*Mannheimia haemolytica*, *M. bovis*	2.23
GB11	4	Dairy (Holstein Friesian)	7	Pure culture	ND	2.24

*BWB, Belgian White and Blue; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ND, not detected.
†Identification with a log score value >2.0 is considered reliable at the species level.

sounds. Before the sampling, each calf had already been treated unsuccessfully with first- or second-line antimicrobial drugs. Thoracic ultrasound examination, performed with a 7.5-MHz linear probe as described previously (*16*), showed a consolidated zone in the lung of ≥1 cm^3 in all animals. A nonendoscopic bronchoalveolar lavage (nBAL) was conducted in all cases, as described previously (*17*). The sampling method was approved by the ethics committee of the Faculty of Veterinary Medicine, Ghent University (approval no. EC 2016/20).

Identification

We inoculated all nBAL samples on an Oxoid Columbia blood agar enriched with 5% sheep blood (http://www.oxoid.com) and on a BD Difco modified pleuropneumonia-like organism agar plate (https://www.bd.com) containing 832,000 IU/L polymyxin, 0.36 g/L ampicillin, 23.1% deactivated horse serum, and 6.5% yeast extract for the isolation of *Mycoplasma* spp. We incubated blood agar plates overnight and pleuropneumonia-like organism agars for 5 days, both at 35°C and in a 5% CO_2 enriched atmosphere. We identified bacterial colonies, grown on both agars, with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry by using the direct transfer method and α–cyano–4–hydroxycinnamic acid as matrix, according to the manufacturer's guidelines. We considered identifications with a log score value >2.0 to be reliable at the species level. We subcultured *G. anatis* isolates on Columbia blood agar enriched with 5% sheep blood (Oxoid) to obtain a pure culture, which we stored at −80°C for further analysis.

Antimicrobial-Susceptibility Testing

For susceptibility testing, we performed the broth microdilution technique for ampicillin, ceftiofur, doxycycline, enrofloxacin, florfenicol, gentamicin, kanamycin, penicillin, spectinomycin, tetracycline, tilmicosin, trimethoprim/sulfamethoxazole, tulathromycin, and tylosin, according to Clinical and Laboratory Standards Institute standards (*18*,*19*). Concentrations of all antimicrobial drugs ranged from ≤0.03 to >128 μg/mL. We performed susceptibility testing of amoxicillin/clavulanic acid by using the gradient strip test. We used *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 as quality-control strains. In addition, we included *E. coli* ATCC 35218 as the quality-control strain for amoxicillin/clavulanic acid testing. We used ampicillin, tetracycline, enrofloxacin, tylosin, florfenicol, spectinomycin, and trimethoprim/sulfamethoxazole as class representatives of the penicillins, tetracyclines, fluoroquinolones, macrolides, phenicols, aminocyclitol/aminoglycosides, and potentiated sulphonamides, respectively, to determine phenotypic resistance for these classes, using Clinical and Laboratory Standards Institute breakpoints for *G. anatis* (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/26/4/19-0962-App1.pdf) (*18*).

Whole-Genome Sequencing

We prepared genomic DNA by using the Bioline Isolate II Genomic DNA kit (Meridian Bioscience, https://www.meridianbioscience.com), following the manufacturer's instructions. We constructed sequencing libraries by using the Illumina Nextera XT DNA sample preparation kit and then sequenced isolates using the MiSeq Reagent v3 kit with a 250-bp paired-end protocol (Illumina, https://www.illumina.com) according to the manufacturer's instructions. We have deposited all generated WGS data in the National Center for Biotechnology Information Sequence Read Archive (*20*) under accession number PRJNA541488. We cleaned and assembled raw reads (Appendix Table 2) and used Kraken 0.10.5 (*21*) to perform k-mer–based classification of cleaned reads against an in-house dump of the complete genomes from the National Center for Biotechnology Information RefSeq Microbial Genomes Database (*22*). We

analyzed paired-end reads and orphaned reads (i.e., reads where only 1 read of the pair survived cleaning) separately by using default settings and then combining the results by concatenating the output files.

Antimicrobial-Resistance Genotyping

We performed genotypic resistance gene detection, as described by Bogaerts et al. (*23*), against the ResFinder database (*24*). We defined AMR gene clusters as resistance genes on the same contig within a sample. We performed detection of mutations linked with increased fluoroquinolone MICs in the quinolone-resistance determining regions of *gyrA* and *parC* by aligning these regions in the *E. coli* K12 reference genome in NCBI (accession no. NC_000913.3) for *gyrA* (accession no. NP_416734) and *parC* (accession no. NP_417491.1) by using the Needle tool for pairwise sequence alignment of the EMBOSS suite (https://www.ebi.ac.uk/tools/psa) (*25*). We used mlplasmids 1.0.0 (https://sarredondo.shinyapps.io/mlplasmids) to predict whether assembled contigs were either plasmid- or chromosome-derived, by using *E. coli* as species model and 1,000 bp as the minimum sequence length (*26*). We then compared contigs predicted to be plasmid-encoded by using blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi), with default settings, against the nucleotide database. We performed transposase detection by using ISFinder (https://www-is.biotoul.fr/index.php) with the blastn tool, using default settings (*27*), to substantiate the presence of transposable elements in close proximity to the AMR gene clusters in the specific contigs of the whole assembly. Last, we used ICEberg 2.0 (http://db-mml.sjtu.edu.cn/ICEberg), with default settings, to detect integrative and conjugative elements (ICEs) or integrative and mobilizable elements (IMEs) in the *G. anatis* assemblies (*28*).

Sample Relatedness

For multilocus sequence typing (MLST), we used an in-house copy of the MLST database for *G. anatis* hosted by the PubMLST platform (http://pubMLST.org/anatis) (*29*), which we pulled in-house using the REST API (*30*), for MLST genotyping. We typed individual loci separately by aligning the assembly for each sample against all allele sequences of that locus by using nucleotide BLAST+ 2.6.0, with default values (*31*). We then performed filtering and best hit identification, as described previously, for AMR gene characterization. Because MLST offered limited resolution in the relationship between samples, we used a single-nucleotide polymorphism (SNP) genotyping approach based on an in-house implementation of the CSI Phylogeny workflow (https://omictools.com/csi-phylogeny-tool) (Appendix Table 3) (*32*), using the NCBI RefSeq entry for *G. anatis* (accession no. NC_015460) as reference to compare diversity among samples. We used MEGA-Computing Core 10.0.4 (https://www.megasoftware.net) to detect the best evolutionary model and construct a maximum-likelihood phylogenetic tree on the basis of the SNP matrix, setting the following options: "missing-data" set to "partial_deletion," "site-cov-cutoff" set to 50, "branch-swap" set to "very_weak," "ml-method" set to "spr3," "action" set to "model," and "bootstraps" set to 100. We then repeated the same workflow by using the genome assembly of isolate GB8 (Appendix Table 3), filtered on contigs ≥1,000 bases with a k-mer coverage of 10–50× as reference. We visualized the resulting phylogenetic trees by using iTOL (*33*) and, afterward, a midpoint rooting. In addition, we constructed a core genome MLST (cgMLST) scheme to investigate the relationship of the isolates in Belgium compared with all genomes for this species publicly available in the NCBI database (Appendix Table 4).

Results

Identification

We compiled all strain origin information and co-infection data (Table 1). The *G. anatis* isolates were all nonhemolytic and were recovered as a pure culture (50% of cases) or the predominant isolate in large numbers (50% of cases). When a dominant culture was obtained, other pathogens were detected to a lesser extent. All calves recovered from the pneumonia because of appropriate antimicrobial therapy, except 1 who was euthanized because of cardiac failure.

Antimicrobial Susceptibility Testing

We observed high MIC values for tylosin, tetracycline, spectinomycin, kanamycin, and enrofloxacin for all isolates, which most likely explains therapeutic failure (Table 2; Appendix Table 1). All isolates exhibited very low MIC values for ceftiofur and amoxicillin/clavulanic acid.

Whole-Genome Sequencing

The number of raw paired-end reads, genome assembly length, N50 (a metric used as a proxy for assembly quality that was defined as the length at which contigs of equal or longer length contained ≥50% of the assembled sequence), and number of contigs ≥1,000 bases was in the same range for all samples, with a median of 372,623 raw paired-end reads, median assembly length of 2,483,037 bases, median N50 value of

Table 2. Overview of phenotypic and genotypic resistance determinants of all investigated bovine *Gallibacterium anatis* isolates, Belgium, 2017–2018*

Isolate	Antimicrobial classes with phenotypic resistance	Identified genotypic resistance determinants
GB2	Macrolides, potentiated sulphonamides, tetracyclines, phenicols, aminoglycosides, fluoroquinolones	*ermB*, *sul2*, *tetM*, *catA1*, *catA3*, *floR*, *aadA1*, *aadB*, *aphA1*, *strA*, *strB*, *gyrA* 83S→Y, *gyrA* 87D→A, *parC* 80S→I
GB3	Penicillins, macrolides, tetracyclines, phenicols, aminoglycosides, fluoroquinolones	bla_{CARB-8}, bla_{TEM-2}, *ermB*, *sul1*, *sul2*, *tetB*, *tetM*, *tetY*, *floR*, *aadA1*, *aadB*, *aphA1*, *strA*, *strB*, *gyrA* 83S→Y, *gyrA* 87D→A, *parC* 80S→I
GB4	Penicillins, macrolides, potentiated sulphonamides, tetracyclines, phenicols, aminoglycosides, fluoroquinolones	bla_{TEM-2}, *ermB*, *dfrA1*, *sul2*, *tetB*, *tetM*,*catA1*, *aac(6")-aph(2")-1*, *aadA1*, *aph(3')-III*, *strA*, *gyrA* 83S→Y, *gyrA* 87D→A, *parC* 80S→I
GB5	Macrolides, potentiated sulphonamides, tetracyclines, aminoglycosides, fluoroquinolones	*ermB*, *dfrA1*, *sul2*, *tetB*, *tetM*, *catA1*, *floR*, *aadA1*, *aadB*, *aphA1*, *strA*, *gyrA* 83S→Y, *gyrA* 87D→A, *parC* 80S→I
GB6	Penicillins, macrolides, potentiated sulphonamides, tetracyclines, phenicols, aminoglycosides, fluoroquinolones	bla_{CARB-8}, bla_{TEM-2}, *ermB*, *dfrA1*, *sul1*, *sul2*, *tetB*, *tetM*, *tetY*, *floR*, *aadA1*, *aphA1*, *strA*, *strB*, *gyrA* 83S→F, *gyrA* 87D→G, *parC* 80S→I
GB7	Penicillins, macrolides, potentiated sulphonamides, tetracyclines, aminoglycosides, fluoroquinolones	bla_{TEM-2}, *ermB*, *sul2*, *tetB*, *tetM*, *catA1*, *catA3*, *aadA1*, *aadB*, *aphA1*, *strA*, *strB*, *gyrA* 83S→F, *gyrA* 87D→G, *parC* 80S→I
GB8	Penicillins, macrolides, potentiated sulphonamides, tetracyclines, aminoglycosides, fluoroquinolones	bla_{TEM-2}, *ermB*, *mphE*, *mrsE*, *dfrA1*, *sul2*, *tetB*, t*etM*, *catA1*, *catA3*, *aadA23*, *aadB*,*aphA1*, *strA*, *gyrA* 83S→F, *gyrA* 87D→A, *parC* 80S→I
GB9	Penicillins, macrolides, potentiated sulphonamides, tetracyclines, aminoglycosides, fluoroquinolones	bla_{TEM-2}, *ermB*, *dfrA1*, *sul2*, *tetB*, *tetM*, *catA1*, *aac(6')-aph(2")-1*, *aadA1*, *aph(3')-III*, *strA*, *gyrA* 83S→Y, *gyrA* 87D→A, *parC* 80S→I
GB10	Penicillins, macrolides, potentiated sulphonamides, tetracyclines, phenicols, aminoglycosides, fluoroquinolones	*ermB*, *sul2*, *tetB*, *tetM*, *catA1*, *floR*, *aadA1*, *aadB*, *aphA1*, *strA*, *qnrD1*, *gyrA* 83S→Y, *gyrA* 87D→A, *parC* 80S→I
GB11	Penicillins, macrolides, potentiated sulphonamides, tetracyclines, aminoglycosides, fluoroquinolones	bla_{TEM-2}, *ermB*, *dfrA1*, *sul2*, *tetB*, *tetM*, *catA1*, *aac(6')-aph(2")*, *aadA1*, *aph(3')-III*, *strA*, *gyrA* 83S→Y, *gyrA* 87D→A, *parC* 80S→I

*Current Clinical and Laboratory Standards Institute breakpoints for *G. anatis* were used to define susceptibility. Identified resistance genes are listed with their name as present in the ResFinder database. For *gyrA* and *parC*, the resulting amino acid changes at positions 83 and 87 (*gyrA*) and 80 (*parC*) are also indicated.

105,124 bases, and median of 58 contigs ≥1,000 bases across all samples (Appendix Table 2). Genome assembly sizes were close to the expected size of ≈2.69 Mb (*34*), indicating high quality of the WGS run. K-mer–based classification of read content for all isolates confirmed the samples to be *G. anatis*, given that this was the only species identified in the sample having a 5% read cutoff.

AMR Genotyping

By using the ResFinder database, we detected various AMR determinants in the WGS data for all isolates (Table 2). In total, we detected 24 different resistance genes across all 10 isolates, and several genes were present in multiple isolates. We found all isolates harbored resistance genes targeting aminoglycosides, phenicols, macrolides, sulphonamides, and tetracyclines. Seven isolates also harbored resistance genes such as bla_{CARB-8} or bla_{TEM-2} targeting β-lactamase–susceptible penicillins. Six isolates contained *dfr*A1, conferring resistance against trimethoprim. Isolate GB10 carried *qnr*D1, a plasmid-mediated quinolone resistance determinant. We found mutations linked with increased fluoroquinolone MICs in the quinolone resistance determining region of *gyrA* and *parC* (*35*) in all isolates, including a single-point mutation in *parC* (Ser-80 to Ile) and 2 mutations in *gyrA* resulting in S-83 to Y or F, and D-87 to A or G, changes. We determined the genotype to phenotype correspondence to be 90% (phenotypic observations might be explained by genotypic detection of corresponding resistance genes). In GB10, we found very high MIC values for penicillin/ampicillin and no corresponding resistance gene. We did find resistance genes without corresponding high MIC values for potentiated sulphonamides in isolate GB3 and for phenicols in isolates GB5, GB7, GB8, GB9, and GB11.

Some resistance genes were closely linked into resistance gene cassettes (Table 3). Overall, we observed a high diversity of resistance genes, both in determinants present in resistance gene clusters and in separate contigs. We detected gene clusters with 3–4 of the same resistance genes found in GB4, GB9, and GB11, and 2 identical resistance genes in GB3 and GB6 (Table 3). In 19 of 20 clusters, we observed a link with transposases in close proximity or localization on putative predicted IMEs, plasmids, or both (Table 3). In addition, we detected a type 4 secretion system not associated with a resistance gene cluster in GB2, GB5, GB7, and GB10 (data not shown).

Table 3. Overview of clustered AMR genes in bovine *Gallibacterium anatis* isolates, Belgium, 2017–2018*

Isolate(s)	Clustered AMR genes†	Linked transposases or IME‡	Predicted contig origin§
GB4, GB9, GB11	*aac6-aph2*, *aph3-III*, *ermB*	Putative IME	Chromosome (0.968–0.971)
GB7	*aadA1*, *aadB*, *catA1*	TnAs3 transposase *A. salmonicida*	Chromosome (0.988)
GB2	*aadA1*, *aadB*, *catA1*, *ermB*, *tetM*	TnAs3 transposase *A. salmonicida*	Chromosome (0.965)
GB3	*aadA1*, *aadB*, *sul1*, *tetM*	TnAs3 transposase *A. salmonicida*	Chromosome (0.98)
GB5	*aadA1*, *catA1*, *dfrA1*, *ermB*, *tetM*	TnAs3 transposase *A. salmonicida*	Chromosome (0.979)
GB4, GB9, GB11	*aadA1*, *catA1*, *dfrA1*, *tetM*	TnAs3 transposase *A. salmonicida*	Chromosome (0.99)
GB10	*aadA1*, *catA1*, *ermB*, *tetM*	TnAs3 transposase *A. salmonicida*	Chromosome (0.977)
GB6	*aadA1*, *dfrA1*, *ermB*, *floR*, *sul1*, *tetM*	TnAs3 transposase *A. salmonicida*	Chromosome (0.986)
GB8	*aadA23*, *catA1*, *dfrA1*, *ermB*, *tetM*	TnAs3 transposase *A. salmonicida*	Chromosome (0.957)
GB8	*aadB*, *aphA1*	Truncated IS6 family transposase	Chromosome (0.848)
GB5	*aadB*, *floR*	IS6 family transposase	Plasmid (0.694); *B. trehalosi* pCCK13698 (75%–99%)
GB7	*aphA1*, *catA3*, *strA*, *strB*, *sul2*	ISapl1 transposase *A. pleuropneumoniae*	Chromosome (0.988)
GB2	*aphA1*, *catA3*, *strA*, *strB*, *sul2*	Truncated IS4 family transposase	Plasmid (0.749); uncultured *Eubacterium* pIE1130 (84%, 99%)
GB10	*aphA1*, *floR*, *strA*, *tetB*	ISVsa3 transposase *V. salmonicida*	Plasmid (0.807); *B. trehalosi* USDA-ARS-USMARC-192 (68%, 99%)
GB3, GB6	*aphA1*, *sul2*	Truncated ISVsa3 transposase *V. salmonicida*	Plasmid (0.898); *P. multocida* USDA-ARS-USMARC-60675 (83%, 99%)
GB4, GB9, GB11	bla_{TEM-2}, *strA*, *sul2*, *tetB*	Tn3 transposase *Salmonella*	Plasmid (0.864–0.895); *S. sonnei* p866 (83%, 99%)
GB3, GB6	bla_{TEM-2}, *tetB*	Tn3 transposase *Salmonella*	Chromosome (0.976)
GB7	bla_{TEM-2}, *tetB*	Tn3 transposase *Salmonella*	Plasmid (0.708); *Salmonella* Heidelberg pN13–01290_23 (100%, 99%)
GB8	*catA3*, *mphE*, *msrE*, *strA*, *sul2*, *tetB*	Truncated ISVsa5 transposase *V. salmonicida*	Plasmid (0.738); *P. multocida* 14424 (71%, 99%)
GB5	*strA*, *tetB*	Not detected	Chromosome (0.526)

*Includes predicted transposases in close proximity of the resistance gene clusters (or predicted IME containing the AMR gene cluster) and the predicated contig origin. AMR, antimicrobial resistance; IME, integrative mobilizable elements.
†AMR genes present on the same contig (genes are listed in alphabetical order).
‡Determined by using ISfinder for transposases and ICEberg for IME.
§Determined by using mlplasmids. Values in parentheses indicate (range of) posterior probability of belonging to either a plasmid or chromosome. For predicted plasmids, the best hit in the National Center for Biotechnology Information nucleotide database is also listed, with its corresponding query coverage and percentage identity, respectively, in parentheses.

Sample Relatedness

To evaluate the relationship between isolates, we performed MLST by using the public *G. anatis* database hosted by the PubMLST platform. However, an exact allelic match could only be identified for 1 locus in GB2, 2 loci in GB3, 2 loci in GB4, 3 loci in GB 5, 2 loci in GB6, 1 locus in GB7, 1 locus in GB8, 2 loci in GB9, 1 locus in GB10, and 2 loci in GB11 (in a total of 8 loci in the scheme). Reliable allele calling for the remaining loci was not possible because of mismatches and different lengths for all samples. Closer inspection revealed that the MLST database only contained 89 isolates corresponding with 81 profiles, suggesting that MLST failed because of the lack of an available background to compare against.

Because MLST was not appropriate for delineating relationships, we performed SNP genotyping by using the NCBI RefSeq reference for *G. anatis* (UMN179). We found 14,583–15,234 SNPs for all samples (Appendix Table 3), resulting in a total SNP matrix of 32,104 positions, indicating large diversity between samples. We repeated the workflow by using the assembly of GB8 (which had the highest original read mapping rate) as a reference; this step ensured that the number of SNPs was not erroneously inflated by taking a reference not suited for SNP genotyping (i.e., a reference too divergent from the actual samples). We found 8,978–11,137 SNPs for all samples (Appendix Table 3), resulting in a total SNP matrix of 25,166 positions, confirming the large genetic diversity among samples. Afterward, we performed model selection and phylogenetic tree reconstruction with MEGA, identifying the general time reversible model as the best fit for both references.

We used GB8 as reference for 1 phylogentic tree (Figure 1) and *G. anatis* UMN179 as reference for another (Appendix Figure). Although branch lengths differed, their underlying topology was identical and well supported by high bootstrap values, indicating that, although some isolates clustered together with fewer differences (GB10 with GB2, GB4 with GB9 and GB11, GB3 with GB6), overall we observed large variation between the different isolates. Notably, for the 4 isolates GB3, GB4, GB5, and GB6 obtained from the same farm (Table 2), only GB3 and GB6 clustered together, whereas GB4 and GB5 were located elsewhere in the phylogeny.

Figure 1. Phylogeny of *Gallibacterium anatis* isolates from cattle in Belgium, 2017–2018, based on single-nucleotide polymorphism genotyping when using GB8 as a reference. Node labels indicate bootstrap support values (expressed as decimals). Branch lengths and the scale bar are expressed as average substitutions per site. The resistance genes detected in each sample are listed to the right according to the legend displayed on top.

We also constructed a cgMLST scheme on the basis of our mining all publicly available *G. anatis* genomes from NCBI, including in total 27 isolates from poultry, complemented with the strains from Belgium (Figure 2). Despite the existence of generally very large distances between all samples, the resulting topology indicated that the strains isolated from cattle in Belgium clustered together and were distinctly separated from all other strains isolated from poultry. Moreover, the subtopology of the isolates from Belgium was concordant with results from the SNP analysis.

Discussion

Our report illustrates the involvement of *G. anatis* in respiratory disease in cattle. Interestingly, isolation of *G. anatis* from cattle was only described for feces (*13*) or was of unknown origin (*13*,*14*). Also, recent microbiome studies on the nasopharyngeal and tracheal bacterial communities of feedlot cattle did not document the presence of *G. anatis* (*15*). The presence of the bacterium in cattle might have been underestimated in the past, and availability of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry might have improved detection rates for *G. anatis*, as seen in poultry (*36*) and humans (*11*). Nevertheless, finding this bacterium in pneumonic animals on multiple farms suggests the possible emerging nature of this pathogen, as suggested in poultry (*37*).

In poultry, clonal outbreaks of *G. anatis* have been described (*38*,*39*), in contrast with our study, where both SNP- and cgMLST-based phylogenetic analysis of the cattle isolates demonstrated a high variety between isolates, even for those retrieved on the same farm. This finding indicates that *G. anatis* strains from the different farms do not originate from 1 single introduction or outbreak and that a large unsampled reservoir of circulating *G. anatis* strains exists in cattle within Belgium. Another explanation for retrieving *G. anatis* in calves with pneumonia might be a direct link with poultry on the affected farms. In our study, no poultry was present, nor was poultry manure used as cattle feed at any farm, although at farm 2 (Table 1), raw eggs were occasionally fed to the calves. Because this practice occurred at only 1 farm, an indirect link with poultry seems unlikely. Moreover, cgMLST analysis indicated that, despite the large variation present in the cattle isolates in Belgium, these isolates still clustered together and were clearly separated from all poultry isolates for which genome information was publicly available. The relatively limited number of currently available *G. anatis* genomes and their large overall distances prevent definitive conclusions, but nevertheless support that no direct or indirect link with poultry exists.

Like other *Pasteurellaceae* species, *G. anatis* most likely acts as an opportunistic bacterium, infecting an already damaged respiratory tract caused by co-infections with viruses or bacteria, as observed in poultry (*37*). Unfortunately, viral involvement in the reported outbreaks in our study cannot be confirmed because we did not perform any viral diagnostics. However, the combined observations we have made suggest that *G. anatis* can act as an opportunistic bacterium in a multifactorial disease complex rather than being a highly virulent pathogen that spreads clonally during a clinical outbreak. To what extent *G. anatis* isolated from cattle in our study can survive in the environment remains unknown.

A second major finding of our study is the multiresistant nature of the retrieved *G. anatis* isolates. All isolates obtained in the study demonstrated acquired resistance against 5–7 different antimicrobial classes, defining them as multidrug-resistant. Although the lack of species-specific clinical breakpoints precludes drawing firm conclusions, the clinical observation of

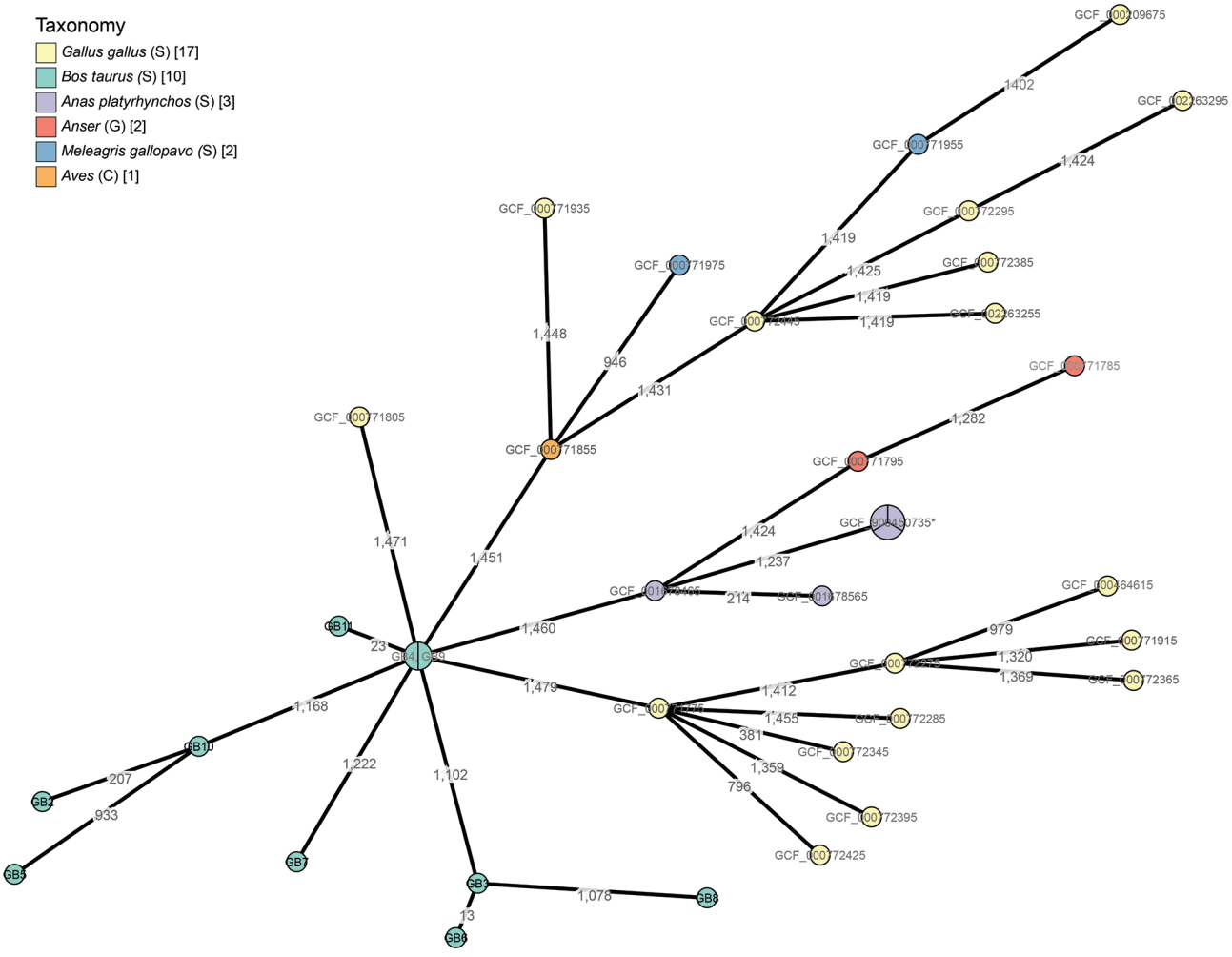

Figure 2. Phylogeny of *Gallibacterium anatis* isolates from cattle in Belgium, 2017–2018, based on a core genome multilocus sequence typing scheme constructed by using the 10 cattle isolates and 27 poultry isolates from National Center for Biotechnology Information (1,516 loci in total). Branch lengths are scaled logarithmically, and branch labels express number of allelic differences between isolates. Nodes scale with the number of isolates that have the same core genome multilocus sequence type. Nodes are colored according to the host organism of the isolate. Asterisk indicates node containing samples GCF_000379785, GCF_000772265, and GCF_900450735 (GB3, GB6, GB8) with the same sequence type. C; class; G, genus; S, species.

unresponsiveness to antimicrobial treatment with various agents also supports this theory. Because antimicrobial susceptibility testing indicated susceptibility for only cephalosporins, amoxicillin/clavulanic acid, or both in all isolates, the isolates can even be defined as extensively drug-resistant (*40*). Also, for *G. anatis* isolated from poultry, a high prevalence of multidrug resistance has been demonstrated (*37*). However, the isolates retrieved in our study also demonstrated acquired resistance against fluoroquinolones, ampicillin, trimethoprim/sulfamethoxazole, florfenicol, and gentamicin. Furthermore, the level and prevalence of multidrug resistance observed in the *G. anatis* isolates we analyzed surpasses previously described multidrug resistance in bovine *Pasteurellaceae* (*41*–*43*).

We detected >20 different resistance genes in the genomes of the *G. anatis* isolates in our study, including determinants conferring resistance to aminoglycosides, phenicols, macrolides, sulphonamides, trimethoprim, tetracyclines, penicillins, and quinolones. Although many of these resistance genes have been described previously in *Pasteurellaceae* obtained from either animals or humans (*43*,*44*), we detected various other resistance genes not previously reported in *G. anatis* or bovine *Pasteurellaceae*. Moreover, 4 resistance genes have so far never been described in *Pasteurellaceae* at all, namely *aad*A23, $bla_{\text{CARB-8}}$, *tet*(Y) and *qnr*D1.

In contrast to recently described bovine multidrug-resistant *Pasteurellaceae* (*43*,*45*,*46*), resistance genes in the *G. anatis* isolates in our study were detected at various

locations in the genome and were seldom contained within ICE, as described previously for *G. anatis* in poultry (*47*). Only 1 gene cluster, carrying 1 or 2 *erm*(B) copies, as well as *aac*6-*aph*2 and *aph3-III* detected in 3 isolates (GB4, GB9, and GB11), was associated with a predicted putative IME. This putative element did not show any remarkable similarities with any of the IMEs in the ICEfinder database for gram-negative bacteria but did show some similarity with ICEs in *Streptococcus pneumoniae* (data not shown). However, for all remaining clustered resistance genes, we observed a link with transposases, some of which were located on predicted plasmids. In addition, the high prevalence and diversity of resistance genes in the bovine *G. anatis* isolates we analyzed suggests that this species might acquire resistance genes relatively easily compared with other *Pasteurellaceae* species. Indeed, *G. anatis* is considered a naturally competent species that has been demonstrated to be less selective in the uptake of foreign DNA compared with other *Pasteurellaceae* species (*48*). As a consequence, these resistance genes might spread to more pathogenic closely related respiratory bacteria like *Mannheimia haemolytica*, *Histophilus somni*, and *Pasteurella multocida*, possibly leading to therapy failure of infectious bronchopneumonia in cattle. We found no relevant virulence genes in the genomes of the strains in Belgium (Appendix Table 5), indicating that such genes are not present or, alternatively, have not yet been described.

In conclusion, *G. anatis* needs to be taken into account as a secondary respiratory pathogen and resistance gene reservoir in cattle. In addition to poultry, cattle hold a potential risk for zoonotic transmission of *G. anatis*, but further research is required to establish zoonotic potential.

Acknowledgements

We thank Marleen Foubert and Arlette Vandekerckhove for their excellent technical assistance.

This study was funded by a PhD Fellowship of the Research Foundation-Flanders (grant no. FWO-1S52616N). The matrix-assisted laser desorption/ionization time-of-flight mass spectrometer was financed by the Research Foundation-Flanders (grant no. FWO-Vlaanderen) as part of Hercules Project G0H2516N (grant no. AUGE/15/05).

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Guaroa Virus and *Plasmodium vivax* Co-Infections, Peruvian Amazon

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During April–June 2014 in a malaria-endemic rural community close to the city of Iquitos in Peru, we detected evidence of Guaroa virus (GROV) infection in 14 febrile persons, of whom 6 also had evidence of *Plasmodium vivax* malaria. Cases were discovered through a long-term febrile illness surveillance network at local participating health facilities. GROV cases were identified by using a combination of seroconversion and virus isolation, and malaria was diagnosed by thick smear and PCR. GROV mono-infections manifested as nonspecific febrile illness and were clinically indistinguishable from GROV and *P. vivax* co-infections. This cluster of cases highlights the potential for GROV transmission in the rural Peruvian Amazon, particularly in areas where malaria is endemic. Further study of similar areas of the Amazon may provide insights into the extent of GROV transmission in the Amazon basin.

Since 1990 in Peru, the US Naval Medical Research Unit No. 6 (NAMRU-6), in collaboration with the Peruvian Ministry of Health, has conducted clinic-based passive surveillance of acute febrile illnesses in Iquitos, the largest city in the Peruvian Amazon. Iquitos is an urban locale in the heart of the Amazon rainforest; the climate is tropical with frequent heavy rainfall. It has a population of ≈400,000, is accessible only by river or air travel, and is situated 120 m above sea level at the confluence of the Nanay, Itaya, and Amazon Rivers in the Loreto Department of northeastern Peru. Iquitos comprises 4 districts: Iquitos, San Juan, Belen, and Punchana. The city and its surrounding periurban and rural areas are home to an abundance of mosquito species and provide a suitable environment for arbovirus and *Plasmodium* spp. transmission. Since 1993, at least 13 arboviruses have been detected in this area, of which dengue virus (DENV), Zika virus, Mayaro virus, and Venezuelan equine encephalitis virus are considered to be of public health importance (*1*).

Guaroa virus (GROV; order *Bunyavirales*, family *Peribunyaviridae*, genus *Orthobunyavirus*) (*2*) is a known cause of febrile illness in tropical regions of Central and South America (*3*). It was first isolated from asymptomatic humans in Colombia in 1956 (*4*) and isolated from symptomatic humans in Brazil in 1964 (*5*). Recent phylogenetic analysis suggests that a common ancestor of GROV and Wyeomyia virus was introduced into South America in the Brazilian Amazon region ≈250 years ago, with subsequent southward spread of GROV to Peru within the past 60–70 years (*6*). A serologic survey in 1965 provided evidence of GROV transmission in Peru shortly after this time (*7*).

More recent antibody prevalence studies on samples collected in Iquitos in 2006 demonstrated an overall GROV seroprevalence of 13% (*3*). The increasing seroprevalence with age suggests that transmission occurred consistently in the region over several years (*3*). In addition to seroprevalence, this study also described 15 symptomatic GROV infections in Peru (including 3 in Iquitos) during 1995–2008, providing further evidence of GROV as a cause of symptomatic disease in the region (*3*).

The only confirmed vector of GROV is *Anopheles* (*Kerteszia*) *neivai* mosquitoes (*8*). This species is an important vector of human malaria in the Pacific lowlands of Colombia (*9*). After a successful elimination effort in the 1960s, malaria reemerged in the early 1990s in the Peruvian Amazon, coincident with the reintroduction of *An. darlingi* mosquitoes and is currently a leading cause of febrile illness (*10*). Subsequently, a resurgence of *P. falciparium* peaked in 1997,

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DOI: https://doi.org/10.3201/eid2604.191104

at which point *P. falciparium* prevalence decreased significantly. The *An. darlingi* mosquito is the primary vector of malaria in rural areas near Iquitos (*11*–*13*). There are numerous reports in the literature of arboviral and malarial co-infections, but reports of GROV and malaria co-infection are scarce, and only co-infection with *P. falciparium* has been reported (*14*,*15*). We describe the clinical and epidemiologic aspects of a GROV outbreak in the rural surroundings of Iquitos and report symptomatic co-infections with GROV and *P. vivax*.

Methods

We identified GROV cases through a passive febrile surveillance system at 12 health centers in urban, periurban, and rural areas in and around Iquitos, which were described previously (*1*). Inclusion criteria were age ≥5 years, oral or tympanic temperature ≥38°C (or axillary ≥37.5°C), duration of symptoms ≤5 days, and no obvious focus of infection. All participants at these sites were initially screened for *Plasmodium* infection by thick smear and were then invited to undergo screening for arboviruses regardless of their smear results. In addition to serum samples obtained during the acute phase, serum samples were obtained during follow-up evaluations 20 days (± 10 days), 3 months (± 15 days), 6 months (± 15 days), and 12 months (± 30 days) after the initial sample. Serum samples collected on day 20 were used to measure convalescent titers.

Of the 12 clinics, 2 are in rural communities with active malaria transmission (Zungarococha and Quistococha communinities), and 3 urban clinics serve as capture areas for communities with active malaria transmission (Bella Vista Nanay, 6 de Octubre, and San Juan). The GROV cases reported in this study were captured during April 5–June 26, 2014, from 3 malaria-endemic communities in the district of San Juan (Santo Tomas, Quistococha, and Zungarococha) (Figure).

We obtained approval for the study protocol (NMRCD.2010.0010) from the Institutional Review Board of NAMRU-6 in compliance with all applicable US federal regulations governing the protection of human subjects and from the Institutional Review Board of the Peruvian Ministry of Health. Informed consent, parental consent, and assent were collected according to Institutional Review Board regulations.

Arboviral Testing

After a negative result by reverse transcription PCR (RT-PCR) for DENV (*16*), we attempted to isolate causative agents with *Aedes albopictus* (C6/36) and African green monkey kidney (Vero 76) cell cultures in combination with an immunofluorescence assay using hyperimmune mouse ascitic fluid (HMAF)

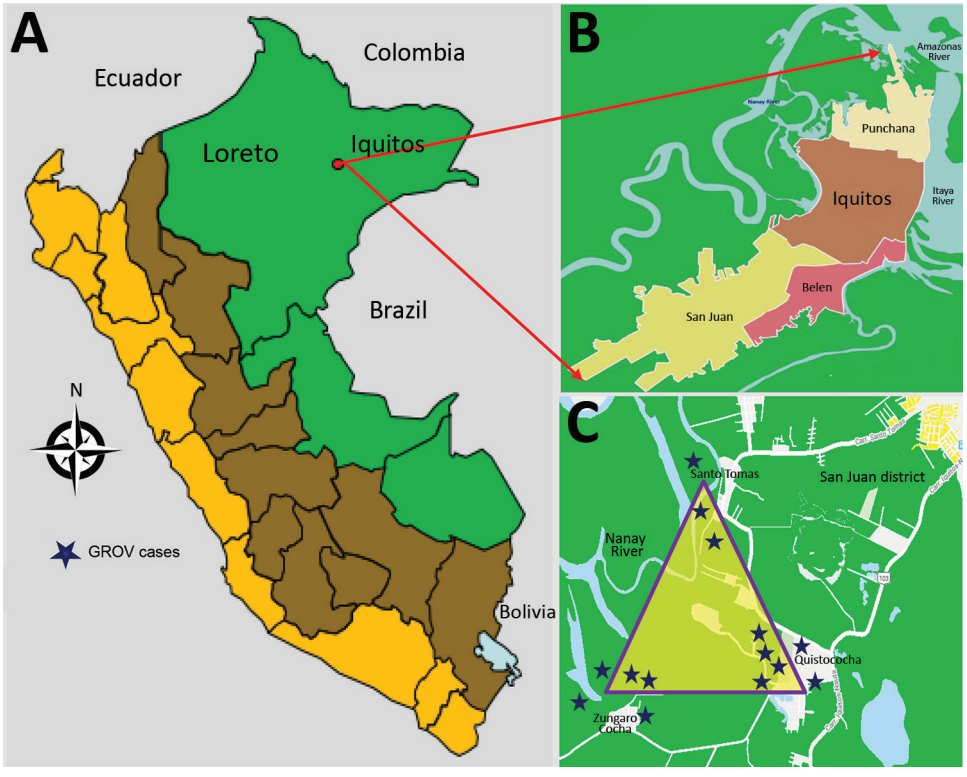

Figure. Geographic distribution of patients with Guaroa virus infection, April–June 2014. A) Peru. B) Iquitos districts. C) District of San Juan. Stars indicate locations of Guaroa virus cases.

raised against flaviviruses, alphaviruses, and bunyaviruses. For bunyavirus detection, we used pooled HMAF against various bunyaviruses (Oropouche 172, Caraparu isolate from Peru, Guaroa isolate from Peru, Maguari R18134, Echarate isolate from Peru and California [EVBSF-283]), followed by the addition of fluorescein-conjugated goat antimouse IgG (*1*). We performed ELISA IgM capture assay with acute and convalescent serum samples for GROV and other endemic arboviruses (Mayaro virus, Venezuelan equine encephalitis virus, Oropouche virus, group C viruses, and DENV). Microtiter plates (96-well format) were coated with goat F(ab')2 antihuman IgM (Jackson Inmuno Research Laboratories Inc., https://www.jacksonimmuno.com) diluted 1:1,000 in phosphate-buffered saline and incubated overnight at 4°C. We diluted participant serum 1:100 and incubated in coated wells for 1 h at 37°C, then added viral antigen and incubated at 37°C for 1 h. We detected viral antigens with HMAF (produced by inoculation of mice with the respective viral strains), followed by horseradish peroxidase–conjugated goat antimouse IgM + IgG (H+L) (Thermo Fisher Scientific, https://www.thermofisher.com). After adding ABTS (2,2′-azino-bis-[3-ethylbenzthiazoline-6-sulfonic acid]) colorimetric substrate, we read plates at 410 nm with a Multiskan Microplate Photometer FC absorbance reader (Thermofisher Scientific) (*1*). We retested samples with detectable IgM at 4-fold serial dilutions (1:100, 1:400, 1:1,600, and 1:6,400). We defined seroconversion as a ≥4-fold increase in IgM between acute- and convalescent-phase samples and considered participants positive for GROV infection if virus was isolated by cell culture or if seroconversion was observed.

RNA Extraction, Sequencing, and Phylogenetic Analysis

We extracted RNA from 14 serum samples using the QIAamp Viral RNA Mini Kit (QIAGEN, https://www.qiagen.com), following the manufacturer's instructions. We performed reverse transcription and amplification using the Access RT-PCR System kit (Promega) and previously described primers Bunya 1 (GTCACAGTAGTGTACTCCAC) and Bunya 2 (CTGACAGTAGTGTGCTCCAC), which amplify a 616-bp amplicon of the S (small) RNA segment. The amplicon covers 462/702 nt of the nucleocapsid coding sequence and 154/226 nt of the 5′-nontranslated region of the viral RNA. We performed reverse transcription at 42°C for 1 h. PCR amplifications comprised 38 cycles of denaturation (94°C for 30 s), annealing (50°C for 40 s), and extension (72°C for 1.5 min) and a final extension at 72°C for 10 min. We then purified amplicons with Centri-Sep columns (Invitrogen, https://www.thermofisher.com) and sequenced directly using the BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems, https://www.fishersci.com), following the manufacturer's protocol. We conducted sequencing on a 3130 XL Genetic Analyzer (Applied Biosystems) platform and analyzed sequences using Sequencher software (Gene Codes Corporation); we queried individual sequences by using the nucleotide database with BLAST (*17*).

Malaria Testing

Microscopy was the primary method of *Plasmodium* identification conducted by the health centers as part of the Ministry of Health's surveillance. Microscopy was also used as part of our study, although to evaluate *Plasmodium* spp. infection more accurately in all GROV-positive cases, we also tested blood from acute-phase samples for *Plasmodium* by PCR regardless of their initial thick-smear results. DNA was extracted from whole blood samples using the DNeasy Blood & Tissue Kit (QIAGEN), following the manufacturer's protocol. *Plasmodium* DNA was amplified using a nested PCR protocol that targets the small subunit ribosomal RNA 18S gene (*18*). Both reactions were conducted in a volume of 50 μL containing 1X Taq polymerase buffer (Invitrogen), 2 mmol/L MgCl2, 125 μmol/L dNTPs (Invitrogen), 0.25 μmol/L of each primer, 1 unit of Taq DNA polymerase (Invitrogen), and 5 μL of DNA sample. We ran both PCR reactions on a Verity Thermal Cycler (Applied Biosystems) as previously described (*18*). We used DNA from *P. falciparum* 3D7 and *P. vivax* Sal-I reference strains as positive controls and human DNA from a person from a non–malaria-endemic area as a negative control. We subsequently ran PCR products on a 2% agarose gel; a band of ≈205-bp indicates the presence of *P. falciparum* DNA, and a PCR product of 120-bp indicates the presence of *P. vivax* DNA.

Results

During the 3-month period when GROV infections were detected, a total of 681 febrile patients were enrolled from all 12 participating clinics around Iquitos. All GROV-infected participants lived in the district of San Juan, in 1 of the 3 malaria-endemic communities: Santo Tomas, Quistococha, or Zungarococha. A total of 121 (18%) of 681 febrile persons resided in 1 of these 3 communities in San Juan. Of those, 14 (12%) tested positive for GROV infection, of whom 6 (43%) were co-infected with *P. vivax*. Of the 14 persons for whom GROV infection was confirmed, 3 were enrolled in urban health facilities, although these 3 persons reside in the rural San Juan district (Figure). During

the 3-month study period, 26 malaria cases were reported in the San Juan district, including the 6 with GROV co-infection.

All 14 GROV-infected persons seroconverted and were negative for all other examined arboviruses; 11 (79%) samples collected from these persons yielded GROV isolates in both Vero-76 and C6/36 (Table 1). Diagnosis of malaria in 6 GROV-infected persons was determined by positive thick smear in 4 cases, positive PCR and thick smear in 1 case, and positive PCR alone in 1 case.

Mean age of the 14 GROV-infected persons was 35.4 years (range 14–64 years). The mean age of the 8 GROV mono-infected persons was 40 years (range 40–64 years), and the mean age of co-infected persons was 29.2 years (range 17–35 years). Nine of the 14 GROV-positive case-patients were male.

Ten samples were positive for GROV by RT-PCR and were sequenced. A BLAST search determined that the DNA sequences had 99% identity with 3 GROV strain isolates collected in Peru during 2004–2008. Multiple alignment analysis of the 10 sequences analyzed showed they were nearly identical, with only 2 nt differences: a synonymous substitution at codon 151 of the nucleocapsid (TTT and TTC) and in the 5′-nontranslated region of the viral RNA at nucleotide position 31 (C and T; Appendix Figure, https://wwwnc.cdc.gov/EID/article/26/4/19-1104-App1.pdf).

All participants reported fever, chills, malaise, body pain, joint pain, and headache (Table 2). Myalgia, anorexia, nausea, and dysgeusia occurred in 8 patients. Only 2 patients reported a rash. Distinct symptom frequencies between persons with GROV mono-infection and co-infected persons were conjunctival injection, dysgeusia, cough, and sore throat. These symptoms occurred more frequently in persons without malaria. These differences in symptoms were not statistically significant (χ^2 with Yates correction significance level 0.05).

Median fever duration for both GROV mono-infection and co-infected persons was 4.5 days. Symptoms with the longest median duration for all participants were body pain, joint pain, headache, and dizziness (all >5 days). Headache was reported during the convalescent phase and for up to 3 months during follow-up in 4 patients with a GROV mono-infection and 2 patients with GROV and malaria co-infection. Duration of symptoms between mono-infected and co-infected persons did not differ significantly. No patients were hospitalized, and all recovered without sequelae.

Discussion

The circulation of many arboviruses in the Peruvian Amazon is well documented, but most cases are not reported (*1*), possibly because the asymptomatic and mild self-limiting infections are common and do not usually result in treatment-seeking behavior (*14*). Other important factors are the absence of diagnostic facilities for detecting arboviruses in areas to which they are endemic, limited access to healthcare for at-risk persons in rural Amazonian populations, and the narrow window of opportunity to collect potentially diagnostic blood samples during the acute phase of the illness (*15*,*19*). Another factor limiting arboviral diagnosis and identification of co-infections with other pathogens is that once malaria is diagnosed, secondary diagnoses are rarely pursued (*15*). For example, in our study GROV infection was diagnosed in 3 patients after they returned to the same health center within 3 days after having tested positive for

Table 1. Laboratory findings and serologic response expressed as inverse titers for patients with GROV infection, Peruvian Amazon, April–June 2014*

Patient	Thick smear malaria result	PCR result		Virus isolated	IgM				
		Malaria	GROV		Acute phase	Convalescent phase†	Month 3	Month 6	Month 12
1	*Plasmodium vivax*	Neg	Pos	GROV	0	1:6,400	0	0	0
2	*P. vivax*	Neg	Pos	GROV	0	0	1:1,600	0	0
3	Neg	Neg	Neg	Neg	1:400	1:1,600	0	VNC	VNC
4	Neg	Neg	Pos	GROV	0	1:1,600	0	0	0
5	Neg	Neg	Pos	GROV	0	1:1,600	0	0	0
6	*P. vivax*	Neg	Pos	GROV	0	1:6,400	0	0	0
7	*P. vivax*	*P. vivax*	Pos	GROV	0	1:6,400	1:400	1:100	0
8	Neg	Neg	Pos	GROV	0	1:6,400	0	0	0
9	*P. vivax*	Neg	Pos	GROV	0	1:6,400	0	0	0
10	Neg	Neg	Neg	GROV	0	1:6,400	0	0	0
11	Neg	*P. vivax*	Neg	Neg	1:100	1:1,600	VNC	VNC	VNC
12	Neg	Neg	Pos	GROV	0	1:400	0	0	0
13	Neg	Neg	Neg	Neg	0	1:1,600	VNC	VNC	VNC
14	Neg	Neg	Pos	GROV	0	1:6,400	1:400	0	0

*GROV, Guaroa virus; neg, negative; pos, positive; VNC, visit not complete.
†At follow-up 20 d (± 10 d) after the acute phase.

Table 2. Clinical, epidemiologic, and demographic characteristics of patients confirmed to have GROV mono-infection and GROV–*Plasmodium* sp. co-infection, Amazonian Peru, April–June 2014*

Patient no.	Age, y/sex	Occupation	Day of illness at enrollment	Infection	Acute-phase clinical manifestations
1	40/F	Housewife	5	Co-infection	Constitutional (malaise, chills, retro-orbital pain, dizziness,headache), gastrointestinal (anorexia, dysgeusia, nausea), musculoskeletal (myalgia, bone pain, joint pain)
2	53/F	Housewife	4	Co-infection	Constitutional (malaise, chills, retro-orbital pain, conjunctivitis, headache), gastrointestinal (anorexia, nausea, abdominal pain), musculoskeletal (myalgia, bone pain, joint pain)
3	64/M	Farmer	3	GROV	Constitutional (malaise, chills, dizziness, retro-orbital pain, conjunctivitis, headache), gastrointestinal (anorexia, dysgeusia, abdominal pain, nausea), musculoskeletal (myalgia, bone pain, joint pain), cutaneous (exanthema)
4	38/M	Farmer	1	GROV	Constitutional (malaise, chills, dizziness, retro-orbital pain, conjunctivitis, headache), gastrointestinal (anorexia, dysgeusia, nausea), musculoskeletal (myalgia, bone pain, joint pain), respiratory (rhinorrhea, sore throat, cough)
5	51/F	Housewife	1	GROV	Constitutional (malaise, chills, retro-orbital pain, conjunctivitis, headache), gastrointestinal (anorexia, dysgeusia, diarrhea, abdominal pain), musculoskeletal (myalgia, bone pain, joint pain)
6	25/F	Housewife	2	Co-infection	Constitutional (malaise, chills, dizziness, retro-orbital pain, conjunctivitis, headache), gastrointestinal (anorexia, dysgeusia, abdominal pain, diarrhea, nausea), musculoskeletal (myalgia, bone pain, joint pain), cutaneous (exanthema), hemorrhagic (gingivorrhagia)
7	22/F	Housewife	2	Co-infection	Constitutional (malaise, chills, retro-orbital pain, headache), gastrointestinal (anorexia, dysgeusia, nausea), musculoskeletal (myalgia, bone pain, joint pain)
8	36/M	Farmer	2	GROV	Constitutional (malaise, chills, dizziness, retro-orbital pain, conjunctivitis, headache), gastrointestinal (anorexia, dysgeusia, nausea), musculoskeletal (myalgia, bone pain, joint pain), respiratory (rhinorrhea)
9	18/M	Army forces	1	Co-infection	Constitutional (malaise, chills, dizziness, retro-orbital pain, headache), gastrointestinal (anorexia, dysgeusia, abdominal pain), musculoskeletal (bone pain, joint pain)
10	29/M	Army forces	4	GROV	Constitutional (malaise, chills, dizziness, headache), gastrointestinal (dysgeusia, abdominal pain, diarrhea), musculoskeletal (myalgia, bone pain, joint pain)
11	17/M	Student	3	Co-infection	Constitutional (malaise, chills, dizziness, headache), gastrointestinal (anorexia, nausea), musculoskeletal (myalgia, bone pain, joint pain)
12	24/M	Student	1	GROV	Constitutional (malaise, chills, retro-orbital pain, conjunctivitis, headache), gastrointestinal (anorexia, dysgeusia), musculoskeletal (myalgia, bone pain, joint pain)
13	64/M	Farmer	5	GROV	Constitutional (malaise, chills, dizziness, retro-orbital pain, conjunctivitis, headache), gastrointestinal (anorexia, dysgeusia, abdominal pain, vomiting, diarrhea, nausea), musculoskeletal (myalgia, bone pain, joint pain), cutaneous (exanthema)
14	14/M	Student	1	GROV	Constitutional (malaise, chills, retro-orbital pain, conjunctivitis, headache), gastrointestinal (anorexia, dysgeusia, nausea), musculoskeletal (myalgia, bone pain, joint pain), respiratory (rhinorrhea, sore throat, cough)

*GROV, Guaroa virus.

P. vivax. Because their febrile symptoms persisted despite treatment with antimalarial drugs, they provided additional samples that tested positive for GROV. Although previous exposure to antimalarial drugs might have affected our RT-PCR results for malaria and might have resulted in a false-negative result, only 2 patients were willing to provide blood samples for arbovirus testing during the same visit in which they tested positive for *P. vivax* by thick smear. The detection of viral and parasitic co-infection demostrates the value of actively looking for viral pathogens in malaria-positive patients at the time they seek medical care during the acute phase, when the probability of identifying a viral infection is highest.

Current knowledge about GROV as a human pathogen is limited, and little is known about its reservoir hosts and vectors, particularly in Peru. *Anopheles* mosquitoes have been implicated elsewhere as vectors for GROV (*5*,*8*,*20*), although few arbovirus are known to be transmitted by *Anopheles* mosquitoes, such as o'nyong-nyong virus, which is transmitted in Africa by *An. funestus* and *An. gambiae* mosquitoes

(*21*). It is possible that a co-infected mosquito could transmit both *Plasmodium* spp. and GROV; however, vector-competence studies of *An. darlingi* mosquitoes with GROV have not yet been published (*22*). The *An. darlingi* mosquito is the most common and competent malaria vector in the Peruvian Amazon (*11*–*13*,*23*,*24*). During 2014, the Peruvian Ministry of Health recorded 65,239 malaria cases in the entire country, most of which resulted from *P. vivax* (84%) and were detected in the Amazon region of Loreto (≈90%) (*23*). The GROV infection reported in this study coincides temporally with the peak of this *P. vivax* outbreak observed in 2014 (*25*), increasing the chances of co-infected mosquitoes, probability of transmission to humans, or both.

Furthermore, malaria rates tend to be high along the Nanay River, located only a few kilometers from the southern Iquitos city limits (*26*). All the GROV cases observed in the present report came from rural communities in the district of San Juan Baustista bordering the Nanay River. Further investigation is needed to understand the nature of these co-infections because they could have occurred solely as a consequence of the elevated levels of circulating *P. vivax*, a possible peak in *Anopheles* populations, or subclinical/persistent malaria with fever caused by GROV infection. In addition, identification of nonhuman reservoirs for GROV is critical to clarify the epidemiology of the disease and distinguish GROV from other arboviruses circulating in and near Iquitos (*27*,*28*).

Consistent with a previous report (*3*), the clinical manifestations of GROV infection in this study were highly nonspecific, and persons co-infected with *P. vivax* were clinically indistinguishable from those with GROV infection alone. It was not possible to determine the relative contribution of malaria and GROV to the symptomatology of co-infected persons; however, 3 of the 5 patients with positive thick smears who were treated with antimalarial drugs returned to the same health center within 3 days after the initial diagnosis because of persistent fever. These symptoms could, at least in part, have been related to the GROV infection consistent with other clinical symptom reports (*3*). Understanding the origin of symptoms in co-infected persons is further complicated because both asymptomatic malaria and arboviral disease are common (*14*,*29*).

The cases of GROV fever we report may underrepresent the true number of total cases in Iquitos during the study period. GROV may be underreported because of overlapping symptoms between pathogens, such as DENV and *Plasmodium* spp., that are more frequently targeted by diagnostic tests. Our study highlights the importance of the febrile surveillance system and its access to advanced diagnostic facilities that enabled detection of these cases. Continued surveillance is necessary not only to monitor the dynamics of well-studied diseases, such as dengue and malaria, but also to capture the emergence of potentially new pathogens with epidemic potential that pose a public health risk. In particular, examining malaria-endemic communities may provide an opportunity to better quantify the incidence of GROV and other endemic or enzootic pathogens.

Acknowledgments

We thank Nora Marin, Flora Vargas, Leslye Angulo, Guadalupe Florez, Christopher Cruz, and Maria Silva for invaluable support in the execution of the study and manuscript preparation. In addition, we thank the personnel of the Direccion Regional de Salud de Loreto for supporting our febrile illness surveillance study.

This study was supported by funding from the US Department of Defense Health Agency—Armed Forces Health Surveillance Branch (AFHSB) Global Emerging Infections Surveillance and Response System (GEIS), Work Unit no. 847705.82000.25GB.B0016. Period of Funding: 2014. ProMIS ID: P0144_20_N6.

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Intensified Short Symptom Screening Program for Dengue Infection during Pregnancy, India

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Mosquitoborne diseases (e.g., malaria, dengue, and chikungunya) are endemic to India and pose diagnostic challenges during pregnancy. We evaluated an intensified short symptom screening program in India to diagnose dengue during pregnancy. During October 2017–January 2018, we screened pregnant women during antenatal surveillance for symptoms of mosquitoborne diseases (fever only, fever with conjunctivitis, fever with rash, or all 3 symptoms) within the previous 15 days. Of 5,843 pregnant women screened, 52 were enrolled and tested for dengue, chikungunya, and Zika viruses by using a Trioplex real-time reverse transcription PCR. Of 49 who had complete results, 7 (14%) were dengue positive. Of these, ocular pain was seen in 4 (57%) and conjunctivitis in 7 (100%). Intensified symptom screening using conjunctivitis, in addition to rash, in pregnant women with fever might improve dengue case detection and can be included in routine symptom screening during pregnancy.

Every year, an estimated 96 million persons worldwide are given a clinical diagnosis of severe dengue infection (*1*). In 2017, a total of 188,401 cases of dengue were diagnosed in India, and the mortality rate was 0.1%. The mortality rate in Maharashtra State was 0.8% in 2017, nearly 8 times higher than the national average (*2*,*3*). The prevalence of dengue infection among pregnant women is not reported, but pregnant women may be uniquely susceptible to dengue infection because of the immune changes that occur during pregnancy (*4*).

In some countries in Southeast Asia, dengue fever is the most common cause of acute febrile illness during pregnancy (*5*). Dengue infection during pregnancy has been associated with poor maternal and infant outcomes, including preterm birth (*6*), hemorrhage, preeclampsia, and caesarean delivery (*7*–*15*). Dengue virus can also be vertically transmitted to the infant, resulting in neonatal dengue, thrombocytopenia, and cerebral hemorrhage (*16*–*19*). Vertical transmission is most likely if the infection occurs in the third trimester or is present during delivery by cesarean section (*20*).

Despite the devastating consequences, the diagnosis of dengue infection during pregnancy remains challenging. This challenge is partially caused by overlapping signs and symptoms of dengue infection with other conditions, such as HELLP (hemolysis, elevated liver enzymes, low platelet count) syndrome, pneumonia, pulmonary embolism, and other febrile illnesses (*21*,*22*). Moreover, molecular assays to diagnose dengue are not widely available. However, there is a remarkable reduction in the case-fatality rate after early diagnosis and access to appropriate medical care (*23*).

This study was planned after detection of Zika virus in India. The goal of this study was to determine the prevalence of dengue, chikungunya, and Zika virus among pregnant women in Pune, India. We also aimed to identify clinical predictors of these infections to improve screening and detection during the antenatal setting.

Methods

Ethics

This study was approved by the Byramjee Jeejeebhoy Medical College Clinical Trials Unit and Johns

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DOI: https://doi.org/10.3201/eid2604.191476

Hopkins University Institutional Review Boards. Written consent was obtained from all participants.

Study Setting and Procedures

During October 2017–January 2018, we surveyed pregnant women who came to the antenatal clinic at Sassoon Government Hospital in Pune. All pregnant women were screened by a postgraduate physician who used a short symptom screening for fever, conjunctivitis, and rash occurring in the preceding 15 days (*24*). Women with any of these complaints underwent a secondary confirmatory screening by a senior physician. At this secondary screening, detailed histories, such as onset and duration of symptoms, reported by the women were collected. All women who had fever and conjunctivitis or fever and rash within the previous 15 days, confirmed in the secondary screening, were approached for enrollment in the study. After obtaining consent, research staff collected data on demographics, obstetric history, and travel history (*25*).

Blood samples were tested for dengue, chikungunya, and Zika viruses at the Indian National Institute of Virology by using Trioplex, a multiplex real-time reverse transcription PCR (RT-PCR) developed by the US Centers for Disease Control and Prevention (*26*). The primary physicians might have also ordered an IgM ELISA and a nonstructural protein 1 antigen test to assess for dengue or chikungunya infection if there was clinical suspicion for either infection. We abstracted this information from medical charts.

We completed follow-up visits at delivery and 6 months postpartum. For women who delivered outside Sassoon Hospital, we abstracted data from their medical records through home visits or postpartum follow-ups. Infants born to enrolled mothers had follow-up visits at birth and 6 months of age to coincide with maternal visits. We planned for additional evaluations if any abnormality was detected.

All data collected were stored on a secure electronic database. This database was specifically designed for our study by Persistent Systems (https://www.persistent.com) on a Salesforce platform (https://www.salesforce.com) by using tablets or laptops.

Sample Size

We did not have a predetermined sample size, but screened all women who came to the center during October 2017–January 2018. A total of 5,843 patients were screened for enrollment.

Results

Among 5,843 pregnant women who attended antenatal visits during the study period, 106 (2%) reported fever, rash, or conjunctivitis in the 15 days preceding their visit in the primary screening. Of these 106 women, we enrolled 52 (49%) who were found to be eligible in the secondary screening by the medical team. These women had the following symptoms: 18 (34%) fever only, 4 (7%) fever plus rash, 18 (34%) fever plus conjunctivitis, and 12 (23%) all 3 symptoms (Figure). We screened out the remaining 54 patients during the secondary screening because they had their symptoms >15 days before the visit, which were our inclusion criteria.

The median age of enrolled participants was 22 years (interquartile range [IQR] 19–25 years). The median gestational age at enrollment was 23 weeks (IQR 18–34 weeks); half of the participants were pregnant for the first time. The most common symptoms reported by participants were headache (n = 33/52, 63%), cough (n = 26/52, 50%), conjunctivitis (n = 26/52, 50%), and rash (n = 13/52, 25%) (Table 1).

Trioplex testing reports were available for 49/52 (94%) participants. Positive results for dengue were obtained for 7/49 (14%) participants. Per routine

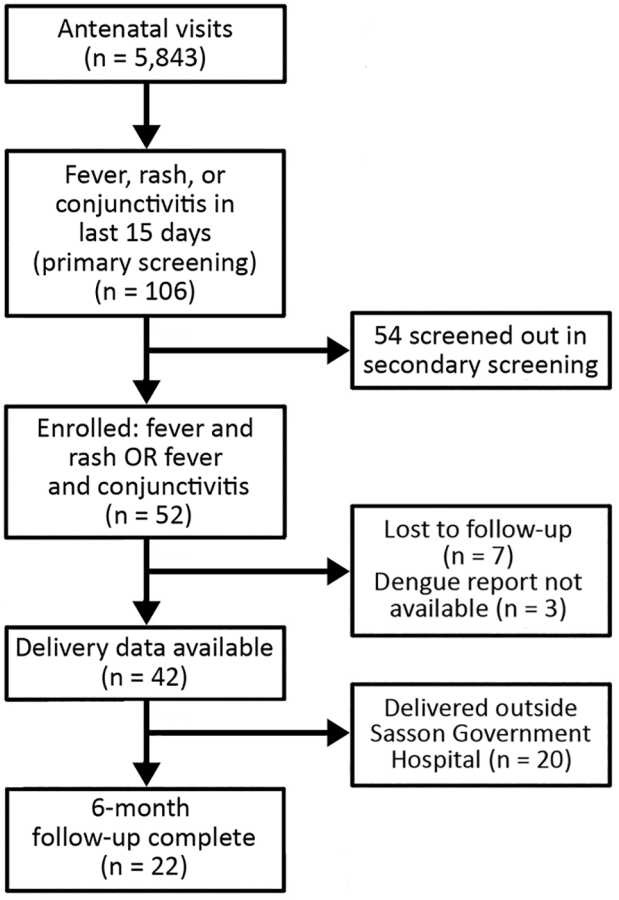

Figure. Screening and enrollment flowchart for participation in an intensified short symptom screening program for dengue infection during pregnancy, India.

Table 1. Clinical characteristics for 52 pregnant women with fever during intensified short symptom screening program for dengue infection during pregnancy, India*

Clinical characteristic	Entire population, n = 52	Maternal dengue PCR positive, n = 7	Maternal dengue PCR negative, n = 42†	OR (95% CI)	p value
Age	22 (19–25)	20 (19–23.5)	22.5 (19.2–25)	0.9 (0.7–1.0)	0.42
Gestational age at enrollment	23 (18–34)	30 (22.5–31.5)	23 (16.5–34)	0.1 (0–0.9)	0.57
Primigravida	25 (48)	4 (57)	21 (50)	1.3 (0.2–10.2)	1
HIV positive	2 (4)	0 (0)	2 (5)‡	0 (0–33.9)	1
Recent travel	15 (29)	1 (14)	14 (33)	0.3 (0–3.2)	0.41
Signs/symptoms					
Myalgia	18 (35)	3 (43)	15 (36)	1.3 (0.2–9.1)	0.7
Body pain	5 (10)	1 (14)	4 (10)	1.6 (0–20.1)	0.55
Arthralgia	13 (25)	3 (43)	10 (24)	2.4 (0.3–16.7)	0.36
Weakness	3 (6)	1 (17§	2 (5)¶	3.5 (0.1–80.7)	0.36
Malaise	2 (4)	1 (14)	1 (2)§	6.2 (0.1–533.1)	0.27
Lymphadenopathy	0 (0)	0 (0)	0 (0)§	NA	NA
Abdominal pain	1 (2)	1 (14)	0 (0)§	∞ (0.2–∞)	0.15
Vomiting	14 (27)	3 (43)	11 (26)	2.1 (0.3–14.6)	0.39
Diarrhea	5 (10)	1 (14)	4 (10)§	1.5 (0–19.6)	0.56
Poor appetite	14 (27)	3 (43)	11 (27)§	2 (0.3–14.1)	0.4
Sore throat	16 (31)	1 (14)	15 (36)	0.3 (0–2.9)	0.4
Eye pain	10 (19)	4 (57)	6 (14)	7.5 (1–65.6)	0.02
Conjunctivitis	26 (50)	7 (100)	19 (46)§	∞ (1.4–∞)	0.01
Rash	13 (25)	2 (29)	11 (26)	1.1 (0.1–8.2)	1
Headache	33 (63)	6 (86)	27 (64)	3.3 (0.3–163.2)	0.4
Cough	26 (50)	3 (43)	23 (56)§	0.6 (0.1–4)	0.69

*Values are no. (%) or no. (range). NA, not applicable; OR, odds ratio.
†Trioplex results were not available for 3 women.
‡Incomplete symptom data for 5 participants.
§Incomplete symptom data for 1 participant.
¶Incomplete symptom data for 3 participants.

clinical care, a few patients underwent immunoassay testing for dengue (n = 4) and chikungunya (n = 3). Of the 7 women who were positive for dengue by Trioplex, 1 (14%) showed negative results by a rapid diagnostic test and 1 (14%) showed positive results by IgM ELISA. Among the remaining 42 women who were negative for dengue by Trioplex, 1 (2%) was negative by a dengue rapid diagnostic test and 1 (2%) was positive by IgM ELISA. All samples showed negative results for Zika and chikungunya viruses by Trioplex. A chikungunya IgM ELISA was performed for 3 participants; 2 (4%) had positive results (Table 2).

We found no major difference in demographic features between participants who were positive for dengue and those who were negative (Table 1). Symptomatically, dengue diagnosis was associated with eye pain (OR 7.5, 95% CI 1–65.6; p = 0.02). All women given a diagnosis of dengue had conjunctivitis (p = 0.01), and 2 women (29%) had rash.

Data collected at the time of delivery were available for 42 (85%) patients; we were unable to obtain delivery date for 7 (15%) patients. The median gestational age at the time of delivery was 38 weeks (IQR 37–39 weeks), which did not differ between mothers who had been given a diagnosis of dengue and those who had not (Table 2). Ten (24%) births were performed by cesarean section. There were 9 (21%) premature infants, 10 (24%) low birth weight infants, 2 (5%) pregnancies complicated by oligohydramnios, and 9 (21%) infants with head circumferences less than the third percentile; none of these findings were associated with maternal dengue infection. There was 1 (2%) stillbirth to a dengue-negative mother. By the time of 6 months follow-up, 3 infant deaths were recorded. Maternal dengue infection was not associated with any of the observed adverse birth outcomes (Table 3).

Discussion

We report a dengue prevalence of 14% among pregnant women with rash or conjunctivitis and a history of recent fevers. However, we did not identify chikungunya and Zika cases in our study. The prevalence of dengue infection in pregnant women, in general, is not reported in the literature, but the

Table 2. Trioplex and standard of care test results during intensified short symptom screening program for dengue infection during pregnancy, India

Trioplex results	Standard of care results
Dengue positive, n = 7	1 rapid diagnostic negative, 1 IgM positive, 5 not tested
Dengue negative, n = 42	1 rapid diagnostic negative, 1 IgM positive, 40 not tested

Table 3. Birth outcomes by maternal dengue infection status among 42 deliveries analyzed by intensified short symptom screening program for dengue infection during pregnancy, India*

Clinical characteristic	All births, n = 42	Maternal dengue positive, n = 6	Maternal dengue negative, n = 34	OR (95% CI)	p value
Gestational age, wk	38 (37–39)	37.7 (37.2–38.1)	39.1 (37.9–39.9)	1.0 (0.7–1.5)	0.28
Cesarean section	10 (24)	1 (17)	9 (26)	0.6 (0–6.1)	1
Premature	9 (21)	1 (17)	8 (24)	0.7 (0–7.3)	1
Low birthweight	10 (24)	2 (33)	7 (21)	1.9 (0.1–16.8)	0.60
Head circumference <3rd percentile†	9 (21)	2 (33)	7 (22)‡	1.8 (0.1–15.6)	0.60
Head circumference <10th percentile†	14 (33)	2 (33)	12 (38)‡	0.8 (0.1–6.9)	1
Stillbirth	1 (2)	0 (0)	1 (3)	0 (0–220.2)	1
Deceased at birth or before 6-mo follow-up§	3 (7)	0 (0)	3 (9)	0 (0–15)	1
Oligohydramnios	2 (5)	1 (17)	1 (3)	6.1 (0.1–530.4)	0.28
Apgar score at 1 min	8 (7–8)	8 (8–8)	8 (7–8)	2.1 (0.4–16.2)	0.36
Apgar score at 5 min	9 (9–9)	9 (9–9)	9 (9–9)	0.9 (0.3–6.7)	0.65

*Values are no. (%) or no. (range). OR, odds ratio.
†Intergrowth standards.
‡Head circumference data were not available for 2 infants.
§Six-month follow-up data were available for 22 infants.

prevalence of laboratory-confirmed dengue among persons with clinically suspected cases in the population in India was reported to be 38% (*27*).

The lower prevalence in our population could reflect our short enrollment period, which did not include the full peak monsoon season because the reports of Zika in India only began in May 2017. Another explanation might be related to the diagnostic method we used. The RT-PCR used in this study identifies dengue infection in the acute phase of infection within 5 days of infection, which coincides with viremia and the febrile phase of illness (*28*). Although RT-PCR is the most sensitive test for diagnosis of acute dengue and can distinguish between dengue and Zika, its high cost and complexity precludes its use for routine care in India. Most studies in India reported diagnosis of dengue by using serologic tests (IgM and IgG), which are less sensitive in early infection but become more sensitive after 5 days of infection (*29*). Regardless, a prevalence rate of 14% is a cause for concern, given possible adverse maternal–fetal outcomes.

A useful finding of our study was the predictive value of ocular findings in the diagnosis of dengue in our pregnant cohort. A separate study of pregnant women with dengue in northern India reported similar findings: 100% of the dengue patients had conjunctivitis, and 57% had eye pain (*30*). According to the World Health Organization, dengue should be considered in patients with a high fever and retroorbital pain (*31*–*34*), but there is no specific mention of conjunctivitis as a trigger for dengue testing. The mechanism behind dengue infection and ophthalmic involvement is not clear, but studies suggest it is related to an immune-mediated process involving specific dengue serotypes (*31*,*32*). Given the results of our studies and others for pregnant women, conjunctivitis should be added to screening for dengue in pregnancy during high-burden months.

In our study, we found that 29% of women with dengue had preterm births and low birthweight infants, which is consistent with recent reviews reporting these factors as the 2 most common adverse pregnancy outcomes (*9*). Although 9 (21%) infants had head circumferences less than the third percentile, this finding was not associated with maternal dengue infection. We also did not find any adverse outcomes related to bleeding in the mother or the neonate; thrombocytopenia was not seen in our group of patients. This finding is different from those of most studies in which postpartum hemorrhage and disseminated intravascular coagulation have been reported. Other obstetric complications, including preeclampsia, eclampsia, placenta previa, or retroplacental hematoma, have been reported in the literature. The gestational age at manifestation of dengue fever results in a major effect on outcomes; manifestations with early or late onset during pregnancy are associated with the worst prognosis (*35*).

Immunologic changes that occur in mid-to-late pregnancy could lead to an increase in the risk for severe dengue if infections occur during pregnancy (*9*,*36*,*37*). In our study, women were screened mainly during the second and third trimesters because the woman usually come for antenatal care during that period. Moreover, none of the patients in this study had placenta previa, abruptio placentae, or severe preeclampsia associated with thrombocytopenia, thus avoiding bleeding and need for transfusions, although preeclampsia was present in 2 of the dengue patients. The 1 stillbirth that occurred was the result of the umbilical cord being wrapped around the neck of the infant and is unlikely to have been related to maternal dengue.

Other studies have also reported a later diagnosis of dengue during pregnancy; a study in Sri Lanka reported 96% of cases in the second or third trimester (*38*). The reason why illness onset usually occurs in mid-to-late pregnancy is unclear. One reason that dengue is not diagnosed easily in early pregnancy is because women do not come to a clinic early or they believe that their symptoms are normal for early pregnancy (e.g., fever, nausea, vomiting). Some patients might not have been aware of their pregnancies in the first several weeks after conception and therefore are less likely to recall a febrile episode than a patient with a known pregnancy. The physiologic and immunologic changes that occur in mid-to-late pregnancy might also contribute to an increase in dengue virus susceptibility. Because dengue infection is common in India, active symptom-based screening of all pregnant women should be incorporated into antenatal care during the high-risk months. Our implementation of an active screening algorithm may have decreased the incidence of adverse pregnancy and birth outcomes.

Our study had several limitations. First, the number of cases detected was small, which might limit detection of major associations with maternal and neonatal outcomes. Because we conducted the study only during September–February, the full temporal pattern of dengue and its complications in pregnancy might have been missed. For this study, because we did not enroll afebrile women with ophthalmic symptoms, we were not able to assess if these symptoms are independently predictive of dengue infection in pregnancy. We plan to incorporate dengue screening into antenatal care in the coming years and with longer infant follow-up to improve the yield of this intervention. However, even with these limitations, we were still able to identify 7 cases of dengue, suggesting that scaling up the study would result in an even higher detection rate.

Although the primary purpose of conducting this study was Zika surveillance, we instead identified 7 cases of dengue on the basis of similar symptoms. This finding suggests that intensified symptom screening by using conjunctivitis, in addition to rash, in pregnant women with fever might increase the efficiency of dengue case detection. The symptom screening is short and can be administered by ancillary medical staff with minimal training to detect cases in the field or in a busy antenatal clinic. Given that the mortality rate for severe dengue fever is 0.8%–2.5% (*2*), this type of active symptom-based screening for dengue might help prevent adverse maternal and fetal outcomes.

Acknowledgments

We thank the Department of Obstetrics and Gynecology at Byramjee Jeejeebhoy Medical College and Sassoon Hospital and Pradip Awate for assistance during the study and the US Centers for Disease Control and Prevention for providing the Trioplex assay.

This study was supported, in part, by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (grants UM1AI069465 and R01HD081929 to A.G., grant K23AI129854 to J.M., grant UM1AI104681 to M.L.R., and grant D43TW009574 to R.B.), as well as the Kellen Foundation, the Ujala Foundation, the Gilead Foundation, and the Wyncote Foundation.

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Prevalence of Antibodies to Crimean-Congo Hemorrhagic Fever Virus in Ruminants, Nigeria, 2015

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Crimean-Congo hemorrhagic fever virus (CCHFV) is a highly transmissible human pathogen. Infection is often misdiagnosed, in part because of poor availability of data in disease-endemic areas. We sampled 150 apparently healthy ruminants throughout Nigeria for virus seropositivity and detected virus-specific IgG in cattle (24%) and goats (2%), highlighting the need for further investigations.

Crimean-Congo hemorrhagic fever (CCHF) is a fatal, zoonotic, tickborne viral infection endemic to Africa, the Middle East, Asia, and Europe. The CCHF virus (CCHFV) is primarily maintained in nature in *Hyalomma* ticks (*1*). Although faunae of different species become infected and sustain a transient viremia, overt disease in animals is not easily recognized. Most CCHF cases occur in humans involved in the livestock industry, including slaughterhouse workers and veterinarians (*2*). This occupational hazard is exacerbated by the asymptomatic infection that CCHFV establishes in animals.

Studies conducted over 3 decades suggest circulation of CCHFV in animals and ticks in Nigeria (*3*,*4*). However, this circulation might not reflect current virus activity in the livestock population of this country. Furthermore, the transborder movement of cattle, goats, and sheep into Nigeria from neighboring countries, such as Burkina Faso and Niger, where CCHF cases have occurred (*5*,*6*), could result in importation of CCHFV-infected animals and ticks. The role of domestic ruminants in CCHFV zoonosis in Nigeria has not been extensively investigated despite a report of CCHF epidemic in the United Arab Emirates caused by imported livestock and ticks from Somalia and Nigeria (*7*). This finding underpins the urgent need for assessment of the current status of CCHF among ruminants in Nigeria. We report the results of a pilot study conducted to investigate the prevalence of CCHFV antibodies in cattle, goats, and sheep from different vegetation zones of Nigeria.

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DOI: https://doi.org/10.3201/eid2604.190354

The Study

During January–May 2015, we collected 150 serum samples (50 each from cattle, goats, and sheep) from live animal markets, abattoirs, and privately owned farms in different states in Nigeria. We collected cattle serum samples from the states of Sokoto (northwest, Sudan savannah zone), Borno (northeast, Sudan savannah), Benue (northcentral, Guinea savannah) and Oyo (southwest, rainforest) (Figure 1). Sokoto and Borno States have potential for transborder spread of diseases, such as CCHF, because they have borders with Niger, Chad, and Cameroon, 3 countries from which cattle are continually transported into Nigeria. We collected goat serum samples from the states of Lagos (southwest, rainforest), Oyo, and Sokoto and sheep serum samples from the states of Ogun (southwest, rainforest) and Oyo (Figure 1).

All animals sampled were adults and apparently healthy at the time of sampling. We heat-inactivated serum samples (56°C, 30 min) and transported them on dry ice to Public Health England (Salisbury, UK) where they were stored at –20°C until used. Approval for this study was provided by the University of Ibadan/University College Hospital Ethics Committee (NHRFC/05/01/2008a).

We performed an ELISA by using recombinant CCHFV nucleoprotein (NP) as described (*8*). We coated half of the ELISA plate wells with purified recombinant CCHFV NP and half with negative control

[1]These authors contributed equally to this article.

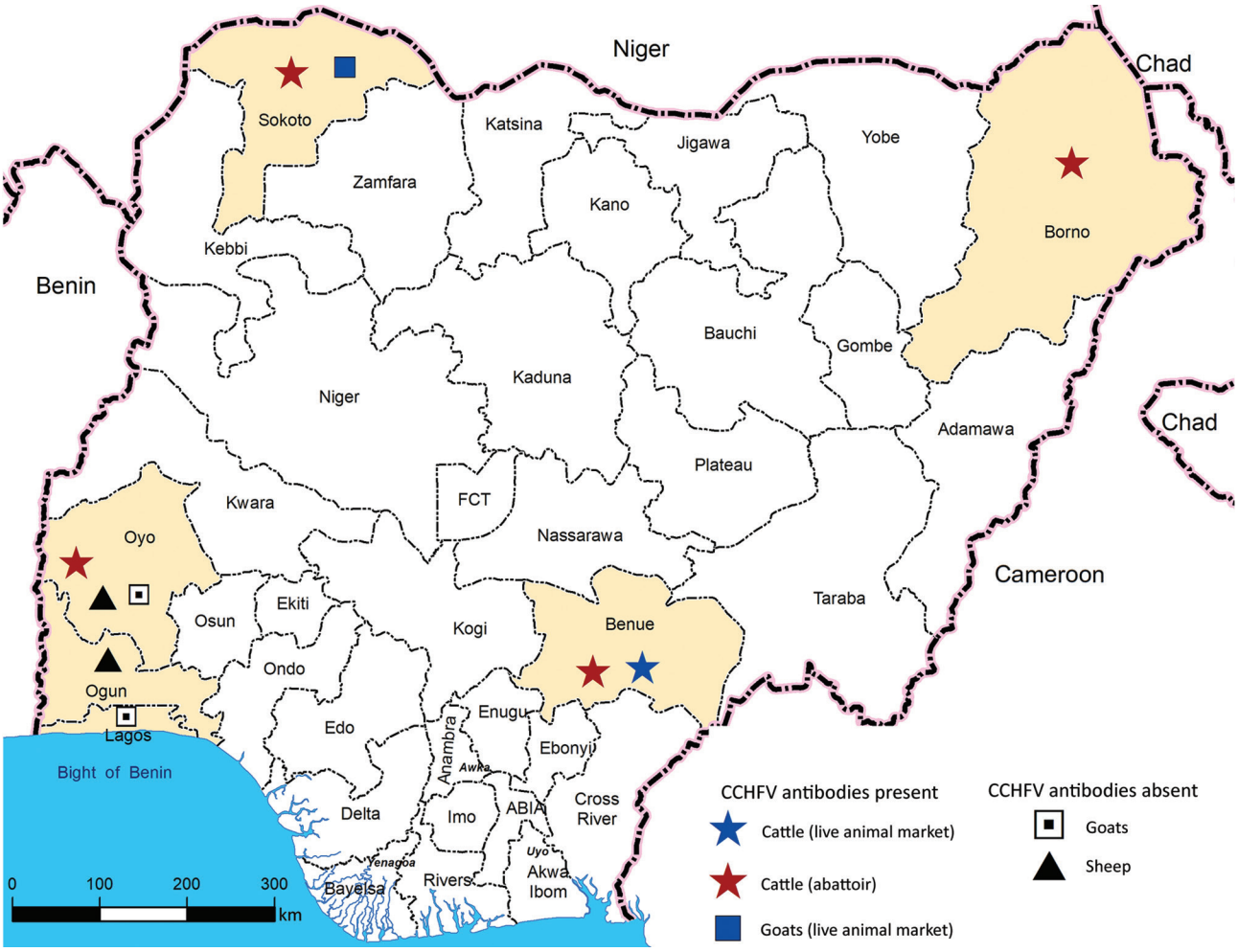

Figure 1. Sample collection sites and distribution of CCHFV antibody-positive and -negative samples in Nigeria. Yellow shading indicates states from which samples were collected. CCHFV, Crimean-Congo hemorrhagic fever virus; FCT, Federal Capital Territory.

antigen and incubated the plates at 4°C overnight. After washing, we incubated the plates at 37°C with blocking solution for 1 h, 4-fold diluted (1:100–1:6400) test serum samples for 1 h, horseradish peroxidase–labeled anti-bovine or anti-goat IgG for 1 h, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) substrate at 25°C for 30 min. Each incubation was followed by a washing step.

We determined sensitivity and specificity of the ELISA initially by creating receiver operating characteristic (ROC) and 2-graph ROC curves with Stat Flex version 5 software (*9*). We estimated an optimal cutoff point by comparing a range of sensitivity and specificity values for a range of cutoff values.

To confirm true-positive ELISA results, we performed an indirect fluorescent antibody test (IFAT) with recombinant CCHFV NP antigens as described (*10*). We reacted serially diluted (1:20–1:80) serum samples with recombinant antigens on multiwell slides, incubated at 37°C for 1 h, and washed. We then incubated slides with Protein G, Alexa Fluor 488 conjugate (https://www.thermofisher.com) at 37°C for 1 h, washed the slides, and examined them by using fluorescent microscopy.

We obtained optical density (OD) values of serum samples tested with the ELISA at a 1:400 dilution and compared the distribution of OD values for IFAT-positive and IFAT-negative samples (Figure 2, panel A). Areas under the 2-graph ROC curve were 0.976 and 1.000 for cattle (Figure 2, panel B), which indicated that the test had a good probability of distinguishing between CCHFV antibody–positive and –negative animals. Because only 1 goat sample was positive by IFAT, the cutoff OD value for goat was not determined by 2-graph ROC analysis.

Seroprevalence rates obtained by the CCHFV IgG-ELISA were 24.0% for cattle, 2.0% for goats, and 0% for sheep (Table). Fourteen (28.0%) of the 50 cattle

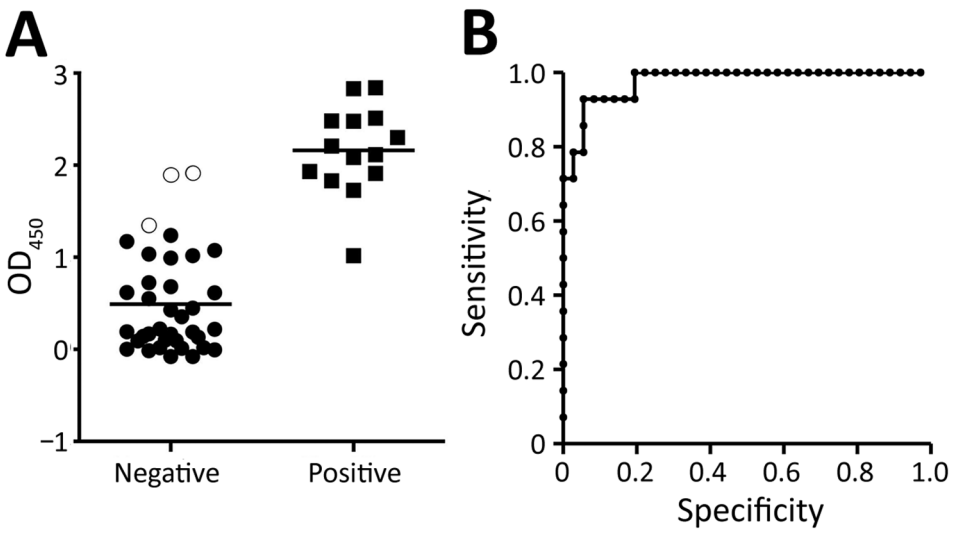

Figure 2. Prevalence of antibodies to Crimean-Congo hemorrhagic fever virus (CCHFV) in ruminants, Nigeria, 2015. A) Distribution of ELISA OD values for serum samples from cattle according to indirect fluorescent antibody test (IFAT) results, Nigeria. The OD at 405 nm for each serum sample at a dilution of 1:400 in the ELISA was plotted against serum samples from CCHFV IFAT antibody-positive and antibody-negative cattle. Three antibody-negative samples (open circles) were above the cutoff OD value in the ELISA and thus considered to be false positive in the ELISA. Other antibody-negative samples (solid circles) were also ELISA negative. Horizontal bars indicate mean OD values. Solid squares indicate IFAT-positive serum samples. B) Receiver operating characteristic (ROC) analysis of an IgG-ELISA specific for nucleoprotein of CCHFV. Area under the ROC curve was 0.9762, which indicates that the test has a good probability of distinguishing between CCHFV antibody–positive and –negative cattle. OD, optical density.

serum samples were positive for CCHFV NP antibodies by IFAT: 3 (15%) from an abattoir in Oyo State, 4 (40%) from a live animal market in Borno State, 1 (10%) from an abattoir in Sokoto State, and 6 (60%) from an abattoir and a live animal market in Benue State. Only 1 (2.0%) of the 50 goat serum samples was positive by IFAT; this positive sample was obtained from an adult female at a live animal market in Sokoto State. We did not test sheep serum samples by IFAT. We found 96.0% concordance between ELISA and IFAT results for cattle.

Conclusions

Serologic and virologic evidence of CCHFV in humans has been reported in Nigeria (*11*). However, to our knowledge, CCHFV presence among domestic animals in the country has not been documented since the studies of Causey et al. (*3*) and Umoh et al. (*4*) conducted >3 decades ago. Detection of CCHFV antibodies in domestic animals is useful because it provides evidence of circulating virus, identifies the location of CCHFV foci, and highlights a potential and increased risk for human infection (*12*). Thus, our findings provide valuable serologic proof of the continued occurrence of CCHFV infection among domestic ruminants in Nigeria because there is no vaccine currently available against this disease (*12*).

We obtained higher CCHFV antibody prevalence rates for cattle, indicating that they were more exposed to the virus than goats and sheep. This finding suggests that in Nigeria, cattle might play a major role in the maintenance, circulation, and epidemiology of CCHFV, an observation that corroborates earlier reports that CCHFV infection appeared to occur most frequently in larger mammals such as cattle, which are the preferred hosts of adult *Hyalomma* ticks (*13*).

Although we found 96% concordance between ELISA and IFAT in this study, the IFAT detected 2 additional positive samples from cattle. This discrepancy could be related to differences in serum starting dilutions used in the 2 tests: 1:100 for the ELISA and 1:20 for the IFAT. Nevertheless, the higher CCHFV

Table. CCHFV IgG ELISA antibody prevalence among cattle, goats, and sheep in Nigeria, 2015*

	Cattle			Goats			Sheep		
		No. (%) positive			No. (%) positive			No. (%) positive	
State	No. tested	ELISA	IFAT	No. tested	ELISA	IFAT	No. tested	ELISA	IFAT
Oyo	20	3 (15.0)	3 (15.0)	13	0	0	30	0	NT
Lagos	NS	–	–	2	0	0	NS	–	–
Ogun	NS	–	–	NS	–	–	20	0	NT
Benue	10	5	6	NS	–	–	NS	–	–
Sokoto	10	1	1	35	1 (2.9)	1 (2.9)	NS	–	–
Borno	10	3	4	NS	–	–	NS	–	–
Total	50	12 (24.0)	14 (28.0)	50	1 (2.0)	1 (2.0)	50	0	–

*CCHFV, Crimean-Congo hemorrhagic fever virus; IFAT, indirect fluorescent antibody test; NS, no samples, NT, not tested; –, no results.

seropositivity obtained in the Sudan savannah (Sokoto and Borno states) and Guinea savannah (Benue State) zones is consistent with a report from Senegal (*14*), which found that CCHFV transmission was most intense in the northern, drier, and sparsely vegetated Sahelian ecozone than in the southern, more humid sub-Guinean (rainforest) zone.

Although *Hyalomma* ticks are the principal transmitters of CCHF in nature, transmission can also occur in their absence (i.e., person-to-person or by infected animals in abattoirs). Moreover, the prevalence of CCHFV antibodies in livestock and humans coincides with the distribution of these arthropods (*15*). Thus, detection of seropositive animals in this study provides good evidence of circulation of the virus among cattle and goats in Nigeria, making them a public health risk. Therefore, our findings highlight the need to fully investigate the transmission dynamics between tick reservoirs and virus-amplifying ruminants across Nigeria to engender improved understanding of virus–host relationships, the characterization of circulating CCHFV, improved diagnostics and monitoring systems, and better disease control.

This study was supported in part by a Grant-in-Aid from the Research Program on Emerging and Re-emerging Infectious Diseases from the Japan Agency for Medical Research and Development; the International Development Group of Massey University, New Zealand; the Regional Training in Animal and Human Health Epidemiology South Asia Program (funded by the European Commission through the Avian and Human Influenza Trust Fund administered by the World Bank and Massey University); and by a Grant-in-Aid for New and Emerging Vector-Borne Diseases from Public Health England.

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Recurrent Herpes Simplex Virus 2 Lymphocytic Meningitis in Patient with IgG Subclass 2 Deficiency

Tanvi Goyal, Imran Ali

We report a case of a patient with a lifetime history of 8 episodes of recurrent lymphocytic meningitis. Our findings suggest that susceptibility to recurrent lymphocytic meningitis might be caused by low serum IgG subclass 2 immunodeficiency.

Recurrent benign lymphocytic meningitis (RBLM) is an uncommon cause of meningitis; its clinical features include episodes of aseptic meningitis followed by complete recovery and unpredictable recurrence (*1*). The most common cause is herpes simplex virus-2 (HSV-2), a member of the family *Herpesviridae*. According to older case series, HSV has accounted for an estimated 0.5%–18% of viral meningitis cases (*2*). A study conducted by Kallio-Laine et al. (*1*) in Finland among 665 patients treated for lymphocytic meningitis indicated a prevalence of HSV-2–associated meningitis of 2.2 cases/100,000 population. RBLM is estimated to occur after primary HSV-2 meningitis in 20%–30% of cases (*3*). Previous research has reported an association of low serum IgG subclass 1 (IgG1) and IgG subclass 3 (IgG3) levels with increased frequency of RBLM (*2*). We report an unusual case of serum IgG subclass 2 immunodeficiency in a patient with 8 lifetime episodes of RBLM.

The Case

The case-patient was a 61-year-old woman with a history of RBLM. She had experienced 7 episodes of viral meningitis during 1978–1998. Her eighth episode occurred >20 years later. During this latest episode, the patient sought care for acute onset of bilateral diffuse headaches, with pain radiating to the neck. Additional symptoms included nausea and photophobia.

Physical examination revealed a temperature of 98.2°F, nuchal rigidity, positive Kernig sign, and no rash. Serum laboratory results showed neutrophilic and lymphocytic leukocytosis (15% neutrophils and 66% lymphocytes, reference value 0 for both). The total nucleated cell count was 283 cells/μL (reference range 0–5 cells/μL). Cranial computed tomography without contrast was unremarkable. Cerebrospinal fluid (CSF) analysis showed pleocytosis with a lymphocytic predominance, elevated protein at 114 mg/dL (reference range 15–45 mg/dL), and normal glucose at 44 mg/dL (reference range 40–70 mg/dL), compared with a serum glucose level of 83 mg/dL. A CSF culture with Gram stain did not show bacterial growth.

The patient also was tested for autoimmune diseases and potential immunodeficiency to determine a possible cause for her recurrent meningitis. Antinuclear antibody was positive with a titer of 1:160; however, the extractable nuclear antigen panel was negative. Chromatin IgG, double-stranded DNA, Smith, and RNA antibodies were all negative by ELISA. Immunoglobulin testing showed decreased IgM at 26 mg/dL (reference range 45–281 mg/dL), indicating an IgM deficiency. IgG subclasses showed decreased IgG2 at 130.3 mg/dL (reference range 242–700 mg/dL) and normal (reference) levels of IgG1, IgG3, and IgG4.

The patient received 1 dose intravenously of each of the following: vancomycin (20 mg/kg), ceftriaxone (2 g), and ampicillin (2 g) until the CSF meningitis panel showed HSV-2, at which time the patient's treatment was switched to intravenous acyclovir (10 mg/kg) every 8 hours for 3 days. She was discharged on day 3, noting improvements in headache and neck pain, with a 7-day course of oral valacyclovir (500 mg) twice daily. Further review showed no history of oral or genital herpes infection.

Conclusions

The syndrome of idiopathic recurrent lymphocytic meningitis was first described in 1944 by French neurologist Pierre Mollaret for 3 patients who had short-lasting episodes of recurrent fever, headache,

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DOI: https://doi.org/10.3201/eid2604.190406

and vomiting caused by aseptic meningitis (*4*). Although the term "Mollaret's meningitis" is reserved for idiopathic recurrent aseptic meningitis (a term established before the advent of PCR), and recurrent meningitis attributable to HSV-2 (or less commonly, Epstein–Barr virus) is called "recurrent viral meningitis," the 2 conditions are clinically indistinguishable (*4*,*5*).

More than 50% of patients with recurrent lymphocytic meningitis attributable to HSV-2 do not report a history of genital herpes (*5*). Moreover, active genital vesicular lesions during episodes of viral HSV-2 meningitis are not a consistent finding (*5*).

Why some patients with HSV-2 infection have recurrent viral meningitis is not known. However, some evidence points toward an immune-mediated pathology in RBLM. Franzen-Röhl et al. (*6*) showed that, contrary to their hypothesis, patients with recurrent HSV-2 meningitis have a stronger HSV-specific cell-mediated immune response compared with patients with recurrent genital HSV-2 infections and healthy seropositive persons. RBLM patients were found to have increased expression of Toll-like receptors (TLRs) and increased interferon (IFN)-α and specific T cell responses, suggesting that the proinflammatory state leads to a more severe clinical disease because of destruction of the blood–brain barrier, tissue remodeling, and vascular leakage (*6*). A case report by Willmann et al. (*7*) demonstrated a TLR-3 deficiency in a patient with RBLM and called for testing of TLR-3 alleles to gain further information on this receptor's relevance to RBLM. Bonnin et al. (*8*) reported a case of RBLM associated with a complement factor 1 deficiency, and Snowden et al. (*9*) reported a case of RBLM associated with hereditary isolated IgG3 deficiency.

IgG1 and IgG3, which largely act against protein components such as tetanus and diphtheria toxin, mediate antibody-dependent cellular cytotoxicity important for clearance of bacterial and viral infections. IgG2 forms immune complexes to trigger complement-independent macrophage and polymorphonuclear cell–mediated phagocytic activity largely against polysaccharide capsules of certain bacteria, including *Streptococcus pneumonia*, *Neisseria meningitides*, and *Haemophilus influenzae* (*10*). Antiviral IgG can prevent infection, decrease virus replication, clear viral infection, and eliminate or lessen the severity of disease (*11*).

IFNs induce antiviral activity in many cell types. The ability of IFN-γ to inhibit replication of herpes simplex–1 viruses in mouse macrophages correlated with the cells' production of nitric oxide (*12*). Inoue et al. (*13*) reported that interferon-γ production by peripheral blood mononuclear cells was decreased among patients with IgG2 deficiency. Furthermore, Kondo et al. (*14*) reported that reduced expression of IFN-γ messenger RNA might play a role in IgG2 deficiency. This research points toward a mechanism by which IgG2 deficiency might lead to recurrent viral meningitis episodes and the associated pathology.

In a study of 21 patients with RBLM, Kallio-Laine et al. (*2*) showed that low serum IgG1 levels were associated with increased frequency of recurrent meningitis episodes. They also reported a trend toward lower serum IgG3 in RBLM patients. However, to our knowledge, no previous research has specifically linked low levels of serum IgG2 with increased frequency of recurrent HSV-2 meningitis episodes.

In addition, although single Ig deficiencies on their own might not necessarily increase susceptibility to infection, combined influences of multiple deficiencies might modulate disease progression and be clinically relevant. Our findings demonstrate a possible immune linkage with increased frequency of RBLM, but more research is needed to determine how this information can be used in the management and treatment of RBLM patients.

Clinicians should suspect HSV-2 in all cases of RBLM, given that HSV-2 is the most common cause. Recognition of RBLM will lead to earlier diagnoses, decreased hospital stays, less unnecessary testing, better clinical treatment, and improved outcomes. Whereas previous research and case reports have shown an association of RBLM with deficiencies in IgG1 and IgG3, our findings suggest that susceptibility to RBLM might also be caused by low serum immunoglobulin IgG2 (*2*,*9*). Although some research has already suggested an immunologic pathology predisposing to recurrent HSV-2 viral meningitis, more research is needed to understand the complete underlying pathophysiology and determine how this information can be used to evaluate, manage, and improve outcomes for patients with RBLM.

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Health-Related Quality of Life after Dengue Fever, Morelos, Mexico, 2016–2017

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We adapted the EQ-5D-3L questionnaire and visual analog scale to assess health-related quality of life (HRQOL) and persistent symptoms in 79 patients with laboratory-confirmed dengue in Morelos, Mexico. The lowest HRQOLs were 0.53 and 38.1 (febrile phase). Patients recovered baseline HRQOL in ≈2 months.

Each year, up to 400 million dengue virus (DENV) infections and ≈40,000 deaths occur globally, costing ≈US $9 billion (*1*–*3*). Accurate estimates of disease are needed to track health progress, evaluate prevention and control technologies, and define research priorities (*4*). However, substantial heterogeneity exists in estimates of disease severity and sequelae (*5*). Research suggests dengue symptoms may persist well beyond the acute febrile phase in some patients (*6*–*8*). Little is known about health-related quality of life (HRQOL) for dengue (*7*,*8*). Despite acknowledgement of symptom persistence since 1997 (*9*), most studies focus on the febrile phase, probably substantially underestimating long-term effects of dengue (*2*,*3*,*6*). We investigated HRQOL of dengue patients during their entire laboratory-confirmed dengue episode.

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DOI: https://doi.org/10.3201/eid2604.190729

The Study

All study participants signed informed consent forms. The Ethics Committee of the National Institute of Public Health (project nos. 1223, 1755) approved the study.

We recruited participants with dengue from inpatient and outpatient facilities in Morelos, Mexico, during 2016–2017. Inclusion criteria were age ≥18 years, visit to a healthcare facility 2–6 days after fever onset, laboratory confirmation of DENV infection, permanent residence in Morelos, and a landline telephone. We excluded patients with cognitive impairment, psychiatric diagnoses, specific chronic diseases, and pregnancy. The final sample comprised 79 patients (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/26/4/19-0729-App1.pdf).

Participants underwent a face-to-face questionnaire interview during the febrile phase and were contacted for follow-up regularly for 1 month. After 1 month, participants were contacted until they did not have dengue symptoms or until 6 months after fever onset (Appendix Table 2). Thus, estimates of HRQOL after 1 month constituted only patients with persistent symptoms.

We used an adapted version of a 3-level EQ-5D (EQ-5D-3L) instrument, a standardized method for measuring health status, to measure patients' HRQOL (*10*), including a visual analog scale (EQ-VAS) to estimate self-reported health status. The EQ-5D-3L questionnaire collects information about patient quality of life in 5 health domains: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression. We also measured quality of life using the EQ-VAS scale (0–100, worst to best health).

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Table 1. Patients with laboratory-confirmed dengue who reported some or extreme problems during the first 30 days after onset of dengue fever symptoms, Morelos, Mexico, 2016–2017*

EQ-5D-3L dimension	No. (%) patients, N = 79			
	Before fever, n = 77	1–6 d, n = 79	7–15 d, n = 71	16–30 d, n = 74
Mobility	1 (1.3)	63 (79.7)	57 (80.3)	59 (79.7)
Self-care	0	43 (54.4)	39 (54.9)	42 (56.8)
Usual activities	2 (2.5)	69 (87.3)	65 (91.5)	65 (87.8)
Pain/discomfort	2 (2.5)	73 (92.4)	66 (93)	63 (85.1)
Anxiety/depression	4 (5.1)	27 (34.2)	28 (39.4)	30 (40.5)

*Patients were >18 years of age. n values indicate number of patients responding to questionnaire during the indicated day range. Health-related quality of life was assessed by an adapted EQ-5D-3L questionnaire (Appendix Table 3, https://wwwnc.cdc.gov/EID/article/26/4/19-0729-App1.pdf) for reporting of problems after 1 month since fever onset (i.e., days 31–60, 61–120, 121–180).

We then created a single EQ-5D-3L index value for the HRQOL (0–1, worst to best health; Appendix Table 4) (*11*). We divided time into day-ranges (0–6, 7–15, 16–30, 31–60, 61–120, and 121–180) because not all participants responded to the questionnaires on the exact same days.

We analyzed changes in HRQOL over time using survival and Cox regression analyses. We defined recovery as baseline HRQOL (before DENV infection) and calculated the time it took each patient to recover. We estimated HRQOL recovery time for subgroups of patients using Kaplan-Meier with log-rank test statistic and identified significant predictors of HRQOL using Cox regression analyses.

The final sample comprised 62% ambulatory and 38% hospitalized patients. Most participants (retrospectively) reported no symptoms before dengue onset. The most affected domains were pain/discomfort, usual activities, and mobility. Almost all participants reported some/extreme problems during the first 6 days (92% pain/discomfort, 87% usual activities, 80% mobility). The proportion of participants reporting problems in any domain increased at 7–15 days after fever onset and remained largely stable until day 30 (Table 1). Among sampled patients, 56% reported dengue-related symptoms ≥30 days; 48%, ≥1 severe symptom; and 73%, ≥1 warning sign. Participants needed an

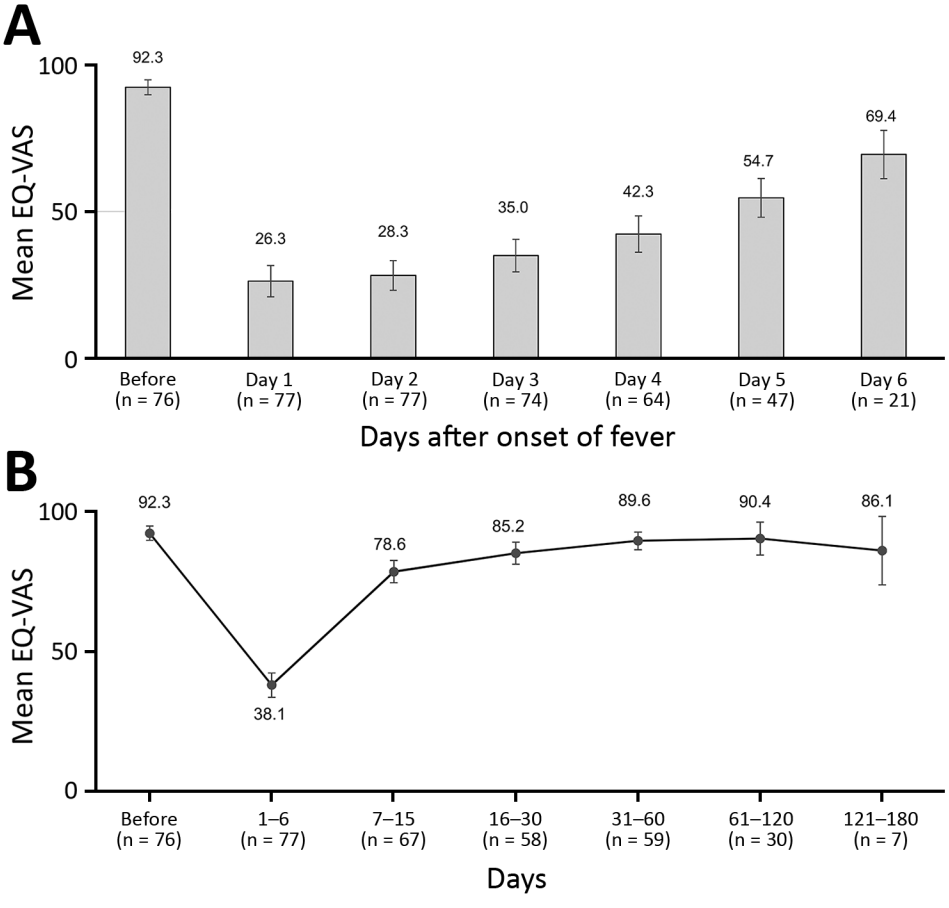

Figure 1. Average self-reported health status, as measured by a 0–100 EQ-VAS, of patients with laboratory-confirmed dengue during the first week after onset of dengue symptoms (A) and from baseline to 121–180 days (B), Morelos, Mexico, 2016–2017. The EQ-VAS scale measures self-reported health, ranging from 0 (worst health status) to 100 (best health status). EQ-VAS is part of the EQ-5D-3L instrument for measuring health-related quality of life. EQ-VAS, visual analog scale. n values indicate number of patients responding to questionnaire during the indicated day range. Error bars indicate 95% CI.

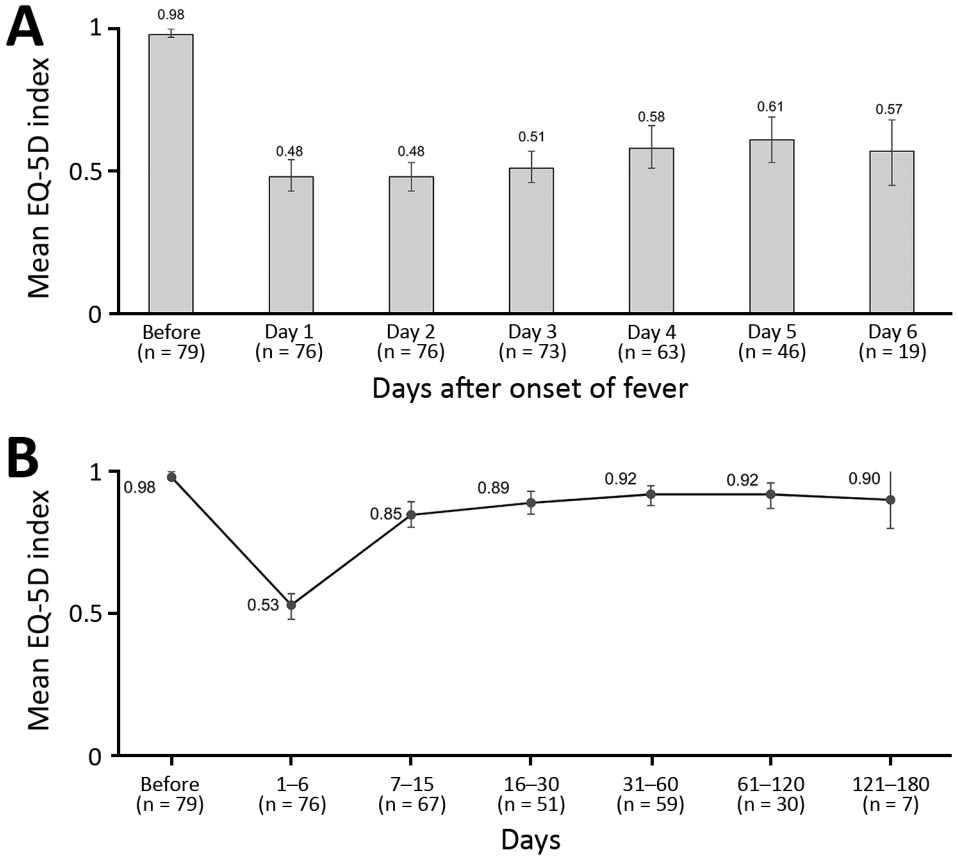

Figure 2. Average health-related quality of life, as measured by the EQ-5D index score, in patients with laboratory-confirmed dengue during days 1–6 of a dengue fever episode (A) and from baseline to 121–180 days (B), Morelos, Mexico, 2016–2017. The EQ-5D scale is a standardized method for measuring health status. n values indicate number of patients responding to questionnaire during the indicated day range. Error bars indicate 95% CI.

average of 46.7 days to completely recover their baseline HRQOL.

We also assessed participants' self-reported health status (EQ-VAS) during the first 6 days (Figure 1, panel A). Participants reported good health at baseline (EQ-VAS 92.3 [95% CI 89.8–94.8]). The worst health was reported during the first day (EQ-VAS 26.3 [95% CI 20.9–31.7]) and second day (EQ-VAS 28.3 [95% CI 23.2–33.3]) and slowly improved until day 6 (EQ-VAS 69.4 [95% CI 61.2–77.7]) but remained well below baseline. When we analyzed the evolution of perceived health until the end of the study (Figure 1, panel B), mean EQ-VAS was 38.1 (95% CI 33.8–42.5) for days 1–6, the lowest observed for any day range. The mean EQ-VAS score then improved until days 61–120 (EQ-VAS 90.4 [95% CI 84.5–96.3]), when it no longer differed significantly from baseline ($\alpha = 0.05$).

We assessed the mean EQ-5D index score before DENV infection (baseline) and during the first 6 days of illness (Figure 2, panel A). Participants showed high baseline scores (EQ-VAS 0.98 [95% CI 0.96–0.99]). The mean EQ-5D index score dropped by >50% to 0.48 (95% CI 0.42–0.49) during the first day and was 0.57 (95% CI 0.46–0.69) on day 6. During the course of the study period, the EQ-VAS was low during the first 6 days (0.53 [95% CI 49–0.58]) and increased to 0.85 (95% CI 0.80–0.89) for days 7–15 (Figure 2, panel B). The index EQ-VAS did not differ significantly from baseline after ≈61 days (0.92 [95% CI 0.88–0.98]).

We tested differences in HRQOL recovery time using Kaplan-Meier curves for individual subgroups (Appendix Figure 1). Survival curves showed slower recovery times for hospitalized participants (≈40% recovered baseline HRQOL) than for ambulatory participants (≈75%) after 30 days ($p = 0.012$). Participants with severe symptoms (≈30%) also showed slower recovery than did participants without severe symptoms (≈75%) after 20 days ($p = 0.001$), as did participants with ≥1 warning signs (≈40%) compared with participants without warning signs (≈85%) after 15 days ($p<0.001$). Participants with higher education had a faster recovery of HRQOL than did participants with less education ($p<0.001$).

We used a Cox regression analysis (Table 2) to identify factors associated with HRQOL recovery (model: proportionality confirmed; mean variance inflation factor = 1.09, all variables variance inflation factor <1.21; final model χ^2 37.8, $p<0.001$; McFadden pseudo-$R^2 = 0.11$). Recovery rates were higher for men than for women (hazard ratio [HR] 1.87; $p = 0.036$),

Table 2. Results of the Cox regression analysis to identify factors associated with recovering baseline health-related quality of life, Morelos, Mexico, 2016–2017

Factor	Hazard ratio (95% CI)	p value
Sex		
F	Referent	
M	1.87 (1.04–3.37)	0.036
Age, y		
≥38	Referent	
18–37	1.74 (0.93–3.23)	0.082
Educational level		
Primary/secondary school	Referent	
High school or higher	2.06 (1.03–4.11)	0.042
Symptoms		
Severe symptoms		
Presence	Referent	
Absence of ≥1	2.82 (1.50–5.33)	0.001
Persistence of symptoms		
No persistence	Referent	
Persistence <30 d	2.28 (1.24–4.19)	0.008
Specific symptoms in the first 15 d		
Presence of specific symptom	Referent	
Absence of skin ache	0.37 (0.19–0.70)	0.002
Absence of scaling skin	0.33 (0.11–0.94)	0.038
Absence of abdominal pain	1.65 (0.79–3.44)	0.182

patients with more education (HR 2.06; p = 0.042), and patients with no severe symptoms (HR 2.82; p = 0.001). In the first 15 days of disease, dengue patients without skin ache had a 63% lower likelihood (HR 0.37; p = 0.002) and patients without scaling had a 67% lower likelihood (HR 0.33, p = 0.038) of recovering to baseline HRQOL.

Conclusions

Dengue significantly reduces HRQOL beyond the febrile phase. Mobility, pain, and usual activities were the most affected domains, consistent with previous studies (*8*,*12*). The proportion of patients reporting problems remained stable among patients with persistent symptoms of dengue. HRQOL decreased abruptly during the febrile phase; most patients then steadily recovered, with some exceptions for those who had not reached baseline HRQOL at 6 months. Other studies have found larger reductions of HRQOL than we found; mean EQ-VAS score was 7 for children 0–14 years of age in Cambodia (*13*) and 10 for hospitalized patients and 20 for ambulatory patients in Brazil (*7*). Our findings were comparable to those of Armien et al. (*14*) in Panama (EQ-VAS 35.2 for children; 31.9 for adults). Female sex was significantly associated with dengue severity in our study, and education (a proxy for socioeconomic status) might be a protective factor. We found skin symptoms to be associated with a faster recovery, possibly because of a lower inflammatory or immune response (*15*).

Our findings are subject to limitations: an adults-only sample; limited socioeconomic characterization of participants; lack of data about previous DENV infections; limitations of the EQ-5D-3L instrument; possible recall bias for baseline HRQOL; response-, recalibration-, and reconceptualization response–shift biases; and a relatively small sample of patients with laboratory-confirmed dengue. Despite these limitations, our findings are relevant for clinical practice and health services research and can help researchers and other stakeholders improve estimates of dengue effects.

Acknowledgments

We thank Jose Ramos-Castañeda's team "Prevención y Control de Enfermedades Transmitidas por Vectores" for its support and cooperation during our research internship in the National Institute of Public Health in Cuernavaca, Mexico. A.S. and I.W. acknowledge the support in the form of professional advice of the National Institute of Public Health. We also thank Clare Hurley for editorial assistance.

This work was supported by Americas Health Foundation Latin American Prizes for Dengue Initiatives to J.R.-C. Data collection was supported in part by a subcontract to the Mexico National Institute of Public Health under a research agreement from Sanofi Pasteur to Brandeis University. The "Stiftung für Begabtenförderung und Berufliche Bildung (sbb)" and the "Deutscher Akademischer Austauschdienst (DAAD)" provided scholarships for our academic project in Mexico.

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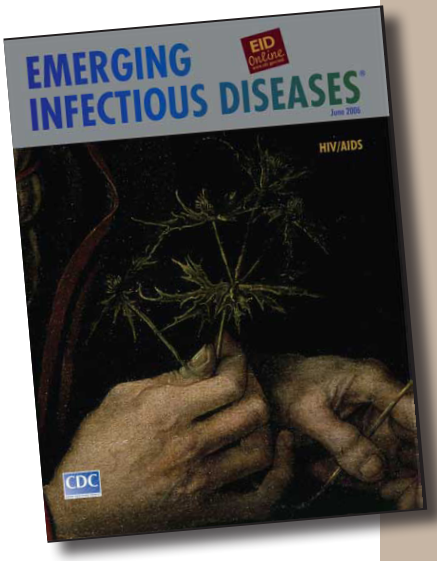

Originally published in June 2006

An acute, self-limited disease characterized by fever, headache, myalgia, and rash caused by any of 4 related but distinct viruses of the genus *Flavivirus* and spread by *Aedes* mosquitoes. Dengue (a Spanish homonym for the Swahili *ki denga pepo*, which describes a sudden, cramplike seizure caused by an evil spirit) is believed to have been first recorded in a Chinese medical encyclopedia from the Chin Dynasty (265–420 AD). The Chinese called dengue "water poison" and knew that it was somehow associated with flying insects.

Sources: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003; Gubler DJ. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev. 1998;11: 480–96; and Halstead SB. Dengue hemorrhagic fever—a public health problem and a field for research. Bull World Health Organ. 1980;58:1–21

https://wwwnc.cdc.gov/eid/article/12/6/et-1206_article

Person-to-Person Transmission of Andes Virus in Hantavirus Pulmonary Syndrome, Argentina, 2014

Daniel O. Alonso, Unai Pérez-Sautu, Carla M. Bellomo, Karla Prieto, Ayelén Iglesias, Rocío Coelho, Natalia Periolo, Isabel Domenech, Gabriel Talmon, Romina Hansen, Gustavo Palacios, Valeria P. Martinez

Andes virus is unique among hantaviruses because it can be transmitted from person to person. This mechanism was previously supported by epidemiologic data and genetic evidence based only on partial sequences. We used full-length virus sequencing to confirm person-to-person transmission of this virus in a cluster of 3 cases in Argentina in 2014.

Pathogenic hantaviruses are members of the family *Hantaviridae* and genus *Orthohantavirus*. These viruses are responsible for hantavirus pulmonary syndrome (HPS) in the Americas. In Argentina, HPS was first described in 1995 during an outbreak in the Andean region of Patagonia, leading to the characterization of Andes virus (ANDV) (*1*). Since then, >1,200 cases have been confirmed in Argentina (*2*,*3*).

Hantaviruses are enveloped, single-stranded, RNA viruses with tripartite negative sense genomes. The small (S, 1.8–2.1 kb) segment encodes a nucleocapsid protein, the medium (M, 3.6–3.8 kb) a glycoprotein precursor, and the large (L, 6.5–6.7 kb) an RNA-dependent RNA polymerase (*4*).

Humans usually become infected with hantaviruses through inhalation of aerosolized excreta produced by infected rodents. ANDV is the unique hantavirus capable of being transmitted from person-to-person (*5*–*7*). Infection by this route takes place during the early prodromal phase, and the incubation period ranges from 9 to 40 days (*8*).

In previous outbreaks, genetic analysis was performed on partial sequences of ANDV, which represented ≈10% of the genome. Thus, the aim of this study was to analyze a cluster of 3 case-patients for whom epidemiologic data were available and compare complete viral genome sequences to assess person-to-person transmission or co-exposure to the same rodent population.

The Study

The occurrence of 3 clustered cases during 2014 led us to suspect person-to-person transmission. The cases were reported during a 43-day period in El Bolsón, Rio Negro. The 3 case-patients had severe disease; 2 of these case-patients (P1 and P2) died (Table 1). P1 and P2, who were twin brothers, had symptoms develop 2 weeks apart, and each sought care at a primary healthcare center (Hospital Area El Bolsón, Rio Negro). P3 was a nurse who attended P2 during this initial hospitalization. After the beginning of the cardiopulmonary phase, each patient was transferred to a high-complexity hospital in Bariloche (Hospital Zonal Bariloche). P1 died 5 days and P2 7 days after symptom onset; P3 survived.

For our investigation, we included 2 unrelated HPS case-patients (NCR1 and NCR2) from the same area for comparison. We confirmed HPS in all 5 patients by using laboratory detection of ANDV-specific IgM and reverse transcription quantitative PCR, as described (*3*). All patients, except P2, had ANDV-specific IgG.

For whole-genome sequencing, we extracted RNA from peripheral blood of patients and prepared

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DOI: https://doi.org/10.3201/eid2604.190799

Table 1. Epidemiologic characteristics of 5 patients with hantavirus pulmonary syndrome, Argentina, 2014*

Patient	Age, y/sex	Date of symptom onset	Date of death	Relationship	Place of residence or work	Risk activity
P1	71/M	2014 May 10	2014 May 15	Twin brother of P2	Los Repollos forest reserve, Río Negro Province	Collecting firewood
P2	71/M	2014 May 25	2014 Jun 1	Twin brother of P1	Los Repollos forest reserve, rural area, Río Negro Province	Collecting firewood, contact with P1
P3	53/F	2014 Jun 16	Survived	Nurse of P2	El Bolsón, semiurban area, no evidence of rodent exposure in her home, nurse at HAEB	Assisted P2 at HAEB on May 27 and 28, before his transfer to HZB
NRC1	41/M	2014 Apr 30	2014 May 5	None	El Blanco, rural area, Cholila, Chubut Province	Daily contact with rodents in poor habitat conditions
NRC2	36/F	2017 May 11	Survived	None	Epuyen, rural town Chubut Province, high school teacher	Camping activities in Los Alerces National Park

*HAEB, Hospital Area El Bolsón (primary healthcare center); HZB, Hospital Zonal Bariloche (high-complexity hospital).

sequencing libraries by using target-enrichment technology and ANDV-specific probes. As expected, we identified the South variant of ANDV in the 5 case-patients. Comparative analysis showed 100% nucleotide identity in the whole genome between the samples from patients P2 and P3 (P2/P3 genome). Patient P1 had 100% nucleotide identity in the complete S and M segments with P2/P3 but had 2 nt changes in the L segment (99.95% nucleotide identity) (Table 2, https://wwwnc.cdc.gov/EID/article/26/4/19-0799-T2.htm); both differences were silent mutations.

On the basis of accurate epidemiologic findings, the only source of infection for P3 was her contact with P2 at Hospital Area El Bolsón. Despite the nucleotide differences between their isolates, co-exposure of P1 and P2 should not be discarded as a source of infection because these persons lived in the same house where they shared the same room and bed. However, even if one considers that these nucleotide changes were 2 silent mutations in the whole viral genome, person-to-person transmission is still the most probable way of infection for P2. A previous study reported a high degree of sequence diversity for the L segment of Puumala virus (*9*), which is consistent with our results.

For further comparison, we obtained the complete sequences of 4 ANDV genomes circulating during a short period in the same area where this virus was first described (*1*). Nucleotide identity among strains from Argentina for the S and M segments ranged from 94.5% to 98.7%; for the L segment, it ranged from 93.6% to 98.7%. These comparisons included reference sequences for a strain from Chile. We observed a higher genetic identity between virus strains from patients P1, P2, P3, and NRC2. However, the site from which virus from NRC1 was isolated was closer to the sites of isolation of viruses from P1, P2, and P3 than to the site of isolation for virus from NRC2 (Figure 1). This finding confirmed the presence

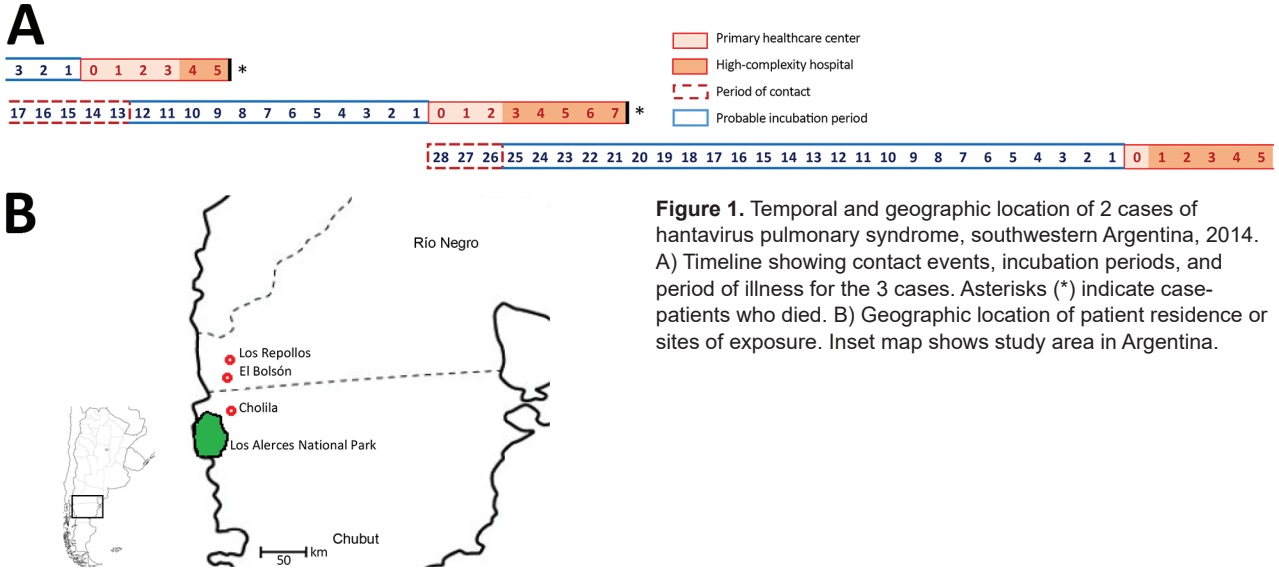

Figure 1. Temporal and geographic location of 2 cases of hantavirus pulmonary syndrome, southwestern Argentina, 2014. A) Timeline showing contact events, incubation periods, and period of illness for the 3 cases. Asterisks (*) indicate case-patients who died. B) Geographic location of patient residence or sites of exposure. Inset map shows study area in Argentina.

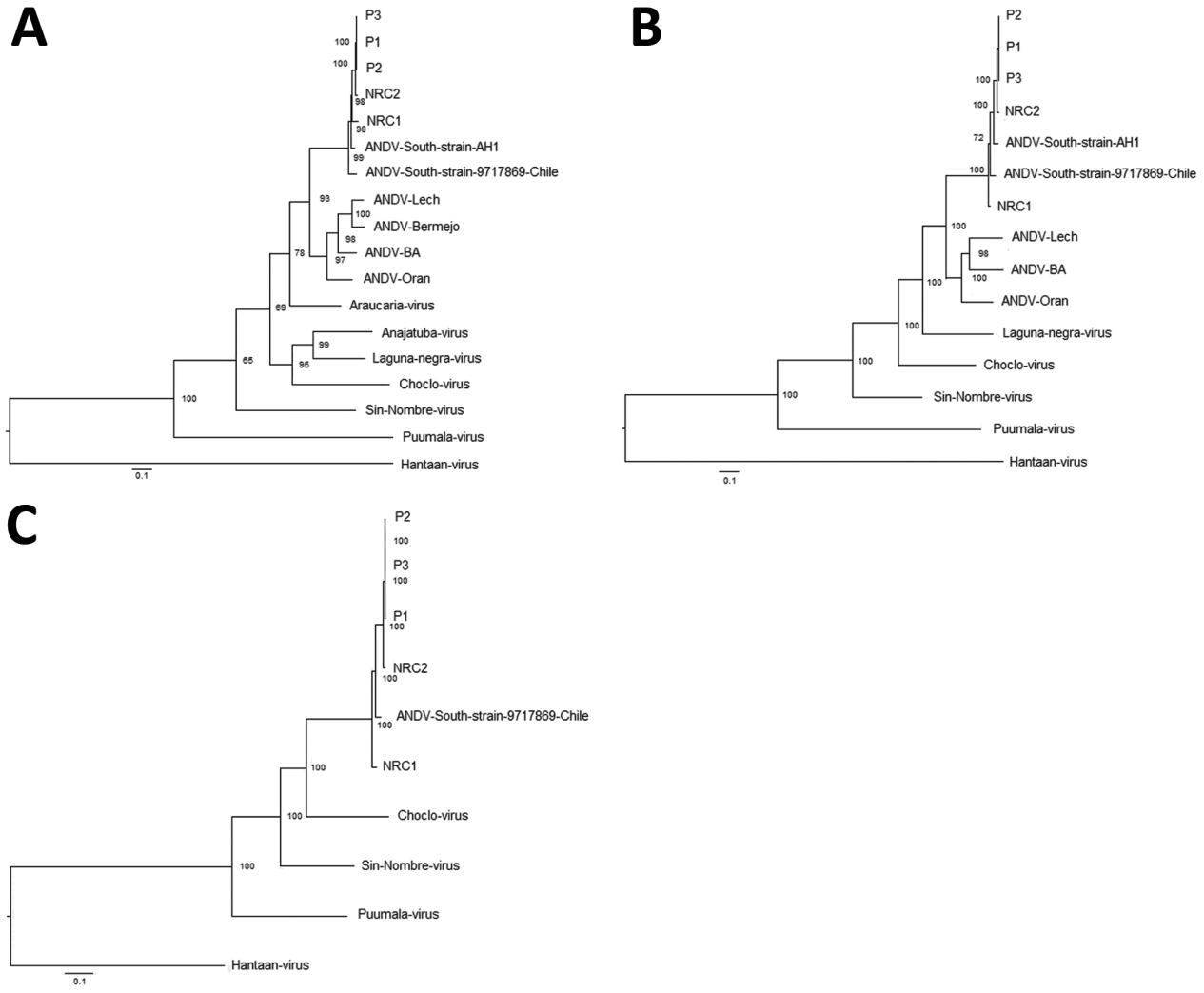

Figure 2. Phylogenetic analysis of hantaviruses based on complete genome of Andes virus (ANDV) isolated from case-patients in Argentina, 2014, and other orthohantaviruses characterized previously. A) Small (S) segment; B) medium (M) segment; C) large (L) segment. We used MrBayes version 3.2.7 (https://nbisweden.github.io/MrBayes) to reconstruct Bayesian maximum clade credibility trees. Numbers along branches are bootstrap values. Bootstrap support was based on 1,000 maximum-likelihood replicates. Scale bars indicate nucleotide substitutions per site. GenBank accession nos.: ANDV-South P1, S: MN850083, M: MN850088, L: MN850093; ANDV-South P2, S: MN850084, M: MN850089, L: MN850094; ANDV-South P3, S: MN850085, M: MN850090, L: MN850095; ANDV-South NRC1, S: MN850086, M: MN850091, L: MN850096; ANDV-South NRC2, S: MN850087, M: MN850092, L: MN850097; ANDV-Orán, S: AF325966, M: AF028024; Laguna Negra virus, S: NC038505, M: NC038506; ANDV-South, S: AF004660, M: AF324901; ANDV-South strain 9717869 Chile, S: AF291702, M: AF291703, L: AF291704; ANDV-Lech, S: AF482714, M: AF028022; ANDV-Bermejo, S: AF482713; Araucaria virus, S: AY740633; Anajatuba virus, S: JX443690; Choclo virus, S: KT983771, M: KT983772, L: EF397003; Sin Nombre virus, S: NC_005216, M: NC_005215, L: NC_005217; Puumala virus, S: NC_005224, M: NC_005223, L: NC_005225; Hantaan virus, S: JQ083395, M: JQ083394, L: JQ083393.

of different subtypes of the ANDV South variant co-circulating in nearby areas. Phylogenetic analysis showed that viruses from case-patients P1, P2, and P3 clustered together; NRC2 had the highest identity values for the 3 genomic segments (Figure 2).

The open reading frames encoding the nucleoprotein, glycoprotein precursor, and RNA polymerase had the same size as sizes of published sequences of ANDV (*10*–*12*). The highest degree of identity for the 4 proteins was for virus from NRC2. We compared predicted amino acid sequences with all available complete sequences of ANDV variants circulating in Argentina (South, Lech, BsAs, and Orán). Virus from P1, P2, P3, and NRC2 had 2 identical amino acid differences (T641I and T938A) in the predicted glycoprotein precursor. These

differences were not found in any other sequences analyzed. Minor amino acid changes could have major effects on virus properties. Whether an amino acid substitution in viruses from HPS case-patients could determine the person-to-person transmission mechanism should be addressed by comparative analysis of higher numbers of complete virus sequences and specific studies on ANDV transmissibility.

Future studies are needed to obtain additional complete viral sequences from rodent populations co-circulating in the same geographic area. This information will enable definitive differentiation of person-to-person transmission from co-exposures in patients with similar activities of risk in disease-endemic regions, which was the case for P1 and P2, both of whom reported collecting firewood a forested area.

Conclusions

We characterized the complete genome of an ANDV strain involved in a person-to-person transmission chain by using target-specific whole-genome sequencing. Our study contributed useful data for clarifying properties involved in the unusual transmissibility of ANDV. These data are crucial for optimal management of HPS case-patients and control of future outbreaks of this lethal disease.

Acknowledgments

We thank the physicians and epidemiologists for collecting samples and providing epidemiologic data.

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Ebola Virus Neutralizing Antibodies in Dogs from Sierra Leone, 2017

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Ebola virus (EBOV) is a highly pathogenic zoonotic virus for which the reservoir host has not been identified. To study the role of dogs as potential hosts, we screened 300 serum samples from dogs in Sierra Leone and found EBOV neutralizing antibodies in 12, suggesting their susceptibility to natural infection.

Ebolaviruses (family *Filoviridae*) comprise highly pathogenic RNA viruses with zoonotic potential. After sporadic introduction from an animal reservoir into the human population, the main route of transmission has been from human to human, causing outbreaks of hemorrhagic fever with case-fatality rates up to 90% (*1*). Although molecular and serologic evidence strongly points toward certain species of bat as reservoir hosts for ebolaviruses (*2*,*3*), a bat-derived Ebola virus (EBOV) isolate has not yet been detected. Despite intensive serologic surveillance focusing on the role of bats, wildlife, and livestock in EBOV ecology (*2*,*4*–*7*), to our knowledge, only 2 reports describe analysis of serum from dogs in Gabon and Liberia after Ebola virus disease (EVD) outbreaks in 2001 (Gabon) and 2014–2016 (Liberia) (*8*,*9*). Although antibodies against EBOV were detected by indirect ELISA, neither EBOV antigen nor viral genome was detected in samples from Gabon. The highest seroprevalence (31.8%) was reported from villages where dogs were reportedly exposed to the virus through contact with human EVD patients or by eating infected animal carcasses (*8*). In Liberia, a multiplex approach indicated that 47 (73%) of 64 dogs had potentially been exposed to filoviruses (*9*). To further investigate the role of dogs in EBOV ecology, we collected 300 serum samples from 174 male (58%) and 126 female (42%) dogs in Moyamba District, Sierra Leone (Figure 1).

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DOI: https://doi.org/10.3201/eid2604.190802

The Study

We sampled only owned and healthy dogs from communities that have been affected by the West Africa EVD outbreak (Appendix, https://wwwnc.cdc.gov/EID/article/26/4/19-0802-App1.pdf). Sampling was performed in rural and urban areas that had been affected by the historically largest EVD outbreak involving the Makona strain during 2014–2016 in West Africa. At the time of sample collection (October–December 2017), of the 300 dogs, 163 were >2 years of age (Table 1). Animals were handled according to a Njala University Institutional Review Board protocol (no. IRB00008861/FWA00018924).

Initially, we screened dog serum samples for the presence of EBOV nucleoprotein (NP)–specific antibodies in an indirect ELISA, as previously described for pigs (*5*), with slight modifications. Using a horseradish peroxidase–labeled protein A/G–specific conjugate, we considered 36 (12%) serum samples to be reactive toward the *Escherichia coli*–derived EBOV-NP (Tables 1, 2). Subsequent Western blot analyses based on insect cell–derived EBOV-NP (*5*) confirmed the presence of EBOV-NP reactive antibodies in 20 (6.6%) samples. Furthermore, we performed virus neutralization tests (VNTs) by using transcription and replication competent virus-like particles (trVLP) and authentic EBOV (variant Mayinga) as described previously (*5*,*10*) (Appendix). We found that 12 (4%) serum samples efficiently inhibited EBOV infection with robust neutralizing titers of 1:16–1:45 and that another 6 samples had weakly positive titers of 1:11–1:13 (Tables 1, 2). Overall, titers from the trVLP-based VNTs with an established cutoff at 80% inhibition of reporter activity were comparable to those of VNTs with live virus (Figure 2).

Conclusions

EBOV seroprevalence detected by ELISA in dogs from EVD-endemic areas in Gabon (25.2%–31.8%) (*8*) was lower than that detected in dogs in our study in Sierra Leone (12%). Technically, differences in detectable seroprevalence could be explained by different preparations used (virus-infected cell lysates in ELISA in

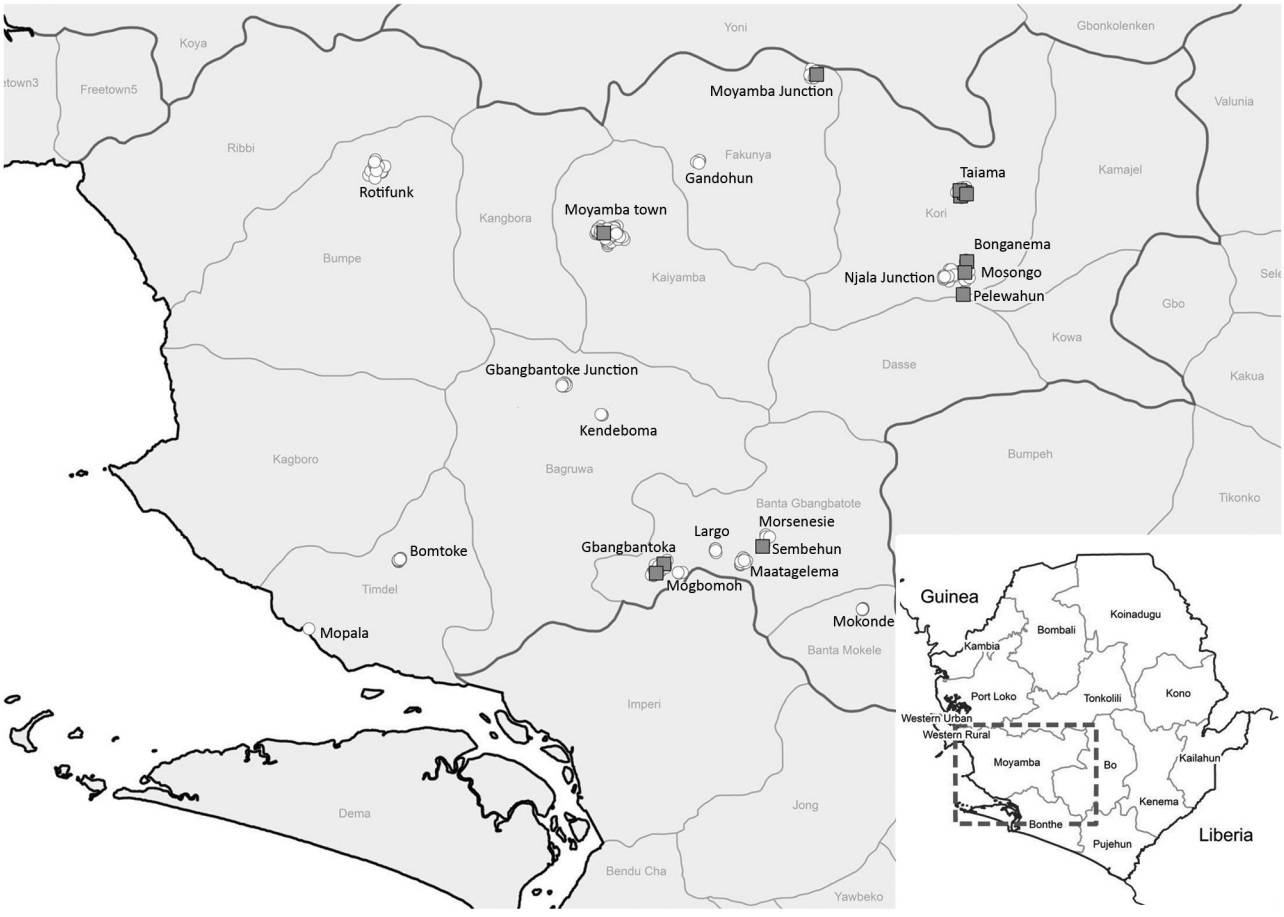

Figure 1. Sampling locations for study of Ebola virus neutralizing antibodies in dogs, Moyamba District, Sierra Leone, 2017. White circles indicate sampling locations; gray squares indicate dog serum samples with virus neutralizing activity. Inset shows location of Moyamba district in Sierra Leone.

Gabon [*8*] compared with single EBOV-NP preparations in our study). Apart from that difference, the observed variation might depend on selected sampling areas, animals, and time points of sampling because stability and persistence of neutralizing and NP-reactive antibodies in dogs after exposure are unknown. In pigs experimentally infected with EBOV, NP-specific antibody titers decreased within 28 days after infection, but neutralizing antibodies seemed to persist longer (*11*). Of note, Marburg virus IgG in convalescent *Rousettus aegyptiacus* bats decreased to undetectable titers at 3 weeks after infection (*12*). Nonetheless, the recent report of EBOV neutralizing antibodies in human survivors up to 40 years after infection (*13*) suggests a rather long-lasting but host-dependent antibody response after infection.

Reactivity of dog serum to EBOV-NP in ELISA and Western blots suggests exposure of the dogs to antigenically related ebolaviruses or Ebola-like viruses, as previously described for pigs (*5*). In our study, a novel ebolavirus, referred to as Bombali virus, which was recently discovered in insectivorous bats from the Bombali

Table 1. EBOV-specific antibodies detected in dog serum samples, by dog age, collected in Moyamba District, Sierra Leone, October–December 2017*

Age, mo.	No. samples tested	EBOV-NP ELISA, no. (%) reactive	Confirmatory EBOV-NP WB, no. (%) reactive	EBOV VNT	
				No. (%) positive	Titers
<12	27	2 (7.4)	0	0	NA
12 –18	60	7 (11.7)	6 (10.0)	5 (8.3)	1:11, 1:11, 1:13, 1:16, 1:27
19–24	50	4 (8.0)	3 (6.0)	1 (2.0)	1:11
25–36	90	11 (12.2)	5 (5.6)	4 (4.4)	1:16, 1:16, 1:19, 1:32
37–48	39	7 (17.9)	5 (12.8)	5 (12.8)	1:11, 1:16, 1:19, 1:23,1:45
>48	34	5 (14.7)	1 (2.9)	3 (8.8)	1:11, 1:19, 1:19
Total	300	36 (12.0)	20 (6.7)	18 (6.0)	

*EBOV, Ebola virus; NA, not applicable; NP, nucleoprotein; VNT, virus neutralization test; WB, Western blot.

Table 2: EBOV-specific antibodies detected in dog serum samples, according to sampling region, Sierra Leone, October–December 2017*

Region	No. samples tested	EBOV-NP ELISA, no. (%) reactive	Confirmatory EBOV-NP WB, no. (%) reactive	EBOV VNT	
				No. (%) positive	Titers
Bomtoke	11	0	0	0	NA
Bonganema	8	3 (37.5)	3 (37.5)	1 (12.5)	1:16
Gandohun	4	0	0	0	NA
Gbangbantoke	24	3 (12.5)	0	3 (12.5)	1:13, 1:19, 1:19
Gbangbantoke Junction	14	0	0	0	NA
Kendeboma	7	0	0	0	NA
Largo	9	0	0	0	NA
Matagelema	16	1 (6.3)	1 (6.3)	0	NA
Mogbomoh	4	0	0	0	NA
Mokonde	14	1 (7.1)	1 (7.1)	0	NA
Mopala	1	0	0	0	NA
Morsenesie	4	2 (50.0)	0	0	NA
Mosongo	26	5 (19.2)	3 (11.5)	2 (7.6)	1:11; 1:16
Moyamba Junction	16	2 (12.5)	2 (12.5)	1 (6.2)	1:23
Moyamba Town	62	1 (1.6)	1 (1.6)	1 (1.6)	1:32
Njala Junction	15	2 (13.3)	1 (6.6)	1 (6.6)	1:11
Pelewahun	14	2 (14.3)	2 (14.3)	1 (7.1)	1:45
Rotifunk	21	2 (9.5)	2 (9.5)	2 (9.5)	1:11, 1:11
Sembehun	7	3 (42.9)	1 (14.3)	1 (14.2)	1:27
Taiama	23	9 (39.1)	3 (13.0)	5 (21.7)	1:11, 1:16, 1.16, 1:19, 1.19
Total	300	36 (12.0)	20 (6.7)	18 (6.0)	

*EBOV, Ebola virus; NA, not applicable; NP, nucleoprotein; VNT, virus neutralization test; WB, Western blot.

District in Sierra Leone (*3*), may account for cross-reactivity of the dog serum to EBOV-NP. The virus neutralization induced by specific binding to the EBOV surface glycoprotein suggests exposure of the dogs to EBOV or to a closely related ebolavirus eliciting cross-neutralizing antibodies. Although in vitro assays using an EBOV glycoprotein-pseudotyped virus revealed that infectivity is restricted in canine cells (*14*), detection of EBOV (cross-)neutralizing antibodies in dogs supports susceptibility to natural EBOV or ebolavirus infection.

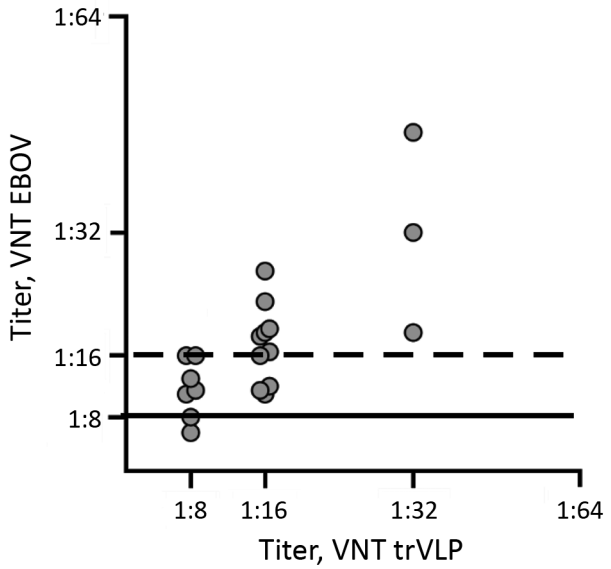

Figure 2. Analysis of dog serum samples (circles) in VNTs for study of EBOV neutralizing antibodies in dogs, Moyamba District, Sierra Leone, 2017. Comparison of dog serum titers obtained in VNTs was based on live EBOV (variant Mayinga) and EBOV trVLP. For VNT using authentic EBOV, serum samples with a titer ≤1:8 (horizontal solid line) are counted as negative; samples with a neutralizing titer >1:8 are considered positive. For trVLP-based VNT, titers equal to 1:16 (horizontal dashed line) are counted as positive. EBOV, Ebola virus; trVLP, transcription and replication competent virus-like particles; VNTs, virus neutralization tests.

The dog with the highest neutralizing titer (1:45) was 48 months of age; other dogs with neutralizing antibodies were 28–72 months of age at the time of blood collection, suggesting exposure during the West Africa EVD outbreak. However, information on past clinical signs in the dogs was not recorded, and the route of exposure or potential infection remains unknown. Exposure of dogs during the EVD outbreak in Gabon was assumed to result from consuming virus-infected carcasses or licking vomitus from EVD patients (*8*). Samples from those dogs, which displayed no clinical signs, tested negative for EBOV RNA (*8*). Furthermore, recent testing of 240 swab samples from dogs from Bombali District revealed no detectable filovirus RNA in the specimens; serologic assays were not performed (*3*).

Although most seropositive dogs in our study were potentially exposed to the virus during the EVD epidemic, 2 dogs with neutralizing antibodies (titers 1:16 and 1:27) were only 16 and 18 months of age, indicating contact with ebolavirus after the World Health Organization officially declared the end of the EVD outbreak in Sierra Leone by mid-March 2016 (*15*). Of note, some of the seropositive dog samples from Gabon were collected from areas without reported human EVD cases (*8*). These findings suggest exposure and immunogenic stimulation of free-ranging dogs by a source other than secretions from acutely

infected patients or infection with a heterologous ebolavirus circulating in wildlife reservoir hosts.

To date, neither evidence of clinical EVD in dogs nor virus shedding with subsequent transmission to humans has been reported. However, whether dogs play an active role in EBOV ecology, represent dead-end hosts, or act as passive virus carriers mechanically spreading the virus after licking and feeding on infected carcasses or fomites remains unknown. Therefore, organ tissues (including salivary glands, bladder, and intestines) or secretions that might lead to virus shedding and transmission should be collected from dogs during any future EVD epidemic.

This report of EBOV neutralizing antibodies in dogs suggests their susceptibility to natural infection by EBOV or antigenically related ebolaviruses. Considering the abundance of dogs and their close association with humans in Africa, the comparably low number of human EVD outbreaks in the past most likely indicates that dogs do not represent a reservoir or intermediate host for EBOV.

Acknowledgements

We thank Stefanie Rößler and Carolin Rüdiger for excellent technical assistance and Patrick Wysocki for preparing the map of sampling locations in Moyamba District. Thanks also to Thomas Müller, Conrad Freuling, and Gereon Schares for providing negative dog serum samples and dog-specific reagents.

This study was financially supported by the Deutsche Forschungsgemeinschaft (German Research Foundation, project no. 197785619 – SFB 1021) and by the German Federal Ministry of Food and Agriculture, on the basis of the decision of the Parliament of the Federal Republic of Germany through the Federal Office for Agriculture and Food.

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Outbreak of *Dirkmeia churashimaensis* Fungemia in a Neonatal Intensive Care Unit, India

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Bloodstream infections caused by uncommon or novel fungal species are challenging to identify and treat. We report a series of cases of fungemia due to a rare basidiomycete yeast, *Dirkmeia churashimaensis*, in neonatal patients in India. Whole-genome sequence typing demonstrated that the patient isolates were genetically indistinguishable, indicating a single-source infection.

During the past decade, outbreaks of bloodstream infections (BSIs) caused by rare and challenging to identify fungi have increased (*1*). Many rare infections occurred among patients admitted to neonatal intensive care units (NICUs). Rarely, yeasts belonging to the phylum Basidiomycota, including genera *Malassezia, Trichosporon*, and *Rhodotorula* (*2*–*4*), have been implicated in outbreaks in NICUs.

Dirkmeia churashimaensis (previously *Pseudozyma churashimaensis*) is a rare basidiomycete, ustilaginomycetous, anamorphic yeast first isolated in 2008 from the leaves of sugar cane (*Saccharum officinarum*) in Okinawa, Japan (*5*). Initially, *D. churashimaensis* was identified as a novel *Pseudozyma* species on the basis of morphological and physiologic aspects and by molecular analysis of the D1/D2 domains and internal transcribed spacer (ITS) regions (*5*). In 2015, Wang et al. used multigene phylogeny and proposed that *P. churashimaensis* represents a new genus, *Dirkmeia* gen. nov. (*6*). *Dirkmeia* gen. nov. is a common endophytic yeast found in the leaf tissues of rice, corn, sugar cane, and pepper plants (*7*). Of note, foliar application of this leaf-colonizing yeast has been reported to control plant viral disease under field conditions (*8*). However, *D. churashimaensis* has not been reported to cause human infections. We report an unusual cluster of 12 cases of fungemia caused by *D. churashimaensis* among NICU patients in a multispecialty hospital in Delhi, India.

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DOI: https://doi.org/10.3201/eid2604.190847

The Study

During June 2016–January 2017, a total of 12 cases of fungemia occurred among neonates admitted to a 24-bed NICU of a multispecialty hospital in Delhi. Cases of BSI were defined as the isolation of *D. churashimaensis* from ≥1 peripheral blood culture in patients with signs and symptoms of sepsis. The first case of fungal sepsis was observed in a preterm infant with very low birthweight (<1,500 g) who experienced asphyxia during birth. The patient's blood culture was positive for yeast on day 6 after birth. Fluconazole treatment was initiated by administering a loading dose of 12 mg/kg bodyweight, after which the patient received 6 mg/kg bodyweight in addition to vancomycin and meropenem. The second case occurred 2 weeks later in a preterm baby with low birthweight (<2,500 g) whose blood culture yielded *D. churashimaensis* on day 2. During the next 6 months, 10 additional cases of *D. churashimaensis* BSI were identified (Table 1).

Isolates grew as yeast-like cream to pale yellow, dry, and wrinkled colonies with fringes on the margin on Sabouraud glucose agar at 35°C after 48 h of incubation; the isolates grew slowly at 37°C over 3 days. Micromorphology showed fusiform yeast cells with hyphae and polar budding with short denticles. Identification by VITEK2 (bioMérieux, https://www.biomerieux.com) yielded *Cryptococcus laurentii* with 88% probability. We conducted carbon assimilation on isolates and noted assimilation of D-trehalose and N-acetyl-glucosamine at 37°C in 48 h.

Because no multilocus sequence or microsatellite typing were available, we used whole-genome sequencing (WGS) and amplified fragment-length polymorphism typing to understand the genetic relationships among isolates. We conducted matrix-assisted laser desorption/ionization time-of-flight mass spectrometry by using Biotyper 3.1 (Bruker Corp., https://www.bruker.com) to identify the yeasts. However, because no database of this yeast is available, we were not able to make an identification. The in-house database created yielded correct identification in the remaining 10 isolates with high score values (>2).

Table 1. Clinical details of patients with *Dirkmeia churashimaensis* fungemia in a neonatal intensive care unit, India*

Pt no.	Isolate ID	GA, wk/sex	DOB; delivery method	Birthweight, g	Date blood sample collected	Day of positive blood test†	Risk factors	Antifungal therapy‡	NICU stay, d; outcome
1	VPCI 2456/P/16§	31/M	2016 Jun 25; LSCS	1,400	2016 Jun 25	6	PT, VLBW, IUGR, thrombocytopenia, CVC, severe asphyxia, sepsis, mechanical ventilation	FLU, 10 d; VAN and MER, 7 d	15; survived
2	VPCI 2478/P/16	29/M	2016 Jul 7; LSCS	1,100	2016 Jul 8	2	PT, VLBW, IUGR, sepsis, thrombocytopenia	FLU, 14 d; VAN and MER, 10 d	12; survived
3	VPCI 2510/P/16	29/F	2016 Jul 19; VD	1,000	2016 Jul 19	4	PT, VLBW, sepsis, thrombocytopenia	FLU, 14 d; VAN and MER, 14 d	18; survived
4	VPCI 2515/P/16§	27/F	2016 Aug 30; VD	1,000	2016 Aug 31	4	PT, VLBW, IUGR, thrombocytopenia, maternal history of preeclampsia, antepartum hemorrhage	FLU, 14 d; AMI and CIP, 12 d	16; survived
5	VPCI 2601/P/16	32/F	2016 Sep 24; LSCS	1,200	2016 Sep 24	3	PT, VLBW, persistent hypoglycemia, severe asphyxia, sepsis, CVC, mechanical ventilation	FLU, 10 d; VAN and MER, 12 d	6; died
6	VPCI 2634/P/16	27/M	2016 Oct 17; VD	750	2016 Oct 17	5	Extremely PT, ELBW, severe asphyxia, CVC, sepsis, mechanical ventilation	FLU, 10 d	8; died
7	VPCI 2699/P/16§	30/M	2016 Nov 9; VD	800	2016 Nov 9	2	PT, ELBW, sepsis, persistent hypoglycemia, severe asphyxia, CVC, mechanical ventilation	FLU, 10 d; VAN and MER, 10 d	11; died
8	VPCI 2759/P/16	33/M	2016 Nov 26; LSCS	1,200	2016 Nov 28	3	PT, VLBW, severe asphyxia, CVC, thrombocytopenia, persistent hypoglycemia, mechanical ventilation	FLU, 14 d; VAN and MER, 10 d	10; survived
9	VPCI 2801/P/16§	27/M	2016 Dec 14; VD	1,100	2016 Dec 14	4	PT, VLBW, severe asphyxia, thrombocytopenia, sepsis, CVC, mechanical ventilation	FLU, 18 d; VAN and MER, 12 d	24; survived
10	VPCI 2845/P/16	27/F	2016 Dec 28; LSCS	1,200	2016 Dec 28	6	PT, VLBW, thrombocytopenia, mechanical ventilation, sepsis, persistent hypoglycemia, CVC	FLU, 10 d; VAN and MER, 8 d	8; died
11	VPCI 2224/P/17§	30/M	2017 Jan 3; LSCS	1,350	2017 Jan 3	4	PT, VLBW, sepsis, persistent hypoglycemia, CVC	FLU, 10 d; VAN and MER, 10 d	14; survived
12	VPCI 2271/P/17§	29/F	2017 Jan 18; VD	1,000	2017 Jan 19	4	PT, VLBW, thrombocytopenia, mechanical ventilation, CVC, sepsis, persistent hypoglycemia	FLU, 10 d; VAN and MER, 12 d	9; died

*AMI, amoxicillin; CIP, ciprofloxacin; CVC, central venous catheter; DOB, date of birth; ELBW, extremely low birthweight (<1,000 g); FLU, fluconazole; GA, gestational age; ID, identification; IUGR, intrauterine growth restriction; LSCS, lower segment cesarean section; MER, meropenem; PT, preterm; Pt., patient; VAN, vancomycin; VPCI, Vallabhbhai Patel Chest Institute (Delhi, India); VD, vaginal delivery; VLBW, very low birthweight (<1,500 g).
†Indicates days after birth.
‡All patients were given a 1-time loading dose of 12 mg/kg bodyweight of FLU and then 6 mg/kg bodyweight.
§Isolates selected for whole-genome sequencing.

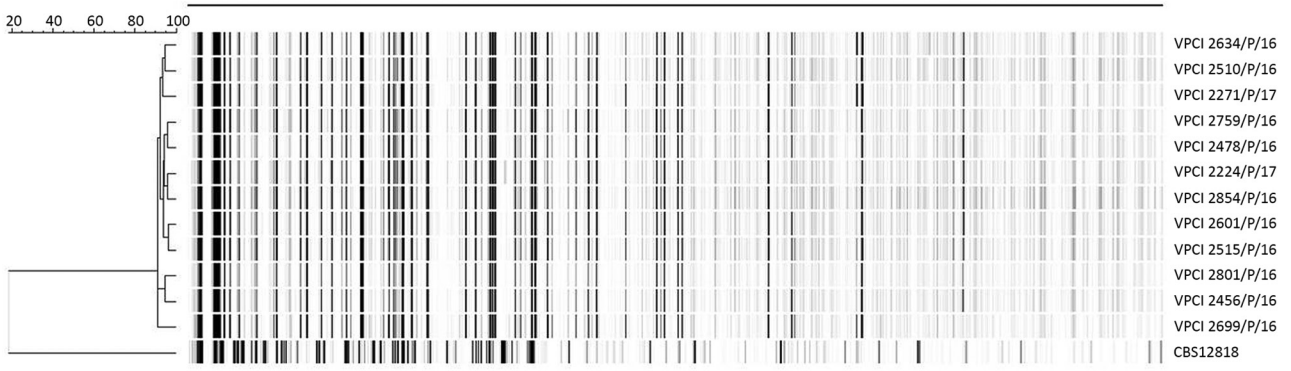

Figure 1. Dendrogram of amplified fragment-length polymorphism analysis of *Dirkmeia churashimaensis* isolated from 12 cases of fungemia in patients in a neonatal intensive care unit, Delhi, India. The dendrogram was constructed by using unweighted pair group method with averages and the Pearson correlation coefficient. Dendrogram was restricted to fragments of 60–400 bp. CBS 12818, a *Pseudozyma aphidis* isolate previously reported from neonatal fungemia in India, was included in the analysis. Scale bar indicates the percentage similarity. VPCI, Vallabhbhai Patel Chest Institute (Delhi, India).

We used isolates ITS and D1/D2 regions to sequence isolates, as described previously (*9*). We searched ITS and D1/D2 region sequences in BLAST (https://blast.ncbi.nlm.nih.gov) and identified isolates from the outbreak as *D. churashimaensis*. The isolates had >99% identity with *D. churashimaensis* sequences from GenBank (accession nos. MN758637–48 and MN158668–79).

We used MEGA 7 (https://www.megasoftware.net) to perform phylogenetic analysis of ITS sequences by using the neighbor-joining method with 2,000 bootstrap values. All isolates from the outbreak clustered together. The reference *D. churashimaensis* isolate formed a distinct cluster but showed 99% nucleotide similarity with isolates from the outbreak. We performed amplified fragment-length polymorphism fingerprint analysis, as described previously (*10*), which yielded identical banding pattern among all 12 isolates, suggesting clonal origin (Figure 1).

We performed WGS on 6 isolates by using IonPGM (IonTorrent; ThermoFisher, https://www.thermofisher.com) next-generation sequencing technology, following the manufacturer's protocol. We deposited sequences into BioProject (accession no. PRJEB35981). We identified average nucleotide identity and SNPs by comparing 6 genomes in MUMmer (http://mummer.sourceforge.net) and compared all the genomes against other publicly available basidiomycete yeast genomes (Table 2) by using progressiveMauve (*11*). We constructed a whole-genome SNP-based phylogenetic tree by using SplitsTree4 (*12*; Figure 2). The assembled genome size of *D. churashimaensis* is ≈21 Mb with a G+C content of 58%. The assembly contained 397–502 contigs ranging in length from 535,979 to 946,852 bp (average contig length 741,415 bp). *D. churashimaensis* isolates were genotypically indistinguishable and had 99.6% similarity among the genomes (average nucleotide identity >99.6%; Table 2). The average number of SNP differences between isolates was 1,074.5 (range 402–1,621), indicating high clonality.

Table 2. Results of average nucleotide identity analysis giving percentage similarity among *Dirkmeia churashimaensis* isolates from patients in a neonatal intensive care unit, India, compared with other basidiomycetes and *Saccharomyces cerevisiae* isolates*

	Isolate ID					
Isolate ID	VPCI 2456/P/16	VPCI 2515/P/16	VPCI 2699/P/16	VPCI 2801/P/16	VPCI 2224/P/17	VPCI 2217/P/17
VPCI 2456/P/16	100	99.86	99.86	99.87	99.86	99.86
VPCI 2515/P/16	99.87	100	99.89	99.91	99.93	99.91
VPCI 2699/P/16	99.86	99.9	100	99.92	99.9	99.91
VPCI 2801/P/16	99.88	99.91	99.92	100	99.89	99.9
VPCI 2224/P/17	99.89	99.91	99.91	99.92	100	99.89
VPCI 2271/P/17	99.88	99.87	99.88	99.89	99.89	100
Cutaneotrichosporon oleaginosus	68.22	68.82	68.51	68.68	68.54	68.57
Cryptococcus neoformans	70.94	69.27	69.99	70.37	70.15	69.95
Trichosporon asahii	69.54	63.37	69.32	69.66	69.78	69.88
Saccharomyces cerevisiae	64.82	63.40	63.56	64.24	64.48	64.52

*ID, identification; VPCI, Vallabhbhai Patel Chest Institute (Delhi, India).

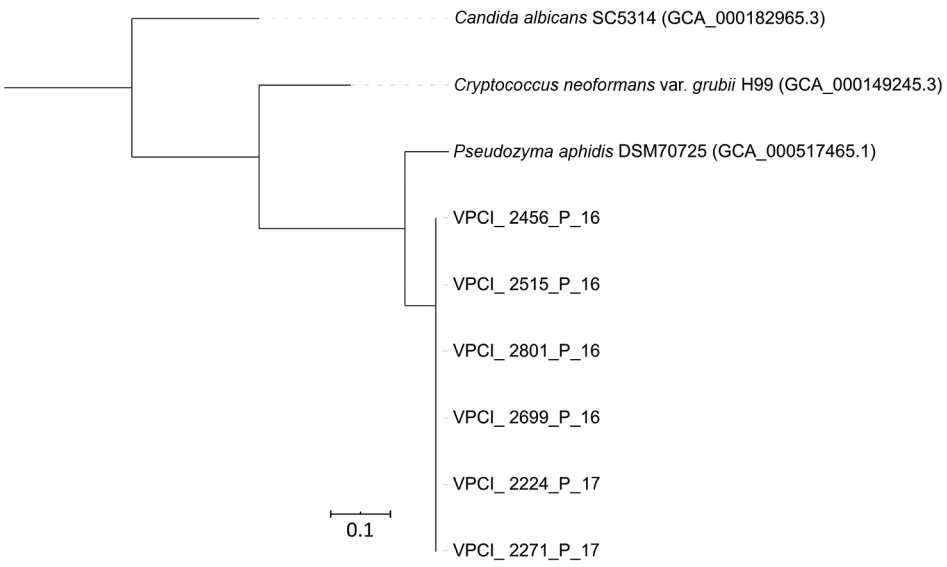

Figure 2. Whole-genome single-nucleotide polymorphism–based phylogenetic tree of 6 *Dirkmeia churashimaensis* isolates from cases of fungemia among patients in a neonatal intensive care unit, India. Other yeast species included for comparison. Scale bar indicates single-nucleotide polymorphism differences per site. VPCI, Vallabhbhai Patel Chest Institute (Delhi, India).

We conducted antifungal susceptibility testing using microbroth dilution method published by the US Clinical Laboratory Standards Institute (*13*). As expected with Basidiomycota, isolates in this outbreak were resistant to echinocandins. Susceptibility testing showed that all isolates were resistant to caspofungin, anidulafungin, and micafungin (MICs >8 μg/mL). However, all isolates had low MICs for azoles, including voriconazole (MIC 0.03–0.125 μg/mL; geometric mean [GM] 0.04 μg/mL), isavuconazole (MIC 0.03–0.125 μg/mL; GM 0.05 μg/mL), itraconazole (MIC 0.03–0.25 μg/mL; GM 0.057 μg/mL), posaconazole (MIC 0.03–0.25 μg/mL; GM 0.092 μg/mL), and fluconazole (MIC 1–4 μg/mL; GM 2.37 μg/mL). Amphotericin B (GM MIC 0.198 μg/mL) and 5-flucytosine (GM MIC0.157 μg/mL) had potent activity.

All patients were treated with fluconazole at a loading dose of 12 mg/kg bodyweight and then 6 mg/kg for 10–14 days; 5 patients died, a case-fatality rate of 42%. All patients had risk factors, such as preterm birth or low or very low birthweight, and 8/12 were intubated (Table 1). The most serious risk factors were central venous catheter (n = 9), thrombocytopenia (n = 8), and severe asphyxia (n = 6). The age at the onset of fungemia ranged from 2 to 6 days, and the attack rate was 0.33 during the 6-month outbreak. The mean gestational age was 29.2 weeks and the mean birthweight was 1.1 kg. Altogether, 11 patients had 1–6 days of antimicrobial drug therapy before isolation of yeast in blood culture.

After the second case of fungemia was identified, infection control measures were implemented and surveillance cultures obtained to trace the source of infection. Doctors, nursing staff, and assistants in the NICU were screened for hand carriage of the yeast, and extensive sampling of fomites including floors, equipment, disinfectants, vials, and infusion pumps was conducted. All environmental cultures were negative, and no other cases of fungemia due to *D. churashimaensis* were identified after continued compliance with infection control measures, including rigorous handwashing practice.

Conclusions

Our report highlights not only clinical importance of rare yeast species in the NICU but also emphasizes that WGS provides a highly sensitive tool for genotyping pathogens without prior knowledge of the genomes (*1*,*14*,*15*). Major healthcare-associated outbreaks of uncommon and novel fungal species have occurred in recent years, including *Candida auris* from a clonal outbreak in India (*9*). Mycologists should be vigilant when they isolate unusual or rare yeasts with potential antifungal resistance. Considerable challenges remain in the diagnosis of rare and unusual yeasts and the risk for misidentification of cases and outbreaks is high. Our findings reinforce the need for awareness of this new fungal risk among the public health community.

Acknowledgments

We thank Pradip Roy for technical assistance and Ferry Hagen for assistance in AFLP.

This work was supported in part by a research grant from the India Council of Medical Research under funding no. OMI/13/2014-ECD-I to A.C., Government of India, New Delhi, India.

J.F.M. has received grants from Astellas, Basilea, F2G, and Merck; has been a consultant to Astellas, Basilea, and Scynexis; and has received speaker's fees from Astellas, Merck, United Medical, TEVA, and Gilead.

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Rift Valley Fever Outbreak, Mayotte, France, 2018–2019

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From November 2018 through July 2019, an outbreak of Rift Valley fever in humans occurred in Mayotte, France; 142 cases were confirmed. Exposure to animals or their biological fluid was reported by 73% of patients. Health authorities have been implementing control measures, including veterinary surveys, vector control interventions, and prevention measures.

The southwestern islands of the Indian Ocean are threatened by arbovirus outbreaks because of their tropical climate, geographic proximity to arbovirus-endemic countries, tourism, and numerous commercial exchanges. One such island is Mayotte, an overseas department of France, located between the eastern coast of Africa and Madagascar. The island is densely populated, with ≈280,000 inhabitants at 690 inhabitants/km^2 (*1*–*3*).

Rift Valley fever (RVF) is a mosquito-borne zoonosis that affects domestic animals and humans. Humans are infected by RVF virus (RVFV) through contact with blood or organs of infected animals, slaughtering or handling infected animals, consuming contaminated meat that was not adequately aged or properly cooked, or consuming raw milk. The virus can also be transmitted through the bite of infected mosquitoes (mainly *Aedes* spp. and *Culex* spp.) (*4*,*5*).

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DOI: https://doi.org/10.3201/eid2604.191147

In Mayotte, epidemiologic surveillance for arbovirus infections among humans was implemented in 2008. This surveillance is based on using real-time reverse transcription PCR (RT-PCR) to test all patients with suspected dengue-like syndrome for dengue, chikungunya, and Rift Valley fever viruses (*6*) and for *Leptospira* spp. (*7*). Each positive case of RVF is reported by the Centre Hospitalier de Mayotte laboratory to the regional health authority (Agence Régionale de Santé Océan Indien) for implementation of control measures (veterinary investigations and vector control). Every confirmed case is investigated, and clinical and environmental data, including information about exposures, are collected.

In Mayotte, epidemiologic surveillance for RVF in livestock was also implemented in 2008 (*8*). A cross-sectional seroprevalence survey of asymptomatic livestock is conducted yearly. At least 350 samples have been tested each year with a commercial ELISA kit (*9*). For correctly identified cattle, sex, breed, and date of birth are available.

RVFV in humans was detected for the first time in Mayotte in 2007 (*10*,*11*). The genomic analysis of the Mayotte isolates placed them within the 2006–2007 eastern African Kenya-1 lineage (*12*), suggesting importation from mainland Africa. Retrospective analyses of livestock serum (collected from 2004 through 2008) showed that RVFV had been in Mayotte since 2004; however, no sequencing was performed at that time (*13*). In addition, a study conducted in 2011 estimated RVFV seroprevalence in the general human population ≥5 years of age to be 3.5% (95% CI 2.6%–4.8%) (*10*).

The Study

The first case of RVF in Mayotte was diagnosed by RT-PCR on November 22, 2018, for a patient living in Mamoudzou. Two weeks later, 4 new cases were diagnosed. No case-patient had traveled during the 2 weeks before symptom onset, and the cases were defined as autochthonous. During the same period,

analyses conducted by the agricultural cooperative of Mayotte and confirmed by the Centre for International Cooperation in Agronomic Research for Development showed that RVF seroprevalence among cattle had increased from 3.6% (95% CI 2.3%–5.6%) in July 2017–June 2018 to 10.1% (95% CI 6.5%–15.3%) in July–September 2018.

From November 22, 2018, through July 31, 2019, RT-PCR at the Centre Hospitalier de Mayotte confirmed 142 cases of RVF in humans. The epidemic curve peaked in week 7, when 18 cases were confirmed. The last confirmed case of RVF was in week 28 (Figure).

Most case-patients were male (3 male:1 female), and median age was 41 years (range 4–75 years). Clusters of cases in humans and animals were located mainly in the central and western areas of the island.

Among the 142 human cases, 126 (88.7%) were investigated; of these, 67.5% of case-patients reported having direct contact with livestock (care, treatment, slaughter) or their biological fluids (including consumption of raw or curdled milk), 15.1% reported living or working near livestock, and 22 (17.4%) reported having none of these exposures. Multiple possible exposures (having direct contact with livestock or their biological fluids and living or working near livestock) were declared by 50 case-patients. However, all case-patients had a connection with the affected areas (living, working, or walking in the central or western part of the island), and 4 lived in environments favorable for mosquitoes and mosquito breeding. Furthermore, most case-patients did not use any type of mosquito control in their houses.

Mosquitoes captured by the Agence Régionale de Santé Océan Indien were predominantly of the genus *Culex*. These mosquitoes were not tested for RVFV. In 100 herds of cattle and 26 herds of small ruminants, PCR detected 165 animals (141 cattle, 17 goats, and 7 sheep) positive for RVFV.

Information about clinical signs and symptoms was available for only 97 human case-patients (Table). Two case-patients had meningitis, and 1 was positive for RVFV by RT-PCR of cerebrospinal fluid. This patient had a stiff neck a week after the onset of signs and exhibited the neurologic sign of initial loss of consciousness. Twelve case-patients, including a pregnant woman in the sixth month of gestation, required hospitalization for at least 48 hours.

Doctors reported 2 severe cases. The first, with a complication of meningoencephalitis, occurred 3 weeks after the first confirmed case. This case-patient was hospitalized for gait disorder, dizziness, confusion, and rapid onset of hemiplegia; the date of symptom onset was March 18. RT-PCR performed on cerebrospinal fluid was negative. The second case-patient experienced an ocular disease 3 weeks after symptom onset. No hemorrhagic fever or deaths related to RVF have been reported since the outbreak onset.

Among livestock, the first confirmed case was reported on December 4, 2018. Clinical signs were reported by the veterinarian at the time of blood sampling. Among the 165 cases confirmed, 121 (100 cattle

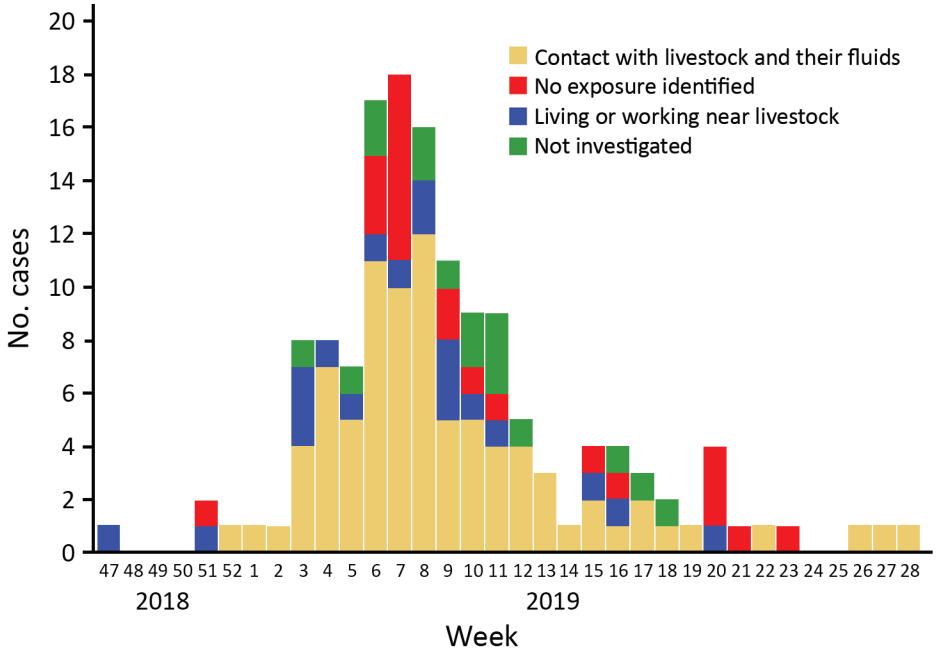

Figure. Sources of exposure for 142 patients with Rift Valley fever, by week of laboratory request, in Mayotte, France, 2018–2019.

Table. Potential Rift Valley fever virus exposures and clinical signs for persons with Rift Valley fever, Mayotte, France, November 22–July 31, 2019

Variable	No. (%) cases
Risk exposure, n = 142	
Investigated	126 (88.7)
Direct contact with animals and their fluids, including milk	85 (67.5)
Living or working near livestock	19 (15.1)
No contact with animals or consumption of products at risk	22 (17.4)
Not investigated	16 (11.3)
Signs and symptoms reported by patients, n = 97	
Fever	87 (89.7)
Arthralgia	61 (62.9)
Myalgia	42 (43.3)
Retroorbital pain	18 (18.6)
Headache	72 (74.2)
Weakness	49 (50.5)
Nausea/vomiting	31 (31.9)
Cough	5 (5.1)
Other signs and symptoms reported by doctors, n = 142	
Meningitis	2 (1.4)
Meningoencephalitis	1 (0.7)
Meningeal syndrome	3 (2.1)
Neurologic signs	2 (1.4)
Ocular complication	1 (0.7)

and 21 small ruminants) had aborted and 44 showed signs such as hyperthermia, nasal discharge, or digestive disorders.

After the first case in a human was diagnosed, the monitoring and managing protocol of the outbreak was shared by the regional unit of the Santé Publique France, the Agence de Santé Océan Indien, and the Centre Hospitalier de Mayotte laboratory. The objective of this protocol was to implement outbreak control actions (e.g., vector control), actively search for symptomatic humans, and conduct clinical and environmental investigations. Two weeks later, the first case in an animal was confirmed and veterinary services conducted surveys.

Public health authorities informed the general population about the situation through media and social networks to encourage persons to take preventive measures against mosquito bites. Farmers and others involved with slaughtering animals are trained to protect themselves from infectious disease agents. In addition, since February 27, 2019, selling raw and curdled milk has been prohibited.

Conclusions

An outbreak of RVF in Mayotte resulted in 142 confirmed cases in humans as of July 31, 2019, and several clusters among livestock were confirmed. The mode of transmission is not well identified for all cases, but most commonly reported were exposure to animals or consumption of raw milk (a common practice in Mayotte). Some evidence indicates that humans may become infected with RVFV by ingesting the unpasteurized or uncooked milk of infected animals (*5*).

Our surveillance system for dengue-like syndrome is particularly sensitive and has enabled detection of the first RVF case-patients requiring hospitalization. However, given the high number of asymptomatic and paucisymptomatic forms of illness reported in the literature, the epidemiologic situation may be underestimated. The extent of the epidemic could be assessed by a seroprevalence survey at the end of this outbreak.

This epizootic occurred in the context of increased illegal imports of animals (goats, sheep, and cows) over several months from potential disease-endemic/epidemic countries. Infected animals, especially sheep, were identified among intercepted animals.

RVFV, which was identified in Kenya, is still present in eastern Africa (*14*), but little information is available about its seroprevalence on the Indian Ocean islands. Mayotte is at risk for introduction and circulation of infectious agents involved in outbreaks in neighboring countries, such as recent infections and circulation of RVFV in the Comoros Islands (*15*).

About the Author

Dr. Youssouf is an epidemiologist and head of the regional unit of the French public health agency in Mayotte. His research interests included epidemiology (especially perinatal epidemiology), infectious diseases, and environmental health.

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Crimean-Congo Hemorrhagic Fever Virus in Humans and Livestock, Pakistan, 2015–2017

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We detected Crimean-Congo hemorrhagic fever virus infections in 4 provinces of Pakistan during 2017–2018. Overall, seroprevalence was 2.7% in humans and 36.2% in domestic livestock. Antibody prevalence in humans was highest in rural areas, where increased contact with animals is likely.

Crimean-Congo hemorrhagic fever (CCHF) is caused by CCHF virus (CCHFV), an emerging zoonotic virus belonging to the order Bunyavirales within the family *Nairoviridae*. The virus is maintained through a tick–vertebrate transmission cycle (*1*); the primary vectors are ticks from the genus *Hyalomma* (*2*,*3*). Wild and domestic mammals, including livestock species such as sheep, goats, and cattle, are amplifying hosts (*2*). CCHFV is listed as a high-priority zoonotic pathogen of humans in the World Health Organization Research and Development Blueprint (https://www.who.int/blueprint/priority-diseases) because of its potential to cause a public health emergency and the absence of specific treatment and vaccines.

Most human infections occur through the bite of infected ticks. Blood and other bodily fluids of infected animals represent an additional source for human infections. In humans, CCHF is manifested by fever, headache, vomiting, diarrhea, and muscular pain; bleeding diathesis with multiorgan dysfunction is seen in severe cases (*4*–*6*). CCHFV is endemic over a wide geographic area, spanning from western Asia to southern Europe and over most of Africa (*2*). Since the earliest identified CCHF case in 1976 (*7*), several outbreaks of CCHFV infection have been reported from Pakistan. Although Pakistan has the fourth highest number of human cases in Asia (*2*), no comprehensive surveillance study has been conducted to determine the disease prevalence in human and animal populations of Pakistan. Therefore, we determined the countrywide risk for CCHFV infection by detecting the virus and antibodies in livestock, ticks, and humans.

The Study

During 2017–2018, we tested 3,710 serum samples from 1,872 humans and 1,838 domestic animals (311 buffaloes, 480 camels, 183 cattle, 440 goats, and 424 sheep) for antibodies against CCHFV (Appendix, https://wwwnc.cdc.gov/EID/article/26/4/19-1154-App1.pdf). We also screened 98 blood plasma samples (24 from goats, 28 from buffalo, and 46 from cows) and 774 ticks (509 *Hyalomma* spp., 134 *Rhipicephalus* spp., 77 *Haemaphysalis* spp., and 54 *Rhipicephalus* [*Boophilus*] spp.), sampled from livestock in Punjab Province, for CCHFV antigen by commercial ELISA (VectoCrimea-CHF-antigen ELISA; Vector-Best, https://vector-best.ru).

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DOI: https://doi.org/10.3201/eid2604.191154

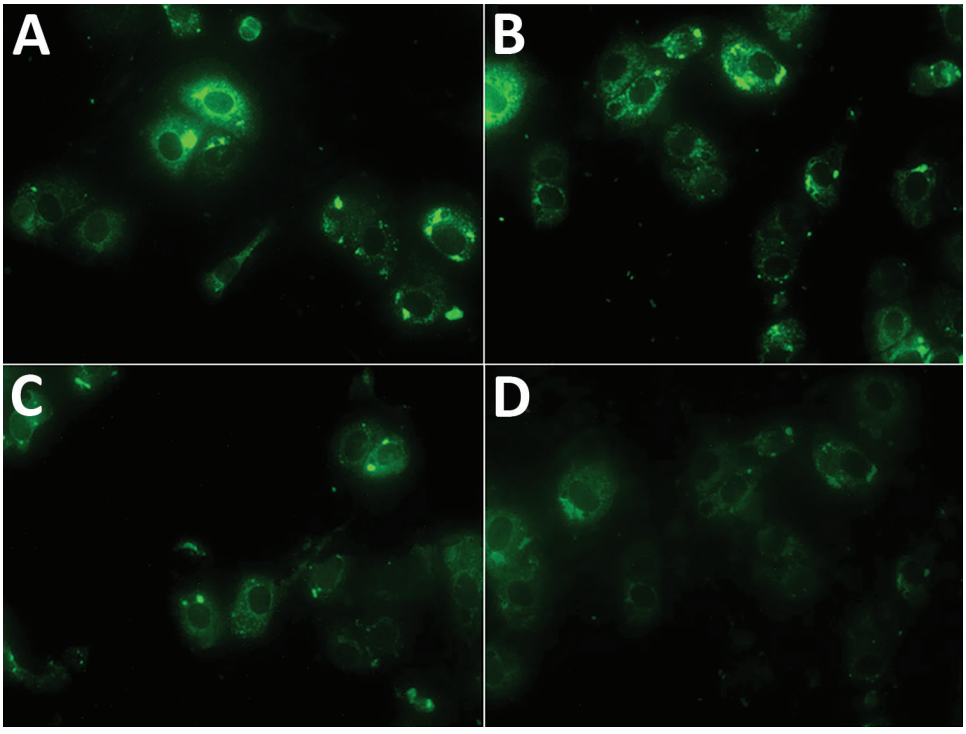

Figure 1. Indirect immunofluorescence assay results for Crimean-Congo hemorrhagic fever virus for 4 samples from humans that were positive by ELISA, Pakistan, 2016–2017. A, B) Samples at 1:100 dilution. C, D) Samples at 1:20 dilution. Original magnification ×100.

We found a total of 51 (2.7%) human samples to be positive for CCHF antibodies by using a 2-step approach, ELISA and confirmatory testing by immunofluorescence assay (Figure 1; Appendix). We observed significantly higher than average prevalence (p<0.01) among samples from Balochistan (5.7%, 95% CI 3.4%–9.3%); and the lowest prevalence among those from Sindh (1.1%, 95% CI 0.5%–2.3%). Samples from Balochistan were almost 6 times (odds ratio [OR] 5.6, CI 2.0–18.0) more likely to test positive than those from Sindh. Seroprevalence increased uniformly with age; we saw the highest level of CCHFV antibodies in persons ≥65 years of age (Table 1). Of the 51 positive samples, 28 (2.7%, 95% CI 1.8%–3.8%) were from female and 23 (2.8%, 95% CI 1.9%–4.2%) from male participants. We observed significantly higher (p<0.01) seroprevalence among livestock farmers (3.2%, 95% CI 2.4%–4.2%) compared with the general population (0.6%, 95% CI 0.1%–2.3%).

Of the 1,838 animals, 666 (36.2%) were positive for CCHF by a commercial ELISA (ID Vet, https://www.id-vet.com). The prevalence of CCHFV antibodies was significantly higher (p<0.01) among camels (56.7%, 95% CI 52.1%–61.2%) than among cattle (44.3%, 95%

Table 1. Univariate analyses of 1,872 human samples positive for Crimean-Congo hemorrhagic fever virus by ELISA, Pakistan, 2017–2018

Category	No. positive/no. tested	Prevalence, % (95% CI)	Odds ratio (95% CI)	p value
Province				<0.001
Punjab	25/930	2.7 (1.8–4.0)	2.6 (1.0–7.7)	
Khyber Pakhtunkhwa	6/128	4.7 (2.1–10.0)	4.6 (1.2–17.5)	
Balochistan	14/247	5.7 (3.4–9.3)	5.6 (2.0–18.0)	
Sindh	6/567	1.1 (0.5–2.3)	1.0	
Age, y				0.451
15–24	7/438	1.6 (0.8–3.3)	1.0	
25–34	19/730	2.6 (1.7–4.0)	1.6 (0.7–4.7)	
35–44	12/388	3.1 (1.8–5.4)	2.0 (0.7–6.0)	
45–54	9/226	4.0 (2.1–7.5)	2.6 (0.8–8.2)	
55–64	3/70	4.3 (1.4–12.5)	2.8 (0.5–12.4)	
≥65	1/20	5.0 (0.7–28.2)	3.2 (0.1–27.3)	
Sex				0.832
F	28/1,055	2.7 (1.8–3.8)	1.0	
M	23/817	2.8 (1.9–4.2)	1.1 (0.6–1.9)	
Occupation				0.006
Livestock farmer	49/1,523	3.2 (2.4–4.2)	5.8 (1.5–49.2)	
General population	2/349	0.6 (0.1–2.3)	1.0	

Table 2. Univariate analyses of 1,838 livestock samples positive for Crimean-Congo hemorrhagic fever virus by ELISA, Pakistan, 2017–2018

Category	No. positive/no. tested	Prevalence, % (95% CI)	Odds ratio (95% CI)	p value
Species				<0.001
Camel	272/480	56.7 (52.1–61.2)	5.6 (4.2–7.6)	
Cattle	81/183	44.3 (36.9–51.8)	3.4 (2.3–5.0)	
Sheep	138/424	32.6 (28.1–37.2)	2.1 (1.5–2.8)	
Buffalo	92/311	29.6 (24.6–35.0)	1.8 (1.3–2.5)	
Goat	83/440	18.9 (15.3–22.8)	1.0	
Province				<0.001
Balochistan	213/359	59.3 (54.1–64.5)	7.6 (5.4–10.6)	
Khyber Pakhtunkhwa	230/439	52.4 (47.6–57.1)	5.7 (4.1–7.9)	
Punjab	159/644	24.7 (21.4–28.2)	1.7 (1.2–2.40)	
Sindh	64/396	16.2 (12.7–20.2)	1.0	
Sex				0.377
F	552/1,504	36.7 (34.3–39.2)	1.1 (0.9–1.4)	
M	114/334	34.1 (29.1–39.5)	1.0	
Age, y				<0.001
≤5	332/1,121	29.6 (27–32.4)	1.0	
>5	334/717	46.6 (42.9–50.3)	2.1 (1.7–2.5)	

CI 36.9%–51.8%), sheep (32.6%, 95% CI 28.1%–37.2%), buffalo (29.6%, 95% CI 24.6%–35%), and goats (18.9%, 95% CI 15.3%–22.8%) (Appendix Tables 1–5). Camels were almost 6 times (OR 5.6) more likely to be positive than other species. As we found for humans, we found significantly higher (p<0.01) seroprevalence of CCHFV antibodies among animals from Balochistan (59.3%, 95% CI 54.1%–64.5%) than among animals from the other regions tested (Table 2).

We built a binary logistic regression model to evaluate possible risk factors for CCHFV seropositivity in animals and humans. The final model (Appendix Table 6) at the animal level indicated that the animals with highest risk for being antibody positive are livestock from Balochistan (OR 12.1, 95% CI 7.7–19.1), buffalo (OR 4.4, 95% CI 2.8–6.8), and animals >5 years of age (95% OR 1.3, CI 1.0–1.7). However, the NR^2 value of 0.277 and the Hosmer-Lemeshow goodness-of-fit test (χ^2 22.005; p = 0.003) indicated that this is a poor model for predicting CCHFV exposure in the sampled livestock population.

In humans, we found the chance of exposure to CCHFV was highest for populations from Balochistan (OR 6.6, 95% CI 2.5–17.5) (Appendix Table 7) and in persons belonging to the herdsman profession (OR 7.3, CI 1.7–30.2). The values of NR^2 (0.070) and Hosmer-Lemeshow goodness-of-fit test (χ^2 1.490; p = 0.684) indicated that our model is a reasonable model for predicting past exposure to CCHFV in the tested human population.

Four plasma samples from buffalo and 4 *Rhipicephalus* tick samples tested positive for CCHFV antigen by ELISA. Of these 8 positive samples, we confirmed 1 tick (T61) and 3 buffalo samples (15B, 16B, and 17B) through partial amplification and sequencing of the small (S) segment (260 bp). The 4 partial sequences of CCHFV S segments (GenBank accession nos. MN135938–MN135941) were 97%–95% identical to virus sequences found in Afghanistan (accession no. JX908640.1), Iran (accession no. KX096702.1), and Oman (accession no. KY362516.1) and clustered together with genotype IV (Asia) (Figure 2). We obtained full-length sequences of the CCHFV S, medium (M), and large (L) segments (accession nos. MN135942–MN135944) from the tick sample by sequencing on a HiSeq3000 (Illumina, https://www.illumina.com). Phylogenetic trees for the S, M, and L segments showed that the T61 strain clustered with genotype IV (Asia) (Appendix Figures 1–3).

Conclusions

This countrywide study of CCHFV in Pakistan strongly suggests virus circulation in specific geographic regions and suggests CCHFV foci and a potential source of human infections. Detection of the antibodies in domestic livestock species (including sheep, goats, cattle, buffalo, and camels) indicates a potential role of these animals in human infections. Demonstration of the virus in animal blood plasma and tick samples by reverse transcription PCR provides strong evidence of active circulation of CCHFV in Pakistan. Furthermore, genetic characterization of the virus reconfirms the circulation of genotype IV in Pakistan (*8*). Of interest, we found no *Hyalomma* tick positive for CCHFV; CCHFV has been reported from *Rhipicephalus* ticks from Iran and clustered together with strains from Pakistan and Iran, indicating that *Rhipicephalus* ticks have been naturally infected with closely related virus in the region (*9*). Our study further confirms the role of *Rhipicephalus* ticks in CCHFV circulation in the region. We observed higher prevalence of CCHFV antibodies in camels than in animals

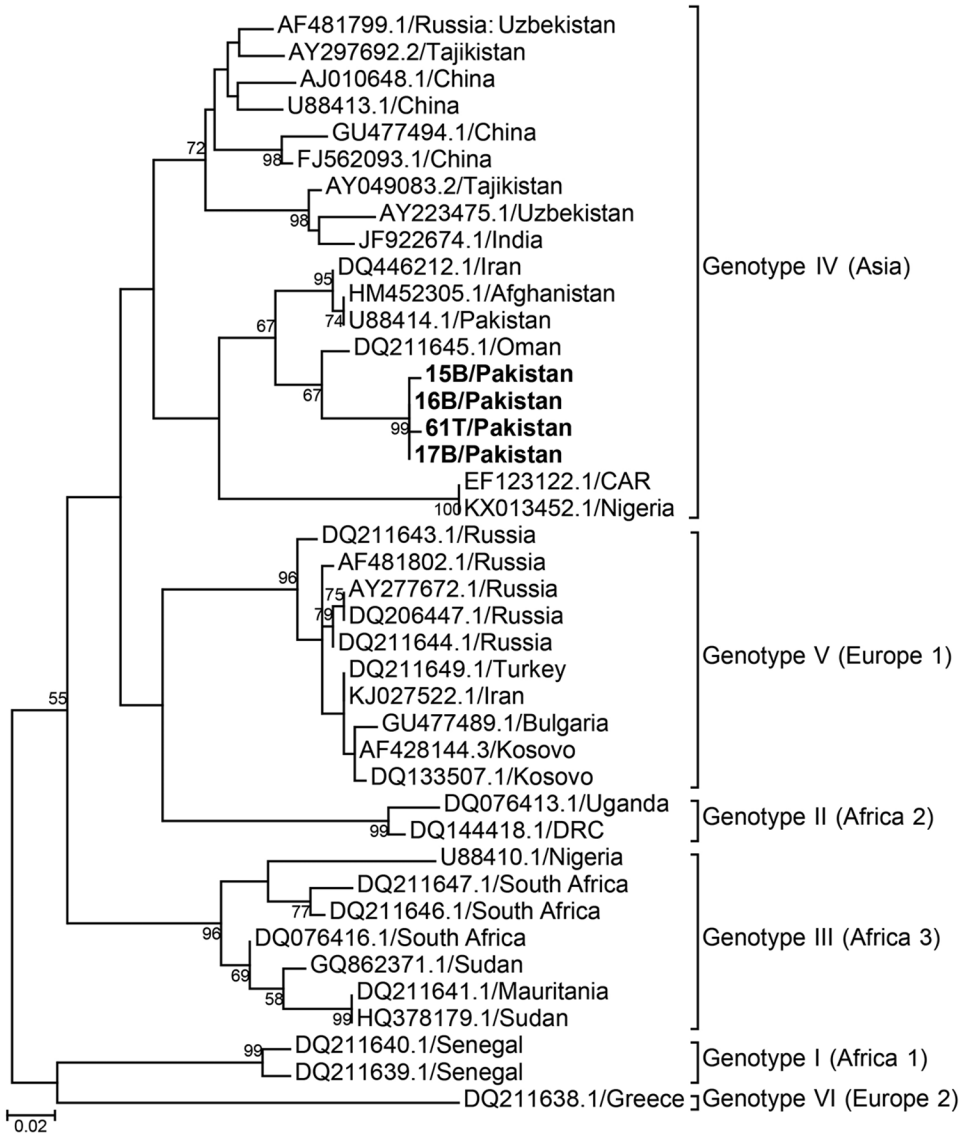

Figure 2. Phylogeny of Crimean-Congo hemorrhagic fever virus, Pakistan, 2016–2017 (bold text), and reference viruses, based on partial small gene sequences. Numbers at branch nodes indicate bootstrap support values. GenBank accession numbers are provided for reference sequences. Scale bar indicates nucleotide substitutions per site.

of other species, indicating the importance of camels in CCHFV ecology in Pakistan.

A high proportion of seropositive humans from Balochistan and Khyber Pakhtunkhwa with a history of exposure to animals is in concordance with earlier reports of CCHF in humans from these areas. The rural economy of Balochistan and Khyber Pakhtunkhwa is based on livestock production, and the increased contact with animals may explain the higher antibody prevalence in humans from these areas. Furthermore, the prevalence of antibodies was significantly higher among herdsmen than among the general population.

In summary, our results indicate the ongoing circulation of CCHFV among animals and humans in some regions of Pakistan. Longitudinal surveys to identify and define the genomic diversity of CCHFV in Pakistan and investigations to explore the exact role of camels in the ecology of this virus would help clarify the risk to the general population and occupational hazards for livestock farmers and veterinarians.

Acknowledgments

We thank the Livestock and Dairy Development Departments in Punjab and Balochistan Provinces, Pakistan. We thank the National Virus Resource Center (Wuhan, China) for providing the virus-infected Vero cells (accession no. IVCAS14.0001).

This work was supported by the National Science and Technology major project (2018ZX0101004), the International Cooperation on Key Technologies of Biosafety along the China-Pakistan Economic Corridor (153B42KYSB20170004), the Intergovernmental Special

Program of State Key Research and Development Plan from the Ministry of Science and Technology of China (2016YFE0113500), and the Science and Technology Basic Work Program from the Ministry of Science and Technology of China (2013FY113500).

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Detection of Zoonotic *Bartonella* Pathogens in Rabbit Fleas, Colorado, USA

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We detected 3 *Bartonella* species in wild rabbit fleas from Colorado, USA: *B. vinsonii* subsp. *berkhoffii* (n = 16), *B. alsatica* (n = 5), and *B. rochalimae* (n = 1). Our results support the establishment of the zoonotic agent *B. alsatica* in North America.

Wild lagomorphs (rabbits, hares, and pikas) are known or potential reservoirs for a number of zoonotic agents, including tularemia (*Francisella tularensis*), plague (*Yersinia pestis*), pasteurellosis (*Pasteurella multocida*), ringworm (*Trichophyton* spp.), and cryptosporidiosis (*Cryptosporidium cuniculus*) (*1*). In 1999, a novel *Bartonella* species, *B. alsatica*, was isolated from the blood of wild rabbits (*Oryctolagus cuniculus*) in eastern France (*2*). *B. alsatica* was later identified as a causative agent of lymphadenitis (*3*) and endocarditis (*4*,*5*) in humans. A case of prosthetic vascular graft infection caused by *B. alsatica* was reported in 2019 (*6*).

The geographic distribution of *B. alsatica* is poorly understood, as is its mode of transmission, although vector-mediated transmission was suggested upon initial characterization of this agent (*2*). *B. alsatica* DNA has been detected in fleas collected from *Bartonella*-infected wild rabbits in France (*7*) and Spain (*8*,*9*), suggesting the potential for fleaborne *B. alsatica* transmission. Our goal was to describe associations between rabbit-associated *Bartonella* and potential flea vectors in the United States to gain insights into transmission of fleaborne zoonoses.

The Study

We collected fleas from live-trapped desert cottontail rabbits (*Sylvilagus audubonii*) in June and July 2005 from 8 sites in Boulder County, Colorado, USA (*10*). We identified fleas to species by light microscopy using dichotomous keys (*10*) and then stored them in 96-well tissue culture plates at –20°C, except for representatives of each species that were removed and submitted to the Centers for Disease Control and Prevention (Fort Collins, CO, USA). In 2015, we extracted DNA from individual fleas using commercial DNA extraction kits (Blood and Tissue Kit; Macherey-Nagel, Inc., *https://www.mn-net.com*), with aliquots of extracted DNA maintained at the University of Richmond (Richmond, VA, USA), and secondary aliquots sent to the Laboratory of Veterinary Public Health, Nihon University College of Bioresource Sciences (Fujisawa, Japan). Both laboratories screened samples for *Bartonella* infection by conventional PCR targeting part of the *ssrA* gene; primers used were ssrA-F (5′-GCTATGGTAATAAATGGACAATGAAATAA-3′) and ssrA-R (5′-GCTTCTGTTGCCAGGTG-3′). The targeted gene was selected because of the robustness of the PCR assay and the ability of the locus to segregate *Bartonella* at the species level (*11*). Nihon University College of Bioresource Sciences also used real-time PCR targeting the *ssrA* gene to confirm *Bartonella* species for those samples. This PCR used a genus-specific TaqMan probe, 6-carboxyfluorescein (FAM)–labeled probe (5′-FAM-ACCCCGCTTAAACCTGCGACG-3′-BHQ1, where BHQ is black hole quencher); primers were the same as for conventional PCR. Samples that tested positive for *Bartonella* DNA by real-time PCR and for which unambiguous sequence data were collected in both laboratories from the target locus (*ssrA*) were reported as *Bartonella* positive. We sequenced all amplicons (301 bp) from conventional PCR and aligned them with *Bartonella* type strains and then subjected them to phylogenetic analysis using MEGA 7.0 (https://www.megasoftware.net).

We collected 141 fleas from 14 desert cottontail rabbits (average fleas per parasitized host 14.3,

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DOI: https://doi.org/10.3201/eid2604.191161

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Table 1. Fleas collected from desert cottontail rabbits (*Sylvilagus audubonii*) in Boulder County, Colorado, USA, and analyzed for presence of *Bartonella*

Rabbit ID no.	Sampling date	No. fleas collected (no. tested)	No. *Bartonella*-positive fleas (no. tested), % positive		*Bartonella* prevalence in fleas, %
			Cediopsylla inaequalis	*Euhoplopsyllus glacialis*	
304	2005 Jul 14	51 (21)	0 (1), 0	0 (20), 0	0
305	2005 Jul 14	2 (2)	0	1 (2), 50	50.0
522	2005 Jul 18	55 (53)	2 (19), 11	6 (34), 18	15.1
633	2005 Jul 18	17 (17)	0 (4), 0	11 (13), 85	64.7
673	2005 Jul 21	4 (2)	0, 0	1 (2), 50	50.0
674	2005 Jul 21	8 (7)	0, 0	2 (7), 29	28.6
794	2005 Jul 28	4 (3)	0, 0	0 (3), 0	0
Total		141* (105)	2 (24)	21 (81)	21.9

*Thirty-six fleas had been processed for *Yesinia pestis* surveillance in a previous project (R.J. Brinkerhoff et al., unpub. data) and thus were not tested for this study.

range 1–54) in the summer of 2005. Of these fleas, 105 (81 *Euhoplopsyllus glacialis* and 24 *Cediopsylla inaequalis*) collected from 7 rabbits sampled at 4 sites (Table 1; specific site locations in *10*) were available for molecular screening for *Bartonella.* The remaining 36 fleas were processed for *Yesinia pestis* surveillance in a separate project (R.J. Brinkerhoff et al., unpub. data) and were not available for *Bartonella* testing.

We detected *Bartonella* DNA in 2 (8.3%) *C. inaequalis* fleas collected from 1 rabbit (ID no. 522) and 21 (25.9%) *E. glacialis* fleas collected from 5 rabbits (ID nos. 305, 522, 633, 673, and 674) (Table 1). All nucleotide sequences matched closely to 3 zoonotic *Bartonella* species, *B. alsatica*, *B. vinsonii* subsp. *berkhoffii*, and *B. rochalimae* (Table 2), and clustered phylogenetically with reference sequences of the type strains with high bootstrap support (Figure). The representative sequences of the 3 *Bartonella* species were registered in International Nucleotide Sequence Database Collaboration with accession nos. PS522-c9 (GenBank accession no. MN654366), PS674-e5 (GenBank accession no. MN654366), and PS674-e6 (GenBank accession no. MN654366). All 3 rabbits (ID nos. 522, 633, 674) from which >1 flea was PCR-positive and available for sequencing produced multiple *Bartonella* species (Table 2).

Conclusions

We detected DNA of 3 zoonotic *Bartonella* species among the 105 rabbit fleas we tested for this study; overall *Bartonella* prevalence in fleas was 21.9% (23/105), which is comparable to previous prevalence estimates from rabbit fleas in Europe (*7*,*9*). This study had several noteworthy findings: *B. alsatica* DNA was detected in North America, and carnivore-associated *Bartonella* species occurred in rabbit fleas. These findings highlight the complexity of pathogen–vector–host associations and demonstrate why vector ecology is necessary for elucidating the evolution and enzootic transmission of vectorborne pathogens. Since *B. alsatica* was described in 1999 (*2*), its DNA has been detected not only in European rabbits (*Oryctolagus cuniculus*) in Spain (*8*) but also in rabbit fleas (*Spilopsyllus cuniculi* and *Xenopsylla cunicularis*) collected in France (*7*) and Spain (*9*) and has been associated with human disease in France (*3*–*6*). In 2019, detection of *B. alsatica* DNA was reported in cat fleas (*Ctenocephalides felis*) infesting cats and dogs in the United Kingdom (*12*).

A recent study reported *ftsZ* and *nuoG* sequences with ≈95% similarity to *B. alsatica* from the spleens of spiny rats (*Thrichomys fosteri*) in Brazil (*13*), the only previous published report of *B. alsatica* in the Americas. The *B. alsatica* sequences in our study were

Table 2. *Bartonella* sequence identities for *ssrA* amplicons amplified from *Cediopsylla inaequalis* and *Euhoplopsyllus glacialis* fleas collected from 5 desert cottontail rabbits (*Sylvilagus audubonii*) in Boulder County, Colorado, USA*

Host no.	Flea nos.	Flea species	Closest *Bartonella* strains/sequence homologies/% (GenBank accession no.)
305	e1	*E. glacialis*	*B. vinsonii* subsp. *berkhoffii* strain Winnie/98.8% (CP003124)
522	e6, e25, e26, e29	*E. glacialis*	*B. v.* subsp. *berkhoffii* strain Winnie/98.8% (CP003124)
	e12	*E. glacialis*	*B. alsatica* strain IBS 382/96.9% (JN029776)
	e32	*E. glacialis*	*B. alsatica* strain IBS 382/96.9% (JN029776)
	c1	*C. inaequalis*	*B. v.* subsp. *berkhoffii* strain Winnie/98.8% (CP003124)
	c9	*C. inaequalis*	*B. rochalimae* strain BMGH (JN029797) 100%
633	e1, e2, e3, e4, e5, e8, e9, e10, e11	*E. glacialis*	*B. v.* subsp. *berkhoffii* strain Winnie/98.8% (CP003124)
	e12	*E. glacialis*	*B. alsatica* strain IBS 382/96.9% (JN029776)
	e13	*E. glacialis*	*B. alsatica* strain IBS 382/96.9% (JN029776)
673	e1	*E. glacialis*	*B. alsatica* strain IBS 382/96.9% (JN029776)
674	e5	*E. glacialis*	*B. v.* subsp. *berkhoffii* strain Winnie/98.8% (CP003124)
	e6	*E. glacialis*	*B. alsatica* strain IBS 382/96.9% (JN029776)

*All samples in the table tested positive for *Bartonella* DNA by *ssrA* real-time PCR (*11*), whereas no samples that were negative by conventional *ssrA* PCR tested positive by real-time PCR.

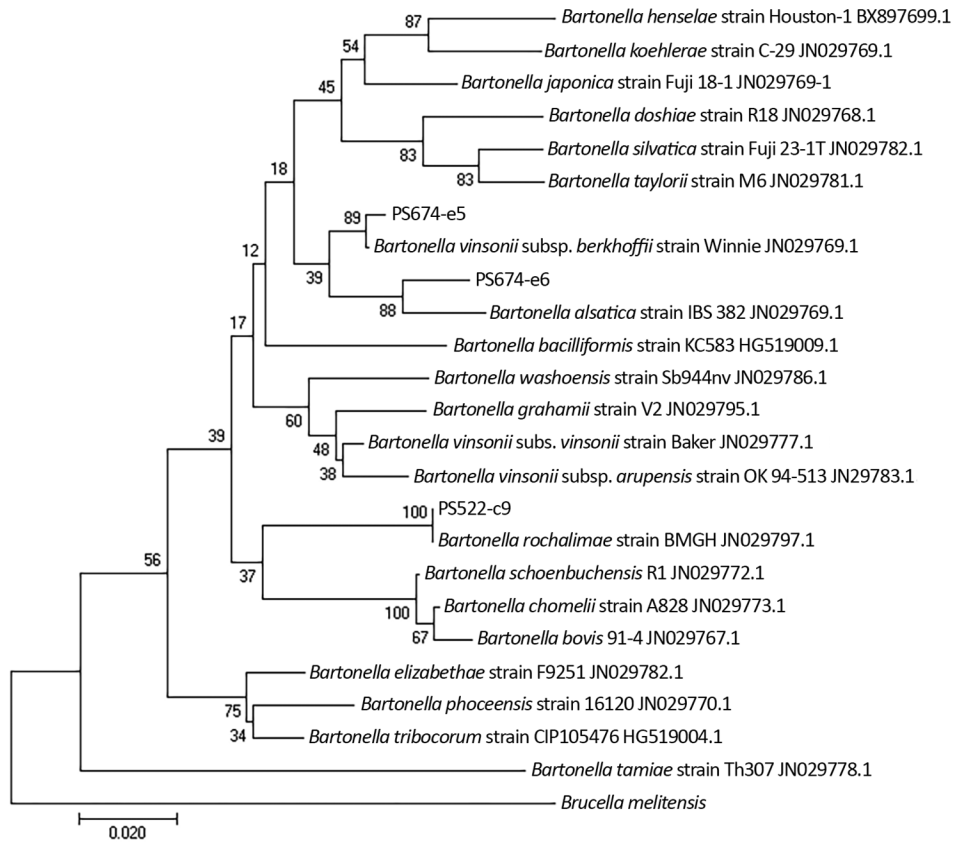

Figure. Phylogenetic relationships of *Bartonella ssrA* sequences detected in study of zoonotic *Bartonella* in rabbit fleas, Colorado, USA, compared with reference sequences. This tree was generated based on 253 bp by maximum likelihood and 1,000 bootstrap replicates using the Kimura 2-parameter evolutionary model with gamma-distributed rates among sites. Sample numbers are found in Table 2. GenBank accession numbers are indicated. Scale bar indicates nucleotide substitutions per site.

more similar to the *B. alsatica* type strain than were the putative *B. alsatica* sequences detected in Brazil. However, the *B. alsatica* sequences in our study were not identical to the type strain, suggesting that divergent *B. alsatica* strains may be circulating in the Americas. Further sampling of lagomorphs and their ectoparasites throughout North and South America is necessary to determine the geographic extent of *B. alsatica*, as well as its genotypic variation and evolutionary history.

We can conclude that both *C. inaequalis* and *E. glacialis* fleas are able to acquire *Bartonella* DNA and that blood-feeding is a likely mode of *Bartonella* acquisition, based on the observation that multiple fleas from the same host tested positive for *Bartonella* DNA. The detection of carnivore-associated *Bartonella* species in rabbit fleas was unexpected; typical reservoirs for *B. rochalimae* and *B. v.* subsp. *berkhoffii* are wild carnivores such as coyotes, foxes, raccoons, and skunks. However, *B. rochalimae* or *B. rochalimae*–like bacteria were found in the blood of brown rats (*Rattus norvegicus*) captured in Taiwan and in California, USA (*14*). Thus, *B. rochalimae* might have the potential to infect rodents as well as carnivores. Both rabbit flea species sampled in this study have been recovered from wild carnivore species in our study system and thus could serve as bridge vectors between carnivores and rabbits (*10*). In Europe, rabbit fleas have also been collected from carnivores (*15*), suggesting potential lagomorph–carnivore *B. alsatica* transmission in other systems as well.

Yersinia pestis, another fleaborne zoonotic agent that periodically causes epizootic events in our system (*10*), may spill over into amplifying hosts from putative reservoirs (mammalian, flea, or both) or from environmental sources. Flea and *Bartonella* (Table 2) exchange between lagomorphs and carnivores suggests that *Y. pestis* could also jump among these groups of mammals. Moreover, desert cottontails co-occur with black-tailed prairie dogs (*Cynomys ludovicianus*), a species associated with epizootic *Y. pestis* emergence, and flea exchange between desert cottontails and prairie dogs has been described (*10*). Given our findings, it is apparent that desert cottontail rabbits are associated with multiple zoonotic *Bartonella* species, including *B. alsatica*, which had not been previously recorded in North America, and that wild lagomorphs may contribute to the maintenance and transmission of several vectorborne zoonoses.

All wildlife sampling was conducted with approval from the Institutional Animal Care and Use Committee at the University of Colorado-Boulder.

Funding for this project was provided in part by a Weinstein grant from the University of Richmond to S.S. and R.J.B., by a Fulbright Scholar Award to R.J.B., and by National Science Foundation grant no. EF0224328. This project was also supported by a University of Richmond Arts and Sciences Summer Fellowship to A.B.S. Additional funding was provided by Nihon University College of Bioresource Sciences.

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Human-to-Human Transmission of Monkeypox Virus, United Kingdom, October 2018

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In September 2018, monkeypox virus was transmitted from a patient to a healthcare worker in the United Kingdom. Transmission was probably through contact with contaminated bedding. Infection control precautions for contacts (vaccination, daily monitoring, staying home from work) were implemented. Of 134 potential contacts, 4 became ill; all patients survived.

Monkeypox is a reemerging zoonosis caused by *Monkeypox virus* (MPXV), a member of the genus *Orthopoxvirus*. MPXV is related to *Variola virus*, the causative agent of smallpox. Although infections with these 2 viruses share many clinical features, monkeypox is generally less severe than smallpox (*1*). Among unvaccinated persons, the monkeypox case-fatality rate can be up to 10%, although case-fatality rates are lower for infection with the West African than the Central African clade of MPXV (*2*). In recent years, the number of cases and geographic spread of monkeypox have been increasing, possibly because of waning immunity to smallpox (*3*–*5*). Before 2018, the only human cases of monkeypox outside Africa occurred in the United States in 2003; that outbreak was associated with rodents imported from Ghana, and human-to-human transmission did not occur (*6*).

In September 2018, Public Health England (PHE) was notified of 2 unrelated cases of monkeypox affecting travelers who had recently returned from Nigeria (*7*). We describe transmission of monkeypox virus from the second of these cases to a healthcare worker (HCW) and the public health measures implemented to prevent further cases.

The Cases

On September 6, 2018, a man with a maculopapular rash, fever, lymphadenopathy, and a 1-week history of feeling generally unwell (patient 2) sought care at a hospital in England (*7*). He was admitted to a single-occupancy room in the acute medical unit. The staff attending the patient wore standard personal protective equipment (PPE), consisting of disposable aprons and gloves. Because a travel-associated infection was considered possible, patient 2 was transferred to an isolation room on September 7, 2018.

Three days later, a clinical diagnosis of suspected monkeypox was made, and infection prevention and control precautions for a high-consequence infectious disease (HCID) were implemented (e.g., enhanced PPE consisting of disposable gown, disposable gloves, filtering facepiece 3 respirator, and face shield or goggles). The patient was transferred to an Airborne HCID Treatment Centre, and monkeypox was confirmed by laboratory testing at PHE (*7*).

Although the risk to the public was considered to be very low, a precautionary approach was adopted. Possible hospital and community contacts of patient 2 were identified and assessed for risk (Table).

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DOI: https://doi.org/10.3201/eid2604.191164

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Because smallpox vaccines provide some cross-protection against monkeypox (*8*,*9*), a single dose of the third-generation smallpox vaccine, Imvanex (modified vaccinia Ankara; Bavarian Nordic, http://www.bavarian-nordic.com), was offered as postexposure prophylaxis (an off-label indication) to contacts at intermediate and high risk. The target vaccination window for these contacts was within 4 days of exposure, up to a maximum of 14 days from exposure. In addition, preexposure prophylaxis with Imvanex (single dose) was offered to HCID staff involved in the care of confirmed case-patients.

For 3 HCWs who had been assessed for risk, including patient 3 (a healthcare assistant), the same single-exposure risk was identified: ≥1 episode of close contact with the bedding and clothing of patient

Table. Public Health England risk assessment and public health recommendations for persons potentially exposed to 2 patients with monkeypox, United Kingdom, 2018*

Risk group	Description	Public health surveillance	Postexposure vaccination with Imvanex	No. persons in risk group†	No. (%) persons in risk group who received postexposure vaccination†
No risk	No known contact (direct or indirect) with a symptomatic monkeypox case-patient‡ OR Laboratory staff handling specimens from a monkeypox case-patient, in a laboratory conforming to UK laboratory standards§	None	Not recommended	Not applicable	0
Low	HCW involved in care of monkeypox case-patient while wearing appropriate PPE (with no known breaches) for all contact episodes OR HCW involved in care of monkeypox case-patient while not wearing appropriate PPE for all contact episodes but not within 1 m of case-patient and with no direct contact with body fluids or potentially infectious material OR Community contact not within 1 m of case-patient	Passive¶	Not recommended	158	0
Intermediate	Intact skin-only contact with a symptomatic (with rash) monkeypox case-patient, their body fluids, or potentially infectious material# or contaminated fomite OR No direct contact but within 1 m of symptomatic monkeypox case-patient without wearing appropriate PPE (including disposable FFP3 respirator or equivalent)	Active#	Vaccination may be considered	125	84 (67)
High	Direct exposure of broken skin or mucous membranes to monkeypox symptomatic case-patient, patient's body fluids, or potentially infectious material** (including clothing or bedding) without wearing appropriate PPE (including disposable FFP3 respiratory or equivalent). Exposure includes inhalation of respiratory droplets or material from scabs from cleaning rooms where a monkeypox case-patient has stayed, mucosal exposure to splashes, penetrating injury from used sharp device or through contaminated gloves or clothing	Active#	Vaccination recommended	5	5 (100)

*Imvanex (modified vaccinia Ankara, Bavarian Nordic, http://www.bavarian-nordic.com) was approved by the European Medicines Agency in July 2013 for active immunization against smallpox in adults. Jynneos (modified vaccinia Ankara; Bavarian Nordic) was approved by the US Food and Drug Administration in September 2019 for the prevention of smallpox and monkeypox disease in adults ≥18 y of age determined to be at high risk for smallpox or monkeypox infection. FFP3, filtering facepiece 3; HCW, healthcare worker; PHE, Public Health England; PPE, personal protective equipment.
†For patients 2 and 3 combined.
‡Case-patients are considered potentially infectious 24 h before the onset of rash.
§See http://www.hse.gov.uk/pubns/books/clinical-laboratories.htm.
¶A person requiring passive surveillance is given information about monkeypox and what to do if illness develops.
#A person requiring active surveillance is given information about monkeypox and instructed to report health status daily to PHE, regardless of symptoms, for 21 d from the date of most recent exposure, and to report any illness immediately. In addition, HCWs with high-risk exposures are to be excluded from work for 21 d after the most recent exposure (note this recommendation was introduced after diagnosis of the third case-patient).
**Potentially infectious biological material consists of skin lesions and detached scabs.

2 before monkeypox was diagnosed. No breaches of standard PPE were identified. All 3 staff members were classified as high-risk contacts and were placed under active surveillance and offered postexposure vaccination. Patient 3 was vaccinated against smallpox on September 14, which was 5 days after the most recent exposure and possibly 6 or 7 days after the earliest exposure to patient 2. Patient 3 had not previously received smallpox vaccine.

On September 22, while off duty, patient 3 noticed a small number of facial lesions and stayed home for the next 2 days but did not report illness to PHE. On September 24, patient 3 sought care with a general practitioner for headache, sore throat, skin lesions on the chin, earache, and eye pain. Patient 3 then reported the illness to PHE.

The general practitioner discussed the case with PHE and provided images of the skin lesions, which were consistent with monkeypox. Further medical assessment of patient 3 at the local hospital was arranged. After assessment and collection of diagnostic specimens, patient 3 remained isolated at home. On September 25, monkeypox was confirmed by PCR testing of multiple sample types, and patient 3 was admitted to an Airborne HCID Treatment Centre.

A total of 134 possible contacts of patient 3 were identified, including staff and patients on the ward where patient 3 worked, family and community contacts, and staff and patients at the general practitioner's office where patient 3 had sought care. Patient 3 had not been working when rash was present; however, as a precaution, all those who had had contact with patient 3 during the 24 hours before onset of the rash (i.e., on September 21) were monitored. Postexposure vaccination was offered to eligible contacts at intermediate and high risk (Table). As an extra precautionary measure, active monitoring, with daily reporting of presence or absence of signs or symptoms, was extended to all outstanding contacts of patient 2 and all new contacts of patient 3. In addition, HCW contacts at high risk were instructed not to attend work for 21 days from the most recent exposure (the incubation period for monkeypox is 5–21 days) (*10*).

A total of 4 contacts of patient 3 became ill within the incubation period and required medical assessment. No further cases of monkeypox were identified in relation to this incident and, after clinical improvement, patient 3 was discharged on October 29, 2018.

Conclusions

Cases of human monkeypox outside Africa are rare; in the United Kingdom, the likelihood of travel-associated monkeypox cases is low (*10*–*12*). To our knowledge, human-to-human transmission of monkeypox outside Africa has not been reported, and human-to-human transmission of the West African clade has been reported for Nigeria only (*4*). Such transmission may occur through close contact with skin lesions of an infected person, via fomites, or by exposure to large respiratory droplets during face-to-face contact (*1*). The transmission reported here occurred from a patient with a travel-associated case to an HCW. The only exposure risk identified during assessment of patient 3 was the changing of potentially contaminated bedding, when patient 2 had multiple skin lesions but before a diagnosis of monkeypox had been considered. The use of standard PPE may not have afforded sufficient protection against monkeypox, particularly if skin lesion debris containing virus had been disturbed and inhaled when bedsheets were changed.

Although patient 3 received postexposure vaccination before symptom onset, vaccination was >4 days after the most recent exposure to patient 2. The optimal timing for postexposure vaccination with Imvanex remains unknown, and the postexposure window period chosen for this incident was informed, in part, by that used during the US outbreak in 2003 (*6*). Patient 3 may have been vaccinated too late to prevent monkeypox.

During this incident, the risk to the public was determined to be very low because effective human-to-human transmission requires close contact with an infected person or virus-contaminated materials. Regardless, monkeypox is considered an HCID in England because it meets the UK criteria (*13*).

Monkeypox cases associated with travel to Nigeria have subsequently been detected in Israel (*14*) and Singapore (*15*). Although monkeypox is rare outside disease-endemic countries in Africa, this incident illustrates the need to be aware of monkeypox as a reemerging and travel-associated infection. Clinicians should consider a potential diagnosis of monkeypox early for patients with compatible symptoms and potential exposure risks, including recent travel to a disease-endemic country. In healthcare settings, implementation of appropriate infection prevention and control precautions as soon as monkeypox is suspected will help prevent secondary transmission.

Acknowledgments

We acknowledge all members of the National Incident Management Team and others involved in the response to this incident. Our thanks go to colleagues at PHE North West Centre, the PHE Rare and Imported Pathogens Laboratory, the PHE Imported Fever Service, PHE Field

Service, PHE Communications team, NHS England and NHS Improvement, Blackpool Teaching Hospitals NHS Foundation Trust, Newcastle upon Tyne Hospitals NHS Foundation Trust, Royal Free London NHS Foundation Trust, Royal Liverpool and Broadgreen University Hospitals NHS Trust, the Animal and Plant Health Agency, the Department of Health and Social Care, the Department for Environment, Food & Rural Affairs, Health Protection Scotland, Public Health Agency of Northern Ireland, Public Health Wales, The Advisory Committee for Dangerous Pathogens, the Joint Committee on Vaccination and Immunisation, Lancashire County Council, Blackpool Council, Wyre Council, Fylde and Wyre Clinical Commissioning Group, North West Ambulance Service, Fylde Coast Medical Services, UK Government Decontamination Service, Braemar Response Ltd, Bavarian Nordic A/S, and the Nigeria Centre for Disease Control.

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At the time of the incident, Dr. Vaughan was an epidemiologist in the Emerging Infections and Zoonoses Section at PHE and a Research Fellow at the NIHR Health Protection Research Unit in Emerging and Zoonotic Infections. She is now a research fellow in Infectious Disease Epidemiology at the London School of Hygiene and Tropical Medicine. Her primary research interests include emerging infections, field epidemiology, and outbreak investigation.

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Pruritic Cutaneous Nematodiasis Caused by Avian Eyeworm *Oxyspirura* Larvae, Vietnam

Do T. Dung, Nguyen T. Hop, Tran H. Tho, Yukifumi Nawa, Pham N. Doanh

A 41-year-old man from Son La Province, Vietnam, sought care for disseminated prurigo-like skin lesions from which nematode larvae were emerging. We morphologically and molecularly identified the larvae as *Oxyspirura* sp. Our findings confirm this nematode species as a zoonotic pathogen for emerging disease.

Various nematode parasites are known to cause cutaneous lesions in humans. Some species, such as nonlymphatic filaria *Onchocerca vorvuls* and *Loa loa*, exploit skin tissues to become mature adults and reproduce (*1*,*2*). Other species, usually animal hookworms such as *Ancylostoma braziliense* and *A. caninum*, as well as other less common species (*2*,*3*), remain larval or immature and accidentally migrate into cutaneous tissues (*1*). Clinical manifestations of cutaneous parasitic infections include migratory nodular lesions or erythema when parasites are in the deeper part of subcutis and serpiginous creeping eruptions when worms migrate through or just under the epidermis. Regardless of clinical manifestation, skin lesions caused by nematode parasites tend to be focal, except when larva currens of *Strongyloides stercoralis* (*2*,*4*) are involved and cause disseminated strongyloidiasis. We describe a case of disseminated cutaneous nematodiasis caused by *Oxyspirura* larvae, adult nematodes of which are known as avian eyeworms.

The Study

In July 2019, a 41-year-old man from Son La Province, northern Vietnam, came to the clinic of the National Institute of Malariology, Parasitology, and Entomology (Hanoi) with symptoms of disseminated pruritic erythema. The patient reported being of Thai ethnicity and told clinicians that he had pruritic lesions for several years. The patient used an herbal lotion from a local traditional medicine practitioner to treat the lesions for a year, but his symptoms did not resolve.

Physical examination revealed numerous erythematous, edematous, and pruritic skin lesions over his entire body skin, except for the soles of his feet. His back (Figure 1, panel A) and abdomen (Figure 1, panel B) were particularly affected. While the skin lesions were being examined, active larvae spontaneously migrated out (Figure 1, panel C) and even jumped out (Video 1, https://wwwnc.cdc.gov/EID/article/26/4/19-1592-V1.htm) from the patient's skin.

Hematology and biochemistry test results showed a slightly elevated total IgE of 171.2 IU/mL (reference range <100 IU/mL), but differential blood count, alanine transaminase, aspartate aminotransferase, creatinine, and other laboratory values were within reference limits. Ultrasonography and chest radiographs did not detect any abnormalities in lungs, liver, gallbladder, pancreas, kidneys, or bladder. A fecal sample was negative for nematode eggs and larvae. The patient noted that ≥3 of his neighbors had similar lesions, and some others suffered from sinusitis and had larvae emerge from their facial skin. The patient provided a video of the severe skin lesions of his neighbor (Video 2, https://wwwnc.cdc.gov/EID/article/26/4/19-1592-V2.htm).

We collected 6 specimens of larvae that emerged from the patient's skin for morphologic and molecular identification. The larvae were 800–850 μm long and 170–200-μm wide (Figure 2, panel A). The larvae had a nerve ring 212–250 μm from the anterior end (Figure 2, panel B), a clear buccal cavity (Figure 2, panel C), and an anus 300–350-μm from the posterior end (Figure 2, panel D). The larvae characteristics were similar to those of *Oxyspirura* spp. (*5*,*6*).

We used 3 larvae for molecular identification by analyzing a partial 18S rDNA sequence. We extracted total DNA from the larvae by using a QIAamp DNA

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DOI: https://doi.org/10.3201/eid2604.191592

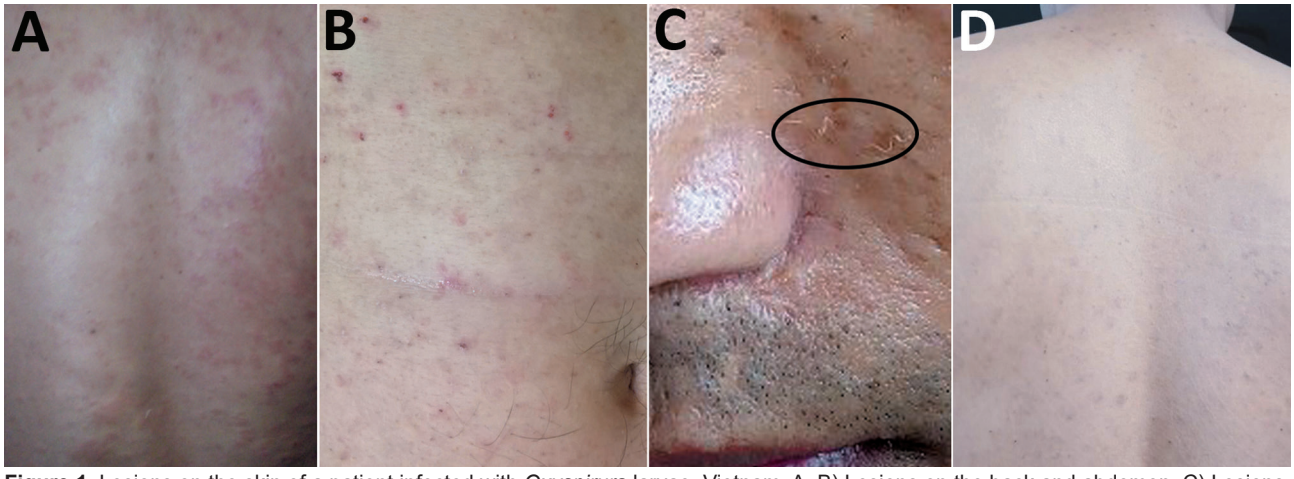

Figure 1. Lesions on the skin of a patient infected with *Oxyspirura* larvae, Vietnam. A, B) Lesions on the back and abdomen. C) Lesions on the face, with visible larvae (oval). D) Lesions on the patient's back 2 months after treatment.

Stool Mini Kit (QIAGEN, https://www.qiagen.com). We successfully amplified an ≈900-bp region by PCR with primer pairs SSU18A and SSU26R (*7*). We directly sequenced both strands by using an Ab3730 (ThermoFisher, https://www.thermofisher.com) and obtained 3 identical 884-bp sequences. We deposited sequences into DDBJ (http://getentry.ddbj.nig.ac.jp; accession no. LC508119) and GenBank (accession no. LC508119).

Consistent with the morphologic identification, a BLAST (http://blast.ncbi.nlm.nih.gov) search revealed that sequences from the larvae had a high similarity (96.6%) with that of *Oxyspirura petrowi* (accession no. LC316613). We downloaded 33 representative sequences with >93% similarity among the 100 highest similar sequences from GenBank and used these sequences to reconstruct a phylogenetic tree by using MEGA7 (https://www.megasoftware.net) and the maximum-likelihood method (*8*). The genomic sequence of the larvae from the patient clustered with *O. petrowi* at a high bootstrap value (93%), confirming that the larvae are of *Oxyspirura* species (Appendix Figure, http://wwwnc.cdc.gov/EID/article/26/4/19-1592-App1.pdf).

Eighty-four species are listed in the genus *Oxyspirura* (*9*). Most are avian eyeworms, and only 2 species were isolated from primates: *O. conjunctivalis* from a lemur and *O. youngi* from Patas monkeys (*6*). Our results confirm *Oxyspirura* larvae as a zoonotic pathogen and a cause of human disease. Despite the large number of nominal species of the genus, *O. petrowi* is the only species for which 18S rDNA sequences are available in GenBank, making identification of the larvae to the species level difficult. An investigation for adult nematode parasites in poultry raised in the patient's community could help identify the zoonotic pathogen in this case.

We treated the patient with albendazole (400 mg/d) for 14 days. His pruritis and lesions persisted but greatly improved after 2 months (Figure 1, panel D).

Skin lesions caused by nematode larvae vary depending on the causative pathogens. Cutaneous larva migrans caused by *A. caninum* canine hookworms

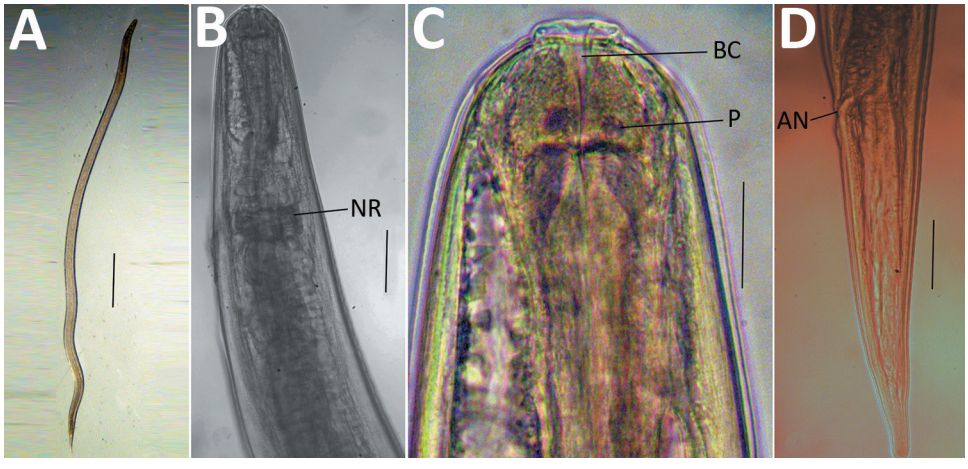

Figure 2. Microscopic images of *Oxyspirura* larvae collected from an infected patient, Vietnam. A) Whole body of *Oxyspirura* larvae; B, C) larvae anterior; D) larvae posterior. Scale bars indicate 100 μm in panel A, 50 μm in panels B–D. AN, anus; BC, buccal cavity; NR, nerve ring; P, papilla.

or *A. braziliense* feline hookworms typically appear as multiple linear or serpiginous lesions on the feet, lower legs, and buttocks (*3*). *Gnathostoma* spp. larvae can cause either creeping eruption or migratory panniculitis (*2*,*10*). In Japan, Spirurina type X larvae in scintillant squids, now identified as the larvae of *Crassicauda giliakiana*, cause cutaneous creeping eruptions (*11*). A zoonotic canine filaria, *Dirofilaria repens*, causes nodular lesions on the skin of humans from Europe to South Asia (*12*). Although rare, larvae of the free-living nematode *Pelodera strongyloides* can infect humans and cause multiple pruritic skin lesions (*13*). Regardless of various clinical features, nematode larvae in humans usually cause focal skin lesions in limited areas (*2*). The case we report is an unusual example of disseminated pruritic lesions caused by *Oxyspirura* larvae.

For transmission, *Oxyspirura* species require arthropod intermediate hosts, such as cockroaches, crickets, and grasshoppers, to develop into infective third-stage larvae (*5*,*14*). The patient in our case affirmed he eats grasshoppers and crickets, which are potential intermediate hosts of the nematode and could have been the route of transmission. Eating insects is a common traditional custom in many countries (*15*), and cutaneous nematodiasis due to *Oxyspirura* larvae is likely in other locations.

Conclusions

We describe a case of systemic cutaneous larval nematodiasis caused by *Oxyspirura* sp. larvae in Son La Province, Vietnam. Because most members of this genus are parasites of birds, investigation for nematodes of poultry in this area and surrounding areas is needed to collect adult worms for species identification. Neighbors of the patient also had the same condition, which suggests that *Oxyspirura* sp. larvae could be a public health concern. Further investigations to determine potential intermediate hosts of this nematode, additional cases of cutaneous larval nematodiasis in the community, and sources of infection will enable the control of infections in animals and humans.

The Medical Ethics Committee of the National Institute of Malariology, Parasitology, and Entomology approved this study (approval no. 975/CV-VSR). We obtained written informed consent from the patient and his neighbor to use the data, video, and pictures in this study.

This study was supported by the National Institute of Malariology, Parasitology, and Entomology, Hanoi, Vietnam, and the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology.

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Whole-Genome Analysis of *Salmonella enterica* Serovar Enteritidis Isolates in Outbreak Linked to Online Food Delivery, Shenzhen, China, 2018

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In July 2018, an outbreak of 10 cases of *Salmonella enterica* serovar Enteritidis infection occurred in Shenzhen, China. Outbreak investigation complemented by whole-genome sequencing traced the source to food ordered online. Our investigation highlights the role of online food delivery platforms as a new mode of foodborne disease transmission.

Salmonella spp. are a leading bacterial cause of acute gastroenteritis globally, resulting in ≈93.8 million cases of gastroenteritis and ≈155,000 deaths each year (*1*). *Salmonella enterica* serovar Enteritidis has been the most common cause of *Salmonella* infections, accounting for >40% of human cases worldwide and >30% in China (*2*,*3*). This serovar frequently has been isolated in foodborne disease outbreaks globally, which are often associated with poultry and related products, such as shell eggs (*4*,*5*).

In China, the use of online food delivery services has gained substantial popularity with the advent of smartphone mobile applications and the development of online food delivery platforms. The number of online food delivery orders has increased rapidly in recent years, accounting for ≈10 billon orders and >100 million monthly active users in 2018 (*6*). However, while such services are convenient, the complex spatiotemporal dynamics of online food delivery networks could result in a new means of spreading foodborne diseases and pose previously unknown effects on public health. We investigated an outbreak complemented by the use of whole-genome sequencing (WGS) to identify and delineate outbreak and sporadic cases to confirm the source of a *Salmonella* Enteritidis outbreak linked to food ordered through an online food delivery platform in Shenzhen, China.

The Study

During June 30–July 3, 2018, a total of 10 cases of diarrheal disease were reported at 2 hospitals in the Nanshan District of Shenzhen, China. This outbreak was suspected to be foodborne illness and was notified to the Shenzhen Center for Disease Control and Prevention (Shenzhen CDC). We collected details of food exposure histories, clinical manifestations, and demographic data through interviews with ill persons.

A total of 21 samples were collected during laboratory and environmental investigations, comprising anal swab specimens from 7 case-patients and 14 samples from the implicated restaurant (6 from chicken legs, 4 from restaurant staff, 2 from kitchenware items, and 2 from other foods). All samples were forwarded to the laboratory-based testing of common foodborne pathogens at Shenzhen CDC as previously described (*7*). *Salmonella* Enteritidis isolates were subtyped by pulsed-field gel electrophoresis (PFGE) using XbaI according to standard PulseNet protocols (https://www.cdc.gov/pulsenet/pathogens/protocols.html).

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DOI: https://doi.org/10.3201/eid2604.191446

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We sequenced genomes on the BGISEQ-500 platform (MGI Tech Co., Ltd., https://en.mgitech.cn) to generate 100-bp paired-end reads and deposited short-read sequence data in GenBank under BioProject PRJNA565566. We used Snippy version 4.3.8 (https://github.com/tseemann/snippy) for single-nucleotide polymorphism (SNP) calling, with *Salmonella* Enteritidis P125109 (GenBank accession no. NC_011294) as the reference genome. We excluded SNPs located in repetitive and recombinogenic regions before phylogenetic analysis, identified repetitive regions using TRF version 4 (https://tandem.bu.edu/trf/trf.html) and self-aligning by blastn (https://blast.ncbi.nlm.nih.gov), and determined recombinogenic regions by using Gubbins version 2.3.4 (*8*). We constructed a maximum-likelihood tree based on genomewide SNPs from an alignment of 300 SNPs using RAxML version 8.2.12 (*9*) under the general time-reversible with gamma distribution model (100 bootstraps).

All 10 case-patients were university students who had diarrhea (≥3×/24 h), fever (≥37.5°C), and high leukocyte counts (≥10.0×10^9 cells/L [reference $4.0–10.0 \times 10^9$ cells/L]); 7 case-patients also reported nausea and vomiting. Case-patients were from 6 different colleges of the same university but lived in different dormitories and did not know each other. However, on the afternoon of June 30, all had eaten food delivery (chicken leg with rice) from the same restaurant near the university, ordered through an online delivery platform during a 6-hour period (noon–6 PM). Foods were precooked an hour before anticipated orders and left at room temperature and then dispatched upon receipt of orders and delivered within 1 hour in ambient temperature (29°C) using a linen storage bag.

From a total of 21 samples, 9 were positive for *Salmonella* Enteritidis, which was isolated from 5 chicken legs and from 4 case-patients and belonged to an indistinguishable XbaI PFGE pattern (JEGX01.SZ0001). Interrogation of existing PFGE patterns within the Shenzhen CDC PulseNet local database showed that 5 *Salmonella* Enteritidis isolates from sporadic cases within 1 month before the outbreak shared the same PFGE pattern. Routine surveillance further identified 5 additional sporadic isolates with the same PFGE pattern within 1 month after the outbreak. However, no clear epidemiologic links were found between any of the 10 sporadic cases and the outbreak.

WGS SNP-based cluster analysis showed that all 9 outbreak-associated isolates were genetically closely related to each other (Figure). Isolates from 5 chicken legs and 4 case-patients differed by ≤1 SNP, confirming chicken legs as the food source of the outbreak. In comparison, the minimum distance between the 10 sporadic isolates and any of the outbreak-associated isolates was 59 SNPs, larger than the common threshold (≤3 SNPs) used for delineating outbreak clusters (*10*), indicating that the sporadic cases were not part of the outbreak.

Conclusions

The burgeoning online food delivery industry has resulted in a landscape of change in food consumption behavior and lifestyle in China. In contrast to traditional restaurant dining, online food delivery could send potentially contaminated food across wide geographic areas throughout a city within a short time to cause large-scale outbreaks. Online food delivery also poses additional food safety risks, including improper handling and storage temperature during transport. As illustrated in this outbreak, the total time elapsed was 2 hours from food preparation to delivery at ambient temperature, potentially enabling *Salmonella* Enteritidis to sufficiently multiply and cause illnesses.

On the basis of the fine-scale delineation by whole-genome SNP-based cluster analysis, we differentiated genetically closely related *Salmonella* Enteritidis isolates that were indistinguishable by PFGE. WGS confirmed chicken legs as the outbreak source and excluded the possible links of additional sporadic isolates to the outbreak and highlights the advantages of using WGS for differentiating highly clonal *Salmonella* Enteritidis isolates. Several studies have demonstrated high epidemiologic concordance and superior discriminatory power of WGS in retrospective outbreak analyses involving *Salmonella* Enteritidis (*10*,*11*).

In this report of a *Salmonella* Enteritidis outbreak investigation linked to online food delivery guided by WGS, we highlighted the food safety challenges posed by a new mode of foodborne disease transmission. One of the new features of this outbreak is that the case-patients who ate food from the same source were isolated from each other, which contrasts with typical foodborne outbreaks that usually involved dining in restaurants or catering events within a family or group settings. Additional cases might therefore be more likely to be missed during the epidemiologic investigation. However, detailed information associated with food orders, such as the ordering and delivery time, the food items ordered, and the names and addresses of the merchant and the consumer,

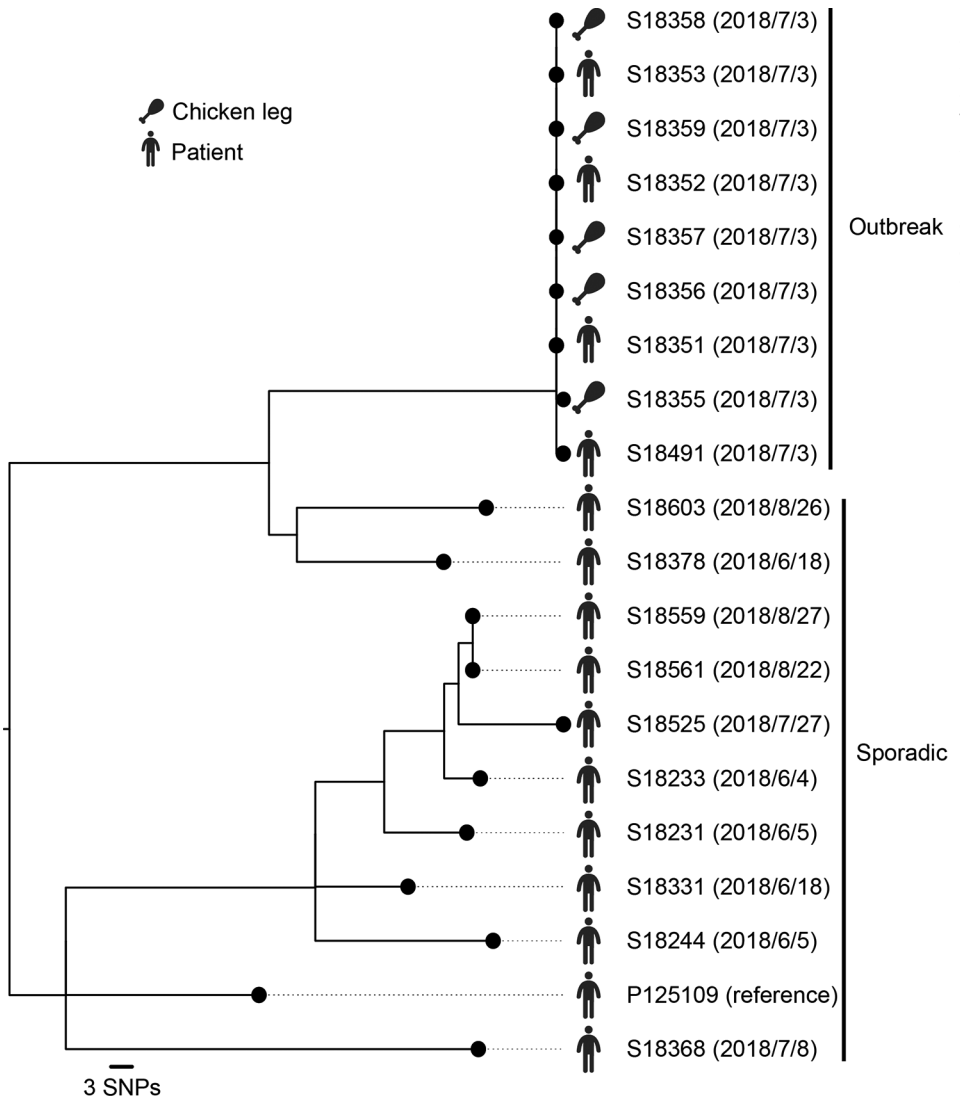

Figure. Clustering of 19 outbreak and sporadic *Salmonella enterica* serovar Enteritidis isolates from an outbreak linked to online food delivery, Shenzhen, China, 2018. Clusters were inferred by constructing a maximum-likelihood tree based on 300 genomewide SNPs. Isolation dates are provided in parentheses adjacent to isolate numbers; reference genome P125109 was used for SNP calling. Scale bar indicates nucleotide substitutions per site. SNPs, single-nucleotide polymorphisms.

would all be electronically recorded over the online food delivery platform. Therefore, such remarkable level of detail would be highly valuable for prospective outbreak investigations. Close collaboration between public health agencies and online food delivery platforms would be essential to facilitate timely intervention of disease propagation and limit the scale of outbreaks efficiently.

Given the continued rapid growth anticipated for the online food delivery industry, in-depth risk assessments should be a research priority to inform appropriate food safety strategies. We explored a rare opportunity to investigate and gained new insights into the transmission dynamics of a *Salmonella* Enteritidis outbreak over an Internet platform, complemented with the pragmatic use of WGS analysis.

This research was supported by the National Key Research and Development Program of China (no. 2018YFC1603902), Sanming Project of Medicine in Shenzhen (no. SZSM201811071), the China National Science and Technology Major Projects Foundation (no. 2017ZX10303406), National Natural Science Foundation of China (no. 81673174), the Shenzhen Municipal Science Technology Program (no. JSGG20160429101631739 and no. JCYJ20160428142519080), and Shenzhen Health and Family Planning Commission (no. 201602004).

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Novel Rapid Test for Detecting Carbapenemase

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DOI: https://doi.org/10.3201/eid2604.181655

We developed a carbapenemase test based on the ability of imipenem to inhibit noncarbapenemase β-lactamases. The test uses bacterial isolates with a fluorescent β-lactamase substrate, producing objective results with 100% sensitivity and specificity in 10 minutes. The assay is inexpensive and consists of only 1 mixing step.

As a potent β-lactamase, carbapenemase can degrade almost all β-lactam antimicrobial drugs, including the carbapenems, regarded as the last line of therapy for many life-threatening infections (*1*,*2*). Various epidemic types of carbapenemase have been reported globally, including *Klebsiella pneumoniae* carbapenemase, Verona integron-encoded metallo-β-lactamase, *Serratia marcescens* enzyme, imipenem-hydrolyzing β-lactamase, New Delhi metallo-β-lactamase, oxacillinase, metallo-β-lactamase, and São Paulo metallo-β-lactamase (*1*). If uncontrolled, the spread of these carbapenemases is expected to increase therapeutic failure and leave many patients with no effective treatment options.

Despite the urgency, timely carbapenemase detection remains a challenge for microbiology laboratories. Phenotypic assays are inexpensive and easily performed, but their use requires 24–48 hours and many lack sensitivity or specificity (*3*). The widespread use of other assays (e.g., molecular tests of carbapenemase genes, mass spectrometry detection of carbapenem hydrolysis) is impeded by the expertise required to perform them and their cost (*4*,*5*). The recently developed (2012) Carba NP test and variants are elegant solutions, but their use requires up to 2 hours (*6*). Further improvements in test rapidity and simplicity are highly desirable, especially for patients in critical condition, who need immediate therapy and infection control action.

We demonstrate that by using fluorescence identification of β-lactamase activity (FIBA), carbapenemase production in bacteria can be detected sensitively and specifically in 10 minutes, with only 1 step. FIBA uses a dark fluorescence probe, β-LEAF (β-lactamase enzyme–activated fluorophore), which turns fluorescent when cleaved by β-lactamases, including penicillinases, extended-spectrum β-lactamases (ESBL), AmpC β-lactamases, and carbapenemases (*7*,*8*). Thus, the rate of fluorescence increase (hereafter called increase rate) is a measure of the bacterial β-lactamase activity and is reduced as the β-lactamase activity is hampered. For a noncarbapenemase β-lactamase, the increase rate will be reduced by the addition of imipenem, which binds the enzyme active site and blocks β-LEAF access (*2*). In contrast, the increase rate for a carbapenemase is relatively unaffected by imipenem addition because carbapenemase is able to rapidly cleave the imipenem and relieve the inhibition (*1*). Accordingly, bacteria that produce carbapenemases can be detected by comparing the increase rate with and without imipenem (Figure; Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/26/4/18-1655-App1.pdf).

FIBA is performed in a 96-well plate. Each well contains 50 μL of 20 μmol/L β-LEAF, 25 μL of phosphate-buffered saline with or without 40 μmol/L imipenem (Cayman Chemical, https://www.caymanchem.com), and 10 μL of either 1 mg/mL polymyxin B nonapeptide or 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (Sigma-Aldrich, https://www.sigmaaldrich.com), which act as weak or strong permeabilizers, respectively. To start the assay, 25 μL of 1×10^{10} CFU/mL bacterial suspension made by colonies grown overnight on BHI agar (Sigma-Aldrich) is added to each well. To monitor the increase rate, fluorescence measurement is then performed at 37°C at 10-s intervals for 10 min with Ex/Em 450/510 nm in the plate reader (Spectramax M5 plate reader, Molecular Devices, https://www.moleculardevices.com). For each bacterial sample, we performed the reactions in duplicate and averaged the results. We objectively interpreted the fluorescence measurements by using an automated Python script (Appendix), which required a few seconds after assay completion.

We tested FIBA on 76 randomly selected infection isolates from either the Centers for Disease Control and Prevention (*9*) or the American Type Culture Collection (https://www.atcc.org). The MICs of these isolates, if not predetermined, were measured by the 2017 Clinical Laboratory and Standards Institute (https://clsi.org) broth dilution method. Genetic test results for β-lactam resistance were provided with the isolates. Among these, 55

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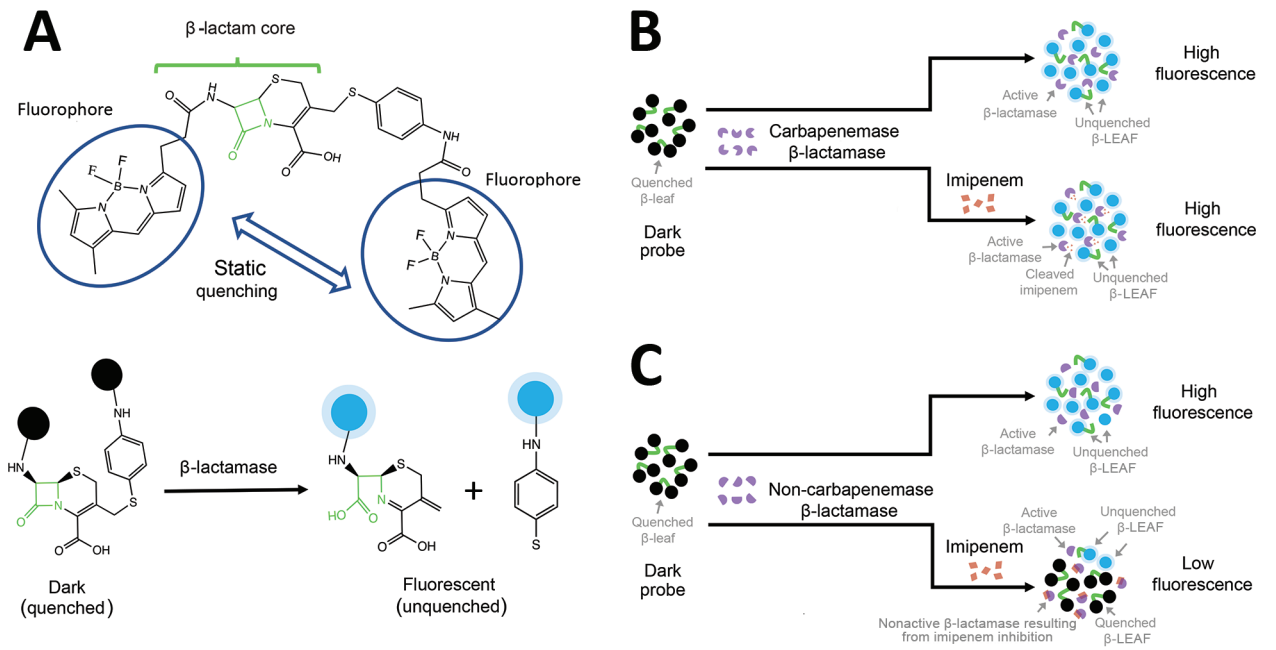

Figure. Schematic illustration of the principle of fluorescence identification of β-lactamase activity. A). The β-lactamase–activated fluorophore probe comprises a cleavable β-lactam core conjugated to 2 fluorophores (circled) that are quenched because of close proximity. This construct was designed to mimic the enzymatic degradation properties of easily cleavable β-lactam antimicrobial drugs. When this probe is attacked by β-lactamase, the probe core is cleaved, leading to the separation of the fluorophores and the recovery of their fluorescent properties (fluorescent state). B) Assay profile for carbapenemase-producing bacteria. C) Assay profile for non–carbapenemase-producing bacteria. Black, quenched fluorophore; blue, unquenched fluorophore turning fluorescent; green, β-lactam core; red, imipenem; purple, β-lactamase. β-LEAF, β-lactamase enzyme–activated fluorophore.

were carbapenemase positive, carrying the major epidemic carbapenemase types including *K. pneumoniae* carbapenemase (n = 20), imipenem-hydrolyzing β-lactamase (n = 2), metallo-β-lactamase (n = 4), New Delhi metallo-β-lactamase (n = 10), oxacillinase (n = 8), *S. marcescens* enzyme (n = 2), São Paulo metallo-β-lactamase (n = 1), Verona integron-encoded metallo-β-lactamase (n = 6), and New Delhi metallo-β-lactamase oxacillinase (n = 2). The other 21 isolates expressed noncarbapenemase β-lactamases, which involved 9 isolates with only ESBL, 3 isolates with both ESBL and porin modification, 6 isolates with only AmpC β-lactamase, and 3 isolates with both ESBL and AmpC β-lactamase. Among these isolates, 3 were carbapenem resistant. The entire panel, which included 28 colistin-resistant strains (MIC ≥4 ug/mL), was classified successfully with FIBA (Appendix Tables 1, 2), resulting in 100% sensitivity (95% CI 94%–100%) and 100% specificity (95% CI 84%–100%).

The primary limitation of this study is the small number of isolates evaluated. However, the breadth of isolates studied here included 8 enzyme types across 16 species, suggesting the generality of the approach.

FIBA can be performed ≈10 times faster than the most rapid carbapenemase test commercially available while maintaining comparable sensitivity and specificity (*6*,*10*). Its automated analysis improves turnaround time and reduces operator variability. With a reagent cost/assay of ≈US $1, FIBA is close in price to phenotypic tests but substantially faster and less labor intensive. Furthermore, the FIBA paradigm is extensible; by replacing imipenem with other known subtype-dependent inhibitors of carbapenemase (e.g., clavulanic acid, EDTA), rapid carbapenemase subtyping may also be possible. Our study demonstrates that low-cost, rapid assessment of carbapenemase can be performed in a 1-step format suitable for large-scale epidemiologic studies, thereby providing a new tool for infection outbreak control.

Acknowledgments

We acknowledge Gerry Nau for helpful conversations.

The research was supported by NIH/FIC R21TW010202 and DOD/AFOSR FA9550-16-1-0479. Patent pending for the β-LEAF design (application no. US20180094292A1).

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Arthritis Caused by MRSA CC398 in Patient without Animal Contact, Japan

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DOI: https://doi.org/10.3201/eid2604.190376

Clonal complex 398 methicillin-resistant *Staphylococcus aureus* (MRSA) is a typical lineage of livestock-associated MRSA. We report a case of intractable arthritis of the shoulder joint caused by a multidrug-resistant Panton-Valentine leukocidin–positive livestock-associated MRSA clonal complex 398 sequence type 1232 clone in a patient in Japan who had no animal contact.

In the past decade, methicillin-resistant *Staphylococcus aureus* (MRSA) has been detected in livestock, including swine, poultry, and veal calves (*1*,*2*). In general, the virulence of animal-derived livestock-associated (LA-MRSA) strains is considered to be lower than that of community-acquired MRSA lineages (*3*). However, LA-MRSA strains can effectively colonize and infect humans, with subsequent transmission in both community and hospital settings. Human colonization with LA-MRSA sequence type (ST) 398 was first recognized among swine farmers in France and the Netherlands in the early 2000s (*4*). According to Larsen et al., clonal complex (CC) 398 MRSA accounted for 21% of MRSA isolated from skin and soft tissue infections in Denmark during 2010–2015 (*5*). However, ST398 MRSA has not been isolated in patients in Japan. We report a case of intractable arthritis of the shoulder joint caused by a multidrug-resistant Panton-Valentine leukocidin (PVL)–positive LA-MRSA CC398 (ST1232) clone in a patient in Japan who had no animal contact.

We performed MRSA identification, staphylococcal cassette chromosome (SCC) *mec* typing, *spa* typing, multilocus sequence typing (MLST), MIC determination, and PCR assays for detecting virulence factors and antimicrobial resistance genes, as described previously (*1*,*6*). The study protocol was approved by the Tokyo University of Pharmacy and Life Sciences Ethics Committee (approval no. 12–09).

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The patient, a 74-year-old man who lives in Tokyo, had received dialysis treatments 3 times a week since April 2018. He reported no overseas travel or animal contact. In September 2018, he felt pain in his right shoulder joint and was admitted to the Tokyo Medical University Hachioji Medical Center. At admission, his leukocyte count was 18,600 cells/μL and his C-reactive protein level was 7.0 mg/dL; we began treatment with cefazolin immediately (day 0). We isolated MRSA from venous blood and joint fluid, and we switched the antimicrobial agent to vancomycin on day 1.

Molecular epidemiologic analysis showed that the MRSA THI2018-120 strain we isolated is classified into SCC*mec* type V and *spa* type t034. Moreover, MLST analysis revealed that the strain was ST1232, a single-locus variant of ST398 that belongs to CC398. When we determined antimicrobial susceptibilities, the THI2018-120 strain exhibited multidrug resistance to oxacillin, gentamicin, clarithromycin, clindamycin, and tetracycline (Table). We detected resistance genes for aminoglycoside (*aacA-aphD*) and tetracycline (*tet*[K]). However, we did not find known macrolide resistance genes, including *ermA*, *ermB*, *ermC*, *ermT, mphC*, and *msrA/B*, or clindamycin resistance genes *lnuA*, *lnuB*, *lnuC*, and *lnuD* (*1*,*6*). We conducted experiments to detect virulence factors, which detected the *lukS/F-PV* genes (Table). In addition, the THI2018-120 strain carried *clfA*, *clfB*, and *fnbA*, which are microbial surface components recognizing adhesive matrix molecules (*6*).

On day 18, we performed surgical debridement. On day 29, the patient had a drug reaction to vancomycin, so we switched the antimicrobial agent to daptomycin. On day 80, we added oral rifampin to the patient's regimen to treat prolonged chronic osteomyelitis. The patient's symptoms improved, and we switched from daptomycin to oral levofloxacin on day 108. On day 119, the patient was discharged when we no longer detected MRSA in pus from drained and nonopen lesions.

Table. Antimicrobial drug resistance for clonal complex 398 sequence type 1232 staphylococcal cassette chromosome *mec* type V methicillin-resistant *Staphylococcus aureus* strain isolated from a patient in Japan*

Antimicrobial drug	MIC, μg/mL	Susceptibility
Ampicillin	4	ND
Oxacillin	8	R
Fosfomycin	0.5	S
Gentamicin	32	R
Levofloxacin	0.25	S
Clarithromycin	64	R
Clindamycin	≥256	R
Tetracycline	128	R
Vancomycin	1	S
Daptomycin	0.5	S

*Antimicrobial resistance genes included *mecA*, *aacA-aphD*, and *tet(K)*. Virulence factors included *lukS/F-PV*, *clfA*, *clfB*, and *fnbA*. ND, breakpoint is not defined by Clinical and Laboratory Standards Institute criteria; R, resistant; S, susceptible.

Human infections with the PVL-positive ST1232 MRSA strain are rare but were reported in New Zealand during 2011–2013 (*1*). In 2015, a fatal infection caused by a PVL-positive ST398 MRSA was reported in a patient who was infected in China but developed symptoms in Japan (*7*).

As mentioned, the virulence of animal-derived ST398 MRSA strains is considered to be lower than that of community-acquired MRSA (*3*). However, we presume that PVL production enhanced the severity of this case. Recent surveillance data suggest that not all cases of MRSA CC398 occurring among humans are related to animals (*8*). Our patient had no connection to animals, which suggests that this strain might be more common in Japan than previously thought. Further investigation, including whole-genome analysis of this isolate, could provide accurate phylogeny with higher resolution. In addition, a more robust estimation of this strain's virulence might elucidate the actual transmission route in this patient.

We previously reported the increased prevalence of the PVL-positive USA300 and USA300-LV clones in Japan, which were disseminated from North and Latin America (*6*). We also reported a case of septic arthritis by a PVL-positive ST772 Bengal-Bay clone, which is a predominant clone in India (*9*). Those data suggest that diverse and highly pathogenic PVL-positive MRSA clones have been entering Japan from abroad. We hypothesize that the PVL-positive ST1232 MRSA strain in this case also was transmitted from abroad. The transmission route of antimicrobial-resistant strains might be not only by humans but also by imported edible meat (*10*). From a One Health perspective, increased monitoring of imported livestock products is needed to prevent antimicrobial-resistant strains entering from other countries.

Acknowledgments

The authors thank Editage (http://www.editage.jp) for English language editing.

This work was supported by the Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (C) (grant no. 18K06797) and the Institute for Social Medicine at Tokyo University of Pharmacy and Life Sciences Grant to H.N.

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Detection of Rocio Virus SPH 34675 during Dengue Epidemics, Brazil, 2011–2013

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DOI: https://doi.org/10.3201/eid2604.190487

Recent seroprevalence studies in animals detected Rocio virus in regions of Brazil, indicating risk for re-emergence of this pathogen. We identified Rocio virus RNA in samples from 2 human patients for whom dengue fever was clinically suspected but ruled out by laboratory findings. Testing for infrequent flavivirus infections should expedite diagnoses.

Brazil has been affected by outbreaks caused by viruses of the genus *Flavivirus*, such as dengue (DENV), Zika, and yellow fever viruses, along with co-infections with other arboviruses (*1*). The amino acid sequences of polyproteins from viruses of this genus are very similar, which has limited the development of detection methods, often resulting in cross-reactions within serocomplexes during serologic testing (*2*). Therefore, tracking in areas where mosquito-specific flaviviruses co-circulate may have led to underestimated infections because of the detection and the hierarchy of disease based on medical importance.

Rocio virus (ROCV) is a potentially emerging neurotropic flavivirus in Brazil; however, because relatively little is known about the biology of this virus, technologies for its detection are limited (*3*–*5*). In 1975, ROCV was found to be related to the causative agent of a fatal outbreak of human encephalitis in Brazil; the case-fatality rate was 13%, and neurologic sequelae affected 20% of patients (*5*). The unexpected outbreak ended in 1980, but little documentation exists with regard to circulation of ROCV in Brazil.

To determine the extent of ROCV circulation in different areas of Brazil, we screened 647 serum samples collected during an outbreak of dengue fever during 2011–2013. The samples came from patients in care units of the public health system, which offer 24-hour outpatient urgent care, and emergency services in the city of Goiânia, central Brazil. The samples were from patients of all age groups and sexes

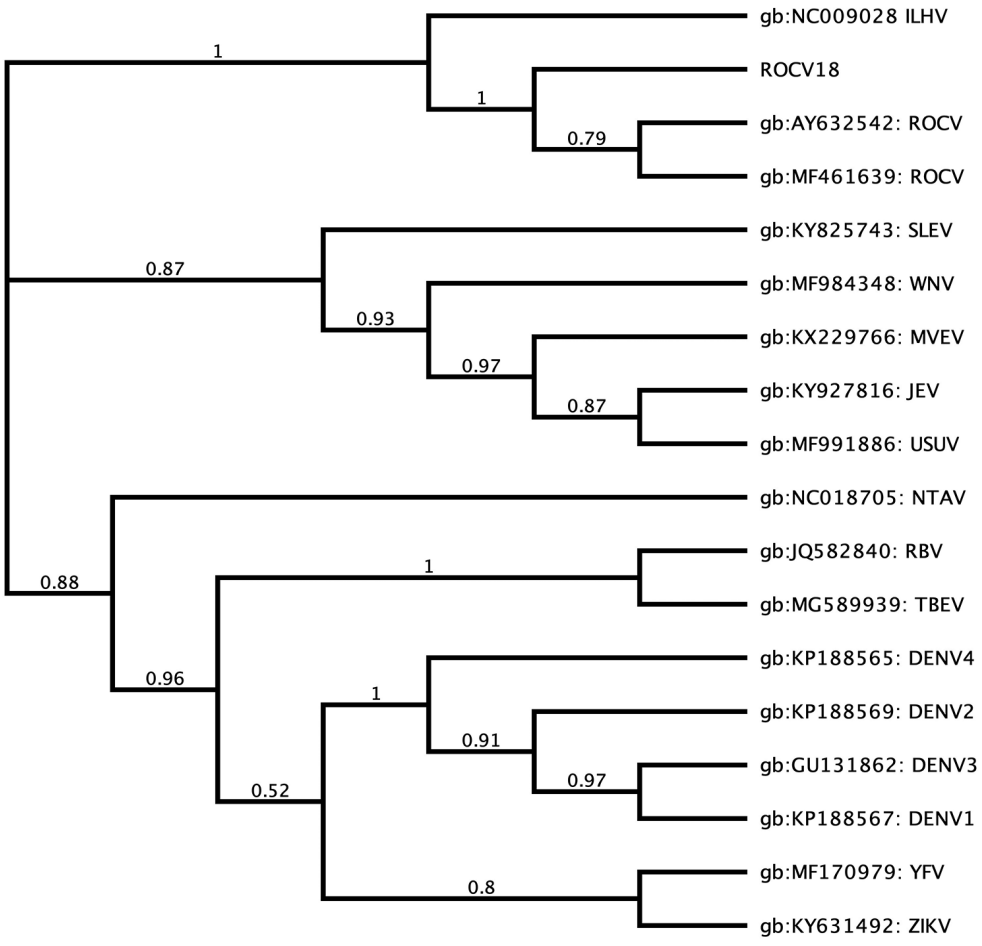

Figure. Phylogenetic analysis of the ROCV nonstructural 5 gene (ROCV18) detected during dengue epidemics in Brazil, 2013, and reference sequences. Tree constructed by using the maximum-likelihood method. Pairwise distances were calculated by using the neighbor-joining algorithm, and node numbers represent bootstrap values (10,000 replicates). GenBank accession numbers are provided. DENV, dengue virus; ILHV, Ilheus virus; JEV, Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; NTAV, Ntaya virus; RBV, Rio Bravo virus; ROCV, Rocio virus; SLEV, Saint Louis encephalitis virus; TBEV, tick-borne encephalitis virus; USUV, Usutu virus; WNV, West Nile virus; YFV, yellow fever virus; ZIKV, Zika virus.

who exhibited signs and symptoms of suspected dengue infection. During the outbreak, the city reported ≈88,000 cases of DENV infection (*6*). Of the 647 samples screened for DENV by use of serologic and molecular methods, 121 were negative for DENV. We subsequently screened those 121 samples for ROCV.

Using nested PCR with genus-specific primers (*7*), we detected the ROCV nonstructural (NS) 5 gene in 2 of the 121 samples. We used the amplified sequences from the ROCV NS5 gene for phylogenetic analysis, which confirmed 100% identity with the consensus sequence of ROCV NS5 in strain SPH 34675, the strain isolated from the 1975 encephalitis outbreak. Furthermore, the detected NS5 ROCV gene (ROCV 18) is related to Ilheus virus from the Japanese encephalitis virus complex and did not change the topology of the phylogenetic tree with other pathogenic flaviviruses, as previously reported (*3*) (Figure).

The 2 ROCV-positive samples were from a 33-year-old female patient and a 47-year-old male patient. The female patient experienced prostration, abdominal pain, diarrhea, and thrombocytopenia (120,000 platelets/mm^3), and the male patient experienced headache, eye pain, pruritus, nausea, and leukopenia (3,560 cells/mm^3) (Appendix, https://wwwnc.cdc.gov/EID/article/26/4/19-0487-App1.pdf). Both patients had fever, myalgia, and arthralgia, but they denied having had chronic diseases and had been vaccinated against yellow fever virus. No information about patients' residence or travel history was available. The patients received ambulatory care, and their clinical outcome was cure.

The molecular diagnostic result for positive ROCV in humans reported in this study corroborates the results of other studies involving serologic tests for ROCV in animals (*8*,*9*) and demonstrates the high probability that ROCV is circulating in different areas of Brazil. Our findings point out the need for clinicians to clearly establish flavivirus infection diagnoses by testing for various and infrequent regional flaviviruses

Acknowledgments

We thank Mariana Pires de Campos Telles for her research.

This work was funded by Fundação de Amparo à Pesquisa do Estado de Goiás (public call no. 05/2012) and was conducted during a scholarship supported by the Program CAPES-Brazilian Federal Agency for Support and Evaluation of Graduate Education within the Ministry of Education of Brazil.

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Epidemiology of Lassa Fever and Factors Associated with Deaths, Bauchi State, Nigeria, 2015–2018

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DOI: https://doi.org/10.3201/eid2604.190678

We report the epidemiology of Lassa fever in Bauchi State, a disease-endemic region, in Nigeria. Since 2015, major increases in Lassa fever attack rate and in the case-fatality rate have occurred in this state. A delay in seeking care by a case-patient for >7 days after symptom onset was the major predictor of death.

In recent years, Lassa fever (LF) outbreaks in Nigeria have become more frequent and larger in magnitude; the outbreak in 2018 was described as the largest in history (*1*). Bauchi State, which had never reported an LF case before 2012, has quickly become one of the high-risk states for LF in this country (*2*). We report LF epidemiology in Bauchi State and identify factors associated with death.

We retrospectively reviewed data for LF cases during January 2015–December 2018 obtained from the platform for integrated disease surveillance and

response for Bauchi State. The source of the data has 100% completeness for variables of interest (sociodemographic characteristics, laboratory results, outcome of illness, health facility of admission, date of onset of illness, date care was sought, date of death, first health center, clinical features at initial examination, outcomes of laboratory investigations, and treatment outcomes).

We analyzed data by using Epi Info version 7.2 software (https://www.cdc.gov/epiinfo/support/downloads.html). We calculated frequencies and proportions and examined the relationship between the outcome variable (death) and the risk factors (including sociodemographics) by using the χ^2 test. We included significant variables ($p< 0.1$) by bivariate analysis and the biologically plausible ones (sex and age) in an unconditional logistic regression model; $\alpha = 0.05$ was considered the level of significance.

A total of 368 suspected LF cases were reported in Bauchi State during January 2015–December 2018, of which 76 were confirmed. The mean ± SD age for confirmed case-patients was 30.7 ± 15.8 years, and most (81.6%) case-patients were 15–64 years of age. This age group had the highest age-specific attack rate (1.8 cases/100,000 persons), and patients <5 years of age had the lowest attack rate (0.2 cases/100,000 persons). Most (54.0%) patients were male; the attack rate was 1.2 cases/100,000 persons for male sex and 1.1 cases/100,000 persons for female sex. The overall case-fatality rate (CFR) was 54.0% (41/76) and was highest (66.6%) for persons <5 years of age (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/26/4/19-0678-App1.pdf). All LF cases were reported from districts contiguously located in the southern parts of the state (Appendix Figure 2).

Overall, more cases were reported in the early and late months of the year compared with the middle months (Appendix Figure 3). The CFR trend for LF showed a major increase from 33.3% in 2015 to 53.3% in 2018 (linear trend χ^2 4.8; $p = 0.03$), and the attack rate increased from 0.1 cases/100,000 persons during 2015 to 0.4 cases/100,000 persons during 2018 (linear trend χ^2 14.0; $p<0.01$). We found by multivariate analysis that a delay in seeking care for >7 days after onset of illness (adjusted odds ratio 6.2, 95% CI 1.40–27.60) or for ≥24 hours after onset of bleeding (adjusted odds ratio 6.4, 95% CI 1.40–29.44) were independent predictors for dying from LF (Table).

This study demonstrated that LF has become a highly fatal disease in Nigeria. With the productive age group being the most affected by LF, its socioeconomic impact in the affected communities should be of concern (*3*). A similar age distribution was reported in a study from the neighboring Plateau State in Nigeria, which reviewed confirmed LF cases reported during 2012–2016 (*4*). However, our findings were different from those for a study from Sierra Leone in 2014, in which children and adolescents were more affected (*5*). This finding was probably caused by a difference in cultural environment between the 2 settings.

The southward geographic distribution of LF cases in the study area might be related to the distinctive Sudan savanna vegetation in that part of Bauchi State, which is characterized by higher annual rainfall (which has been shown to influence the incidence of

Table. Independent predictors of dying from Lassa fever, Bauchi State, Nigeria, 2015–2018*

Variable	No. (%) died, n = 41	No. (%) survived, n = 35	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Sex				
M	21 (51.2)	20 (48.8)	0.8 (0.32–1.95)	0.8 (0.18–3.70)
F	20 (57.1)	15 (42.9)		
Age group				
Productive	33 (53.2)	29 (46.8)	0.9 (0.26–2.75)	1.2 (0.22–6.68)
Dependent	8 (57.1)	6 (42.9)		
Place of residence				
Rural	27 (48.2)	29 (51.8)	0.4 (0.13–1.19)	0.4 (0.08–2.27)
Urban	14 (70.0)	6 (30.0)		
First place of admission				
Tertiary hospital	27 (46.6)	31 (53.4)	**0.2 (0.07–0.85)**	0.2 (0.02–1.45)
Other	14 (77.8)	4 (22.2)		
Bleeding episode†				
Yes	34 (60.7)	22 (39.3)	2.2 (0.73–6.66)	NI
No	7 (41.2)	10 (58.8)		
Duration between seeking care and onset of illness, d				
>7	25 (73.5)	9 (26.5)	**4.5 (1.69–12.08)**	**6.2 (1.40–27.60)**
≤7	16 (38.1)	26 (61.9)		
Duration between seeking care and any bleeding episode, h‡				
≥24	25 (80.6)	6 (19.4)	**7.4 (2.21–24.81)**	**6.4 (1.40–29.44)**
<24	9 (36.0)	16 (64.0)		

*Bold indicates significance ($p<0.05$). NI, not included in a regression model; OR odds ratio.
†n = 73 for this variable because of missing values. Variable not included in the regression model ($p>0.1$).
‡n = 56 for this variable because not all case-patients had a bleeding episode.

the disease) compared with the Sahel savanna vegetation in the central and northern parts of Africa (*6*,*7*). Furthermore, the intensive agricultural activities in the southern districts and the common postharvest practice of drying crops in open spaces in these hilly areas probably favor food contamination by the disease vector.

Our finding that a delay in seeking care of ≥24 hours after onset of bleeding was a strong predictor of death among cases is a concern. LF has some common early symptoms similar to those of other febrile diseases, especially malaria, that are frequently encountered in most LF-endemic settings (*8*). An LF diagnosis is often delayed because health workers suspect these other febrile diseases (*8*). Furthermore, Bauchi State has the highest CFR in Nigeria, nearly double the national average (28.9%) (*2*,*9*). A similarly high CFR was reported in a previous study in Sierra Leone in a region affected by conflicts where the health infrastructure was poor (*5*). Finally, the designated LF treatment center in Bauchi State lacks adequately trained personnel and other essential resources to effectively manage complications once they occur. If one considers that a delay in seeking care has been demonstrated to be a predictor of death in this study, the high CFR in Bauchi State could have been lower if the treatment center was better equipped or if cases could be diagnosed earlier.

Acknowledgments

We thank the Bauchi State Ministry of Health for providing support during this study.

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Plague Epizootic Dynamics in Chipmunk Fleas, Sierra Nevada Mountains, California, USA, 2013–2015

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DOI: https://doi.org/10.3201/eid2604.190733

We describe *Yersinia pestis* minimum infection prevalence in fleas collected from *Tamias* spp. chipmunks in the Sierra Nevadas (California, USA) during 2013–2015. *Y. pestis*–positive fleas were detected only in 2015 (year of plague epizootic), mostly in *T. speciosus* chipmunks at high-elevation sites. Plague surveillance should include testing vectors for *Y. pestis*.

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To better forecast vectorborne infection dynamics, characterizing disease cycles in both hosts and vectors is critical. The rate of infection of vector species can serve as a good indicator for risk during epizootic events, especially in areas with high human–wildlife overlap, but vectors are often poorly sampled. *Yersinia pestis*, the bacterium that causes plague, is carried by multiple flea species in western North America, where sciurids are often the primary reservoirs (*1*). Although human plague cases in this area are rare, in 2015, two cases were linked to exposures in Yosemite National Park, California, USA (*2*). In the investigation conducted to determine the source of these exposures, multiple *Y. pestis*–positive flea and rodent species were documented, and the lodgepole chipmunk (*Tamias speciosus*) was the host that was most frequently seropositive (*2*).

Plague surveillance in the western United States typically involves serologic testing of rodents and carnivores. Positive serologic results indicate prior plague activity. A *Y. pestis*–positive flea, however, indicates current plague transmission and is more likely to trigger control activities (*3*). Here, we sought to characterize *Y. pestis* infection in fleas of alpine (*T. alpinus*) and lodgepole (*T. speciosus*) chipmunks in Yosemite National Park and surrounding areas during 2013–2015. We focused on *T. speciosus* chipmunks because of their documented role in the 2015 epizootic (*2*) and on *T. alpinus* chipmunks because they co-occur with *T. speciosus* chipmunks (*4*) and little is known about their role in plague ecology. Our goals were to describe the proportion of *T. speciosus* and *T. alpinus* chipmunks harboring *Y. pestis*–positive fleas and the minimum infection prevalence of *Y. pestis* in fleas collected from these species across multiple sites and in years with and without known epizootic activity.

During June–October 2013–2015, we collected fleas from tagged chipmunks. Using a metal-pronged comb, we combed each animal 5 times down the dorsum, the tail, and each hind leg and placed collected fleas into vials containing 100% ethanol. These procedures were approved by the University of California, Berkeley, Animal Care and Use Committee (Berkeley, California, USA).

We identified key flea specimens (N = 122) (*5*–*9*) and then cleared, dehydrated, and mounted them on microscope slides (Denver Museum of Nature and Science accession nos. ZP.2000–176). For the remaining fleas, we microscopically observed and identified the species (*5*) using keys (*6*–*9*) and mounted some fleas as references. For each host, we pooled all conspecific fleas, which resulted in 162 pools (with 291 fleas total) from 121 *T. alpinus* chipmunks and 538 pools (with 1,096 fleas total) from 389 *T. speciosus* chipmunks (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/26/4/19-0733-App1.pdf). We used molecular methods to detect *Y. pestis* DNA in flea pools (Appendix).

We found *Y. pestis*–positive fleas exclusively in 2015 at 5 of the 6 sites surveyed (Figure; Appendix Table 2). In 2015, 7.29% (14/192) of *T. speciosus* hosts carried ≥1 *Y. pestis*–positive flea. The minimum infection prevalence of *Y. pestis* in *T. speciosus* chipmunk–hosted fleas was 3.28% (assuming 1 positive flea per positive pool, 18 positive pools/548 total fleas in 280 pools tested). All 3 of the flea species (*Ceratophyllus ciliatus mononis*, *Eumolpianus eumolpi*, and *E. eutamiadis*) most commonly found on *T. speciosus* and *T. alpinus* chipmunks were found to be positive for *Y. pestis* (Appendix Table 1) (*10*). In 2015, a total of 5.13% (2/39) of *T. alpinus* hosts carried ≥1 *Y. pestis*–positive flea (Appendix Table 2). The infection prevalence (not minimum infection prevalence because each positive pool contained a single flea) of *Y. pestis* in *T. alpinus* chipmunk–hosted fleas was 2.47% (2 positive pools/81 total fleas in 50 pools tested). Unfortunately, these fleas were too damaged to identify morphologically, and molecular species identification was not possible.

Y. pestis–positive flea pools were detected at 5 of 6 high-elevation (2,650–3,200-m) study sites in 2015. Many of these sites are areas of high human activity, with popular hiking trails or established campgrounds. In 2015, plague risk assessments, including testing flea pools and rodent carcasses for *Y. pestis* DNA and rodent serology, also took place at lower elevation sites (1,778 ± 553 m) in and around the park; these surveys detected *Y. pestis* at 4 of 17 locations (*2*).

Altogether, our data indicate a dramatic shift in *Y. pestis* prevalence in fleas during a plague epizootic year in California. Our results support integrating flea testing, especially those at high-elevation sites, into regular surveillance.

Acknowledgments

We thank numerous field assistants, Mary Joyce Pakingan and Sabrina Horrack for assistance with laboratory work, and Jon Pigage for providing flea identification support.

T.T.H. was supported by a National Science Foundation graduate research fellowship during this work.

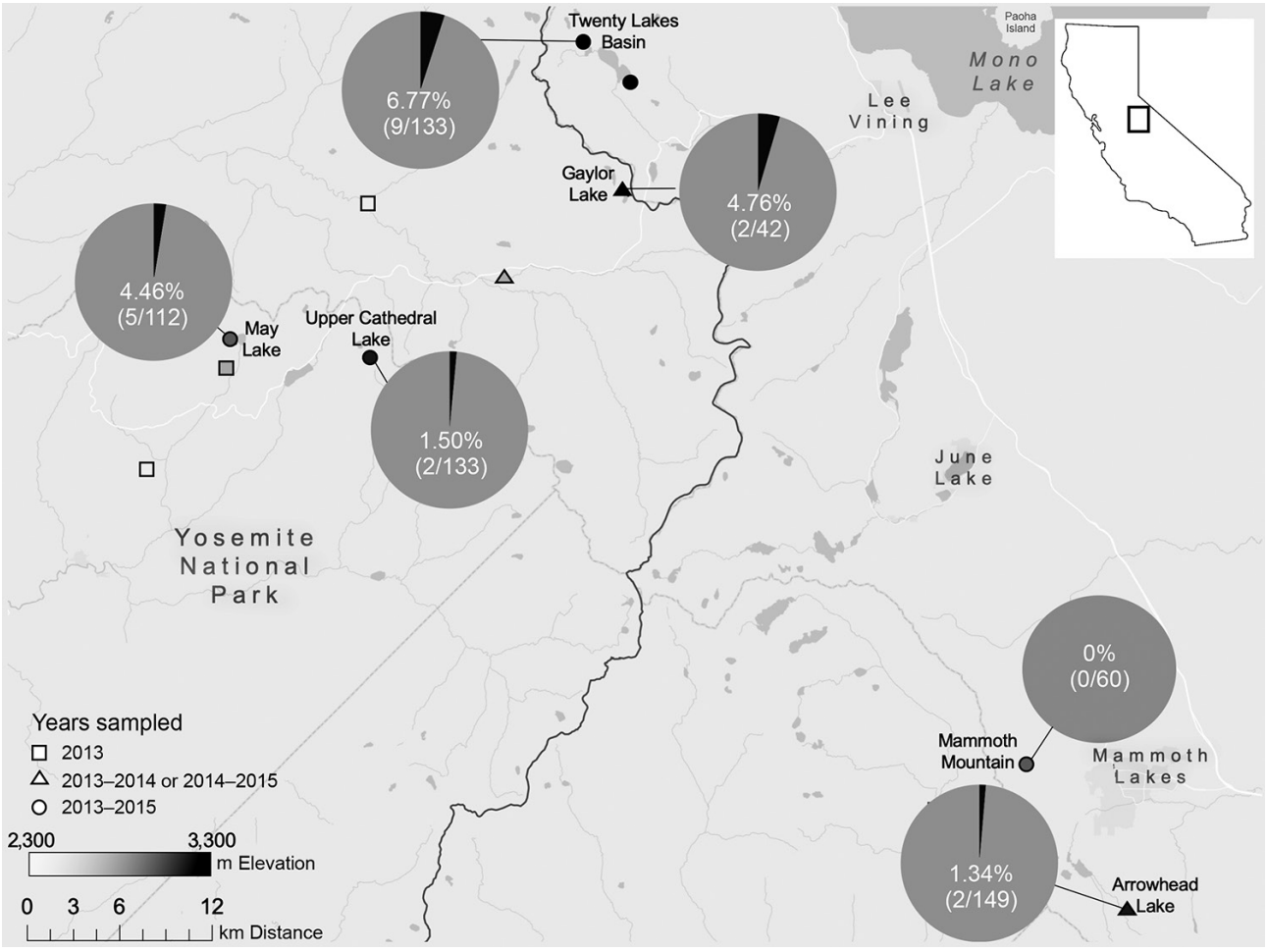

Figure. Minimum *Yersinia pestis* infection prevalence in fleas harbored by *Tamius speciosus* and *T. alpinus* chipmunks, by study site, Sierra Nevadas, California, 2013–2015. We visited sites for different numbers of years: 1 study year (2013 only), 2 study years, or all 3 study years. Plague prevalence was zero in 2013 and 2014, and map shows plague prevalence only in 2015. Pie charts show percentage of minimum infection prevalence (no. *Y. pestis* DNA–positive pools/no. fleas in pools tested). Sites without pie charts were either not visited in 2015 or had no flea pools collected there in 2015 because of low chipmunk prevalence. The irregular black line shows the eastern border of Yosemite National Park. Inset shows location of study sites in California. See Appendix Tables 1, 2 (https://wwwnc.cdc.gov/EID/article/26/4/19-0733-App1.pdf) for more details on fleas tested.

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Brucella melitensis in Asian Badgers, Northwestern China

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DOI: https://doi.org/10.3201/eid2604.190833

We isolated *Brucella melitensis* biovar 3 from the spleen of an Asian badger (*Meles leucurus*) in Nilka County, northwestern China. Our investigation showed that this isolate had a common multilocus variable-number tandem-repeat analysis 16 genotype, similar to bacterial isolates from local aborted sheep fetuses.

[1]These authors contributed equally to this article.

Brucellosis can be transmitted between domestic animals and wildlife (*1*). *Brucella melitensis* has been isolated from wildlife, such as chamois (*Rupicapra rupicapra*) (*2*), Alpine ibex (*Capra ibex*) (*3*), and Iberian wild goat (*Capra pyrenaica*) (*4*). Badgers are major predators in forests and consume a broad spectrum of food items, including small terrestrial vertebrates and their cadavers (*5*), which might result in contact with pathogens from tissues of these vertebrates. We report an Asian badger (*Meles leucurus*) in China naturally infected with *B. melitensis* biotype 3.

This study was approved by the Animal Ethics Committee of Shihezi University (approval no. AEC-SU2017–04). In 2017, a total of 7 illegally hunted and dying badgers in Nilka County, northwestern China, were confiscated by the local government.

We identified the animals as Asian badgers by using a PCR targeting the 16S rDNA gene (GenBank accession no. MH155253). We collected different organs or tissues, including heart, liver, spleen, lung, kidney, small intestine, large intestine, and blood, from all badgers. We separated serum from blood samples by centrifugation at 1,000 × *g* for 15 min and tested serum by using the rose bengal test (RBT) and serum agglutination test (SAT) (*6*). To detect *Brucella* antigens, we used immunohistochemical staining of liver and spleen tissue sections by pipetting mouse anti–*Brucella melitensis* IgG diluted 1:100 in 30% bovine serum albumin/phosphate-buffered saline onto each section. For comparison, we collected samples from 14 aborted sheep fetuses from Nilka County.

We extracted genomic DNA from all samples by using a commercial kit (Blood and Cell and Tissue Kit; BioTeke, http://www.bioteke.com). We used the partial *omp*22 gene (238 bp) encoding 22-kD outer membrane protein to identify the *Brucella* genus and the *IS*711 gene to identify *Brucella* species. We used PCRs that have been described (*7*). We used *Brucella* reference strains (*B. melitensis* 16M and *B. abortus* 2308) as positive controls and double-distilled water as a negative control.

We homogenized spleen samples of badgers and the 14 aborted sheep fetuses and inoculated these homogenates onto individual *Brucella* agar plates, which we then incubated at 37°C in an atmosphere of 5% CO_2 for 5 days. We tested putative *Brucella* colonies using by H_2S production, dye inhibition, agglutination by monospecific serum, and sensitivity to bacteriophages (Appendix Table, https://wwwnc.cdc.gov/EID/article/26/4/19-0833-App1.pdf). We analyzed colonies by using a multilocus variable-number tandem-repeat analysis (MLVA) typing assay (*8*).

Only serum from badger no. 2 was positive for smooth *Brucella* antigen by RBT and SAT; the specific

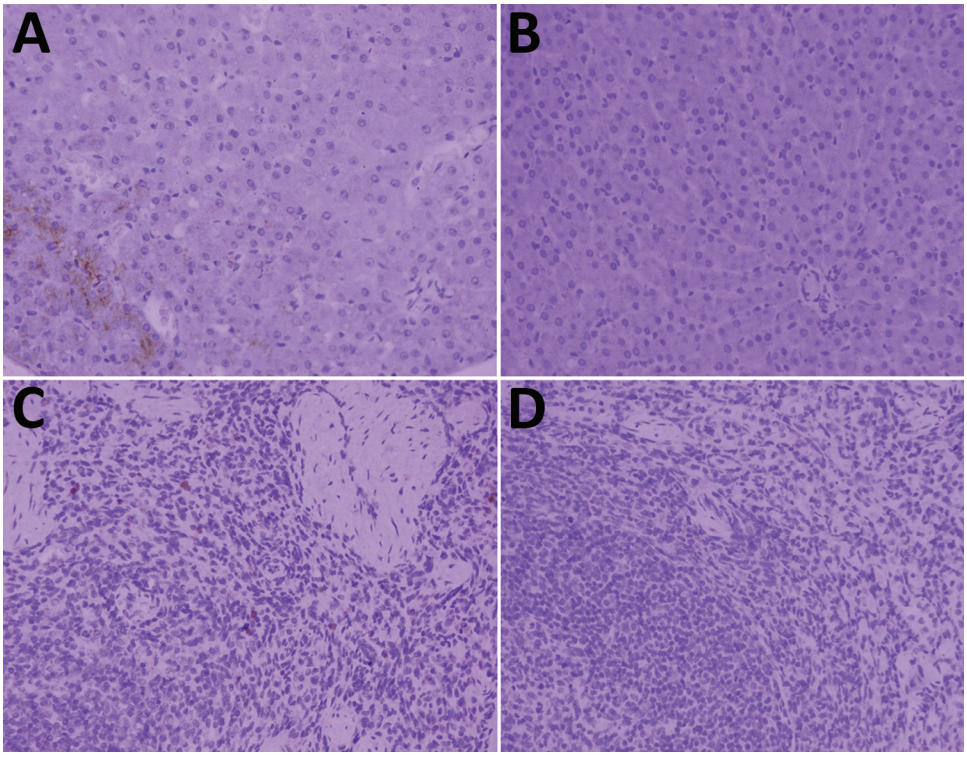

Figure. *Brucella* antigen in liver and spleen from Asian badger infected with *Brucella melitensis*, northwestern China. A) *Brucella* antigen in liver of Asian badger no. 2; B) liver of an uninfected Asian badger; C) *Brucella* antigen in spleen of Asian badger no. 2; D) spleen of badger without *Brucella* antigen. Diaminobenzidine staining; original magnification ×400.

antibody titer was 1:160 (≈125 IU/mL). We successfully amplified 2 genetic markers (regions of the *omp*22 and *IS*711 genes) from blood, heart, liver, spleen, lung, kidney, small intestine, and large intestine from badger no. 2 but not from samples of other badgers. In addition, we isolated *B. melitensis* biotype 3 from badger no. 2 and 5 aborted sheep fetuses according to phenotypic identification (Appendix Table). MLVA-16 typing indicated that the isolates from badger no. 2 and aborted sheep fetuses had a common MLVA-16 type (1-5-3-13-2-2-3-2-4-40-8-8-4-3-7-7). In addition, immunohistochemical staining with a brown chromogen (diaminobenzidine) identified *Brucella* antigens in liver and spleen of badger no. 2 (Figure).

B. melitensis is isolated mainly from goats and sheep, in which it causes fetal abortion (*1*). The Asian badger is a semihibernating, burrowing animal species that has not been reported to harbor this pathogen. In a previous study, Li and Hu reported that 0.30% (12/4,015) of sheep in Nilka County, China, were serologically positive for smooth *Brucella* antigen by RBT and 9.75% (145/1,485) were serologically positive for smooth *Brucella* antigen by SAT (*9*). The habitats of Asian badgers and the grazing areas of sheep and goats partially overlap, which can be most likely explained by observations of shepherds that Asian badgers eat aborted fetuses or their placentas during lambing season in winter. In this study, *B. melitensis* biovar 3 isolates, designated as XJ1802 and XJ1804 strains, were found in aborted sheep fetuses and an Asian badger. MLVA-16 typing indicated that they shared a common MLVA-16 type (Appendix Figure). This finding suggests that the Asian badger is a *Brucella* spillover host that becomes infected from sheep that act as a reservoir host.

Another study reported that coyotes were infected probably through ingestion of aborted fetuses and placentas in enzootic brucellosis areas (*10*). In our study, we detected *Brucella* DNA from blood, heart, liver, spleen, lung, kidney, small intestine, and large bowel of badger no. 2 and identified *B. melitensis* biovar 3 from spleen tissue. This finding suggests that pathologic changes in multiple organs or tissues caused by *B. melitensis* might occur.

In the future, it will be essential to evaluate the clinical status of Asian badgers naturally infected with *B. melitensis*. In addition, more extensive surveillance is necessary to expand our knowledge on the epidemiologic interface between wildlife and domestic animals in the context of *Brucella* infections.

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Multicenter Study of Azole-Resistant *Aspergillus fumigatus* Clinical Isolates, Taiwan[1]

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[1]Results from this study were presented in part at the 30th International Congress of Chemotherapy and Infection, November 24–27, 2017, Taipei, Taiwan.

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DOI: https://doi.org/10.3201/eid2604.190840

In a multicenter study, we determined a prevalence rate of 4% for azole-resistant *Aspergillus fumigatus* in Taiwan. Resistance emerged mainly from the environment (TR_{34}/L98H, TR_{34}/L98H/S297T/F495I, and TR_{46}/Y121F/T289A mutations) but occasionally during azole treatment. A high mortality rate observed for azole-resistant aspergillosis necessitates diagnostic stewardship in healthcare and antifungal stewardship in the environment.

Worldwide emergence of azole-resistant *Aspergillus fumigatus* since the late 2000s threatens human health (*1*). Azole resistance in *A. fumigatus* might develop during patient therapy with medical azoles or through exposure to azole fungicides in the environment; environmental exposure predominantly involves TR_{34}/L98H and TR_{46}/Y121F/T289A mutations in *cyp51A* (*1*).

Taiwan is an island country in eastern Asia that is geographically separated from mainland Eurasia and has a long history of azole fungicide use. To delineate the influence of clinical and environmental use of azoles on resistance, we conducted a multicenter study that investigated 375 *A. fumigatus* sensu stricto isolates collected during August 2011–March 2018 from 297 patients at 11 hospitals in Taiwan (Appendix Table 1, Figure 1, https://wwwnc.cdc.gov/EID/article/26/4/19-0840-App1.pdf).

We confirmed the presence of azole resistance by using the Clinical Laboratory Standard Institute method (Appendix Table 1) (*2*). Isolates resistant to ≥1 medical azoles (itraconazole, voriconazole, posaconazole, and isavuconazole) were defined as azole-resistant *A. fumigatus* and examined for resistance mechanisms, microsatellite-based phylogenetic relatedness, and growth rates following previously described methods (*3*,*4*).

Overall, 19 isolates from 12 patients were azole-resistant *A. fumigatus*. These isolates had resistance rates of 4.0%/patient and 5.1%/isolate analyses (Appendix Tables 2, 3). Ten (83.3%) patients harbored azole-resistant *A. fumigatus* that had environmental mutations, including TR_{34}/L98H (5 isolates, 5 patients), TR_{34}/L98H/S297T/F495I (7 isolates, 4 patients), and TR_{46}/Y121F/T289A (1 isolate) mutations. This observation

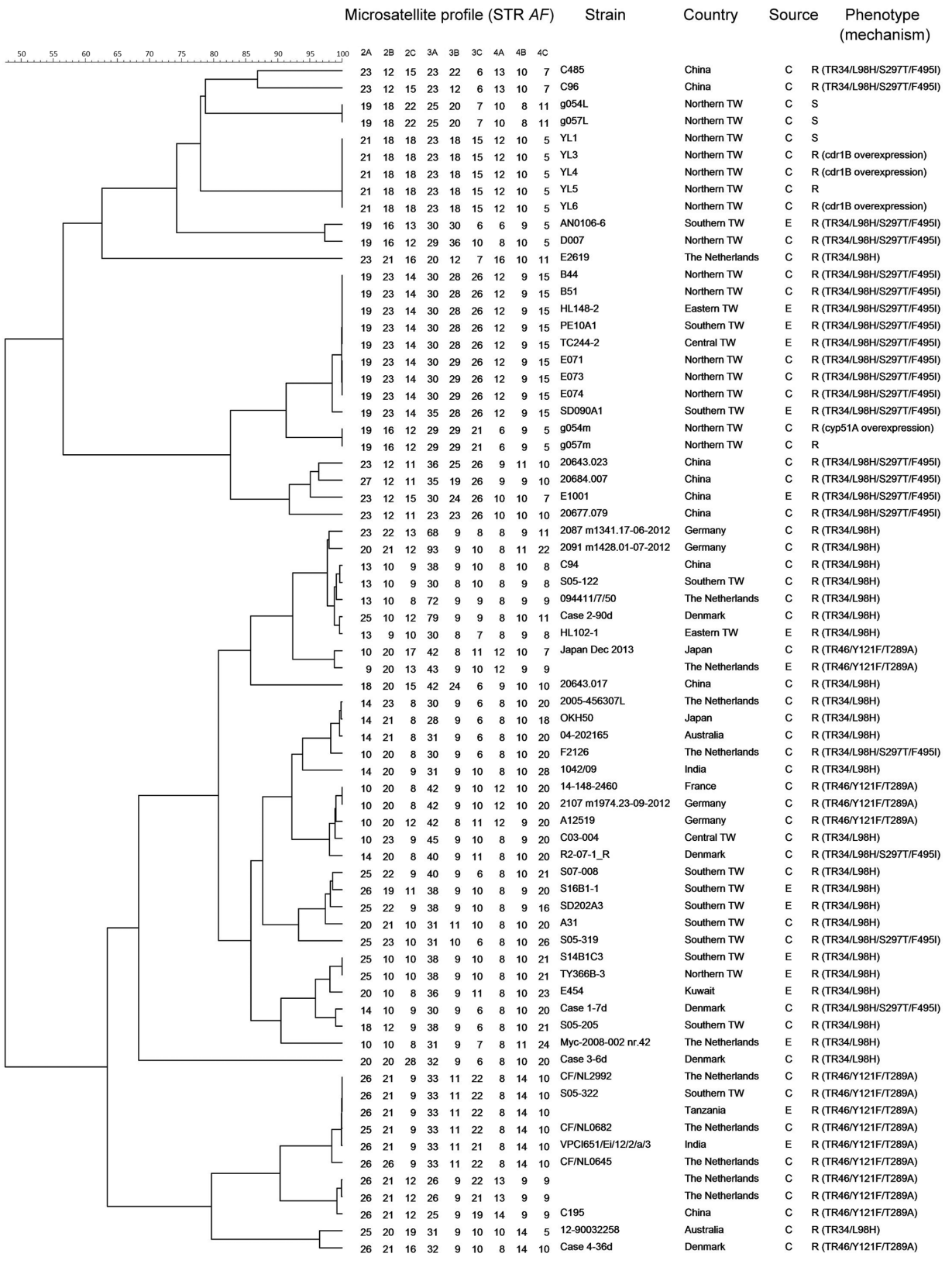

Figure. Genetic relatedness among *Aspergillus fumigatus* isolates based on microsatellite genotyping, Taiwan. Scale bar indicates percentage relatedness. *AF*, *A. fumigatus*; C, clinical; E, environmental; R, azole-resistant; S, azole-susceptible; STR, short tandem repeat; TW, Taiwan.

is consistent with the estimated global prevalence of azole resistance in *Aspergillus* (3%–6%) and the predominance of environmental resistance mechanisms in azole-resistant *A. fumigatus* (*1*,*5*).

Phylogenetic analysis showed that TR_{34}/L98H/S297T/F495I isolates from 2 patients with pulmonary aspergillosis (isolates B44 and B51 in 2012, isolates E071, E073, and E074 in 2015) (Figure) belonged to a local microsatellite genotype widely distributed in the environment of Taiwan (*3*), indicating that this clone has locally evolved and adapted to the environment. The TR_{34}/L98H isolates were genetically clustered with local environmental isolates or clinical isolates from China and Europe (Appendix Table 4). The TR_{46}/Y121F/T289A isolate (S05–322) recovered in 2018, which colonized a patient without overseas travel, was genetically identical to a clone prevalent in the Netherlands and Tanzania (*6*), raising the concern of the intercountry transfer of resistant isolates.

All TR_{34}/L98H/S297T/F495I, TR_{34}/L98H, and TR_{46}/Y121F/T289A isolates exhibited cross-resistance to difenoconazole and tebuconazole (both triazole fungicides) without fitness cost, demonstrated by normal growth rates (Appendix Figure 2). The TR_{34}/L98H/S297T/F495I isolates and TR_{46}/Y121F/T289A isolates were also resistant to prochloraz (an imidazole fungicide) (Appendix Table 2). The prevalence of TR_{34}/L98H/S297T/F495I isolates in Taiwan might be attributed to widespread use of prochloraz over the past 3 decades. Studies have suggested an association between use of imidazole fungicides and emergence of azole-resistant *A. fumigatus* with TR_{34}/L98H/S297T/F495I mutations (*7*,*8*).

In Taiwan, the annual consumption of difenoconazole and tebuconazole has exceeded that of prochloraz since 2012 (Appendix Figure 3), further creating a favorable environment for maintenance and spread of TR_{34}/L98H, TR_{34}/L98H/S297T/F495I, and TR_{46}/Y121F/T289A isolates. Thus, the One Health approach to implement environmental antifungal stewardship is warranted to minimize ongoing resistance selection in the fields.

Six azole-resistant *A. fumigatus* isolates with wild-type *cyp51A* were obtained from 2 patients. Four pan–azole-resistant urinary isolates were sequentially recovered from a patient (no. 11) with *A. fumigatus* renal abscesses who was receiving voriconazole for >3 months in whom an initial urine isolate was susceptible to azole; all 5 isolates were genetically identical.

Overexpression of *cdr1B* (a drug efflux transporter) and an S269P mutation in *hmg1* (a hydroxymethylglutaryl-CoA reductase) were identified in 4 resistant isolates but not in the initial susceptible isolate (Appendix Table 5, Figure 4), suggesting their roles involved in azole resistance (*4*,*9*). Another 2 pan–azole-resistant respiratory isolates were recovered from a patient (no. 12) who had pulmonary aspergillosis and was receiving voriconazole for 4 months. Azole-susceptible and azole-resistant isolates co-existed in this patient, which echoes the international recommendation suggesting testing multiple colonies (≥5) from a single culture (*1*). *Cyp51A* overexpression and an F262 deletion in *hmg1* (*hmg1*$^{F262\_del}$) were identified in these 2 resistant isolates. Although *hmg1*$^{F261\_del}$ was recently reported in azole-resistant *A. fumigatus* from a voriconazole-exposed patient (*4*), whether *cyp51A* overexpression and *hmg1*$^{F262\_del}$ act synergistically to cause resistance warrants further studies.

Finally, reduced colony sizes were observed in all 6 azole-resistant *A. fumigatus* isolates with wild-type *cyp51A* (Appendix Figure 2). Thus, attention should be paid to select colonies of various sizes for susceptibility testing from patients with azole exposure.

Overall, 4 patients harboring azole-resistant *A. fumigatus* with environmental mutations and 2 patients harboring azole-resistant *A. fumigatus* with wild-type *cyp51A* showed development of invasive aspergillosis, and all had aspergillosis-related deaths. High mortality rates for azole-resistant aspergillosis we observed (6/6, 100%) and for those from a previous report (*10*) emphasize the need for a proposed integrated algorithm for management and control of azole-resistant aspergillosis (Appendix Table 6).

In conclusion, we report a health threat that arose from clinical and environmental use of azoles; environmental use contributed at a larger and global scale. These data necessitate diagnostic stewardship in the clinic and antifungal stewardship in the environment.

Acknowledgments

We thank Cheng-Chen Cheng, Li-Fang Chen, Li-Yu Yang, and Ya-Feng Wu for laboratory assistance; Hui-Chun Chang for collecting clinical information; and the following hospitals for sharing *Aspergillus* spp. clinical isolates: Changhua Christian Hospital, Chi Mei Medical Center-Liouying, Chung Shan Medical University Hospital, Ditmanson Medical Foundation Chia-Yi Christian Hospital, Far Eastern Memorial Hospital, Hualien Tzu Chi Hospital, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung Medical University Chung-Ho Memorial Hospital, Kaohsiung Veterans General Hospital, Mennonite Christian Hospital, National Cheng Kung University Hospital, National Taiwan University Hospital, Show Chwan Memorial Hospital, Taichung Veterans General Hospital, Tainan Sin Lau Hospital, and Taipei City Hospital Heping Fuyou Branch.

This study was supported by the National Health Research Institutes of Taiwan (grants IV-105-PP-08 and IV-106-PP-08), the Ministry of Science and Technology of Taiwan (grants MOST 105-2628-B-400-004-MY2), and Gilead Sciences, Inc. (grant IN-US-131-3918).

Y.-C.C. has received honoraria for speaking and advisory board membership from Pfizer, Gilead, Merck, and Astellas, and was involved as a steering committee member of regional education programs at Gilead (Asia CARE) and Pfizer (Medical Mycology Training Network). W.-LL. has received honoraria for speaking from Pfizer and Gilead.

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Knowledge of Infectious Disease Specialists Regarding Aspergillosis Complicating Influenza, United States

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DOI: https://doi.org/10.3201/eid2604.190953

In an online survey, we found that nearly one fifth of physicians in the United States who responded had seen or heard about a case of invasive pulmonary aspergillosis after severe influenza at their institution. However, <10% routinely used galactomannan testing to test for this fungus in patients with severe influenza.

Invasive pulmonary aspergillosis (IPA) occurs primarily among immunocompromised patients with a history of organ or stem cell transplantation, chemotherapy, or immunosuppressive medications. However, a multicenter retrospective study in the Netherlands and Belgium suggested that patients

with severe influenza (i.e., requiring intensive care unit [ICU] admission) are also at risk for IPA (*1*). In that study, 19% patients with severe influenza showed development of IPA. More than half of these patients were not immunocompromised, and mortality rates were twice as high among ICU patients with IPA compared with those without IPA.

Corticosteroids, which have been associated with higher mortality rates and are used for influenza patients (*2*), are a known risk factor for IPA and have been associated with IPA in severe influenza (*3*). However, 44% of patients who showed development of IPA in the study in the Netherlands and Belgium had not received these medications (*1*). Although case reports exist (*4*,*5*), clinicians might not consider IPA as a cause of worsening respiratory function or sepsis because influenza is not considered a classical risk factor for IPA and because of the complexity inherent in diagnosis (*6*). In the study in the Netherlands and Belgium, IPA cases were diagnosed by galactomannan antigen testing of bronchoalveolar lavage fluid (*1*). Although galactomannan testing might be useful in the ICU setting (*7*), it is unclear how often galactomannan testing is performed in the United States.

To clarify clinical practices regarding diagnosis of IPA in patients with severe influenza, the Emerging Infections Network (EIN) surveyed infectious disease specialists in the United States. EIN is a provider-based emerging infections sentinel network supported by the Centers for Disease Control and Prevention and the Infectious Diseases Society of America (*8*). During May–June 2018, EIN distributed a 6-question poll to its >1,500 member listserv (https://ein.idsociety.org); 114 responded.

Twenty-nine (26%) respondents were familiar with reports of aspergillosis after severe influenza, and 21 (18%) had seen or heard about ≥1 case at their institution (Table). Among 108 responding clinicians, 33 (31%) always or very often used lower respiratory tract specimens for diagnostic testing in patients with severe influenza. Only 8 (8%) of 107 clinicians always or very often used galactomannan testing in patients with severe influenza in the ICU and worsening respiratory function.

Most respondents were unaware of concerns about IPA in severe influenza, suggesting that physicians might not consider it in their differential diagnosis. In addition, most respondents reported infrequent use of lower respiratory specimens and galactomannan testing in patients with severe influenza, which might limit ability to detect IPA.

Although our response rate and possible selection bias might limit our ability to draw conclusions, ≈20% of respondents had seen or heard about an IPA case at their institution. IPA in patients with severe influenza might be more common than appreciated based on small numbers of previously published cases in the United States (*4*,*5*). Additional research and

Table. Summary results of survey on invasive pulmonary aspergillosis accompanying severe influenza among a network of infectious disease specialists, United States, May–June, 2018*

Survey characteristic	No. (%)
Region where respondents are from, n = 114	
Midwest	25 (22)
Northeast	27 (24)
South	33 (29)
West	29 (25)
Familiar with reports of aspergillosis after severe influenza infection, n = 114	
Yes, familiar with reports	29 (26)
No, not familiar with reports	83 (73)
Seen or heard about a case of aspergillosis in the setting of severe influenza at place of work, n = 114	
Yes, 1 case	15 (13)
Yes, >2 cases	6 (5)
No	93 (82)
For patients with influenza requiring ICU admission, how commonly are lower respiratory specimens (e.g., bronchoalveolar lavage, bronchial wash) obtained, n = 108	
Never	2 (2)
Rarely	28 (26)
Sometimes	45 (42)
Very often	28 (26)
Always	5 (5)
When treating patients with severe influenza in the ICU and worsening respiratory function, how often do you order galactomannan testing (e.g., in serum or bronchoalveolar lavage), n = 107	
Never	30 (28)
Rarely	45 (42)
Sometimes	24 (22)
Very often	4 (4)
Always	4 (4)

*n values indicate number of participants who responded. ICU, intensive care unit.

surveillance are needed to understand the association between IPA and severe influenza and performance of galactomannan testing in patients with severe influenza. Nonetheless, it is essential for clinicians to consider IPA in patients with severe influenza who do not improve with treatment, even in those who are not immunocompromised.

This study was supported by Cooperative Agreement 1 (U50 CK000477) funded by the Centers for Disease Control and Prevention.

All coauthors contributed to study design. S.E.B. and P.M.P. collected data; M.T. and S.E.B. analyzed data; M.T., B.R.J., and K.D.B. interpreted data; M.T., T.M.C., B.R.J., and K.D.B. wrote the paper; and S.E.B. and P.M.P. supervised the study.

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West Nile Virus in Farmed Crocodiles, Zambia, 2019

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DOI: https://doi.org/10.3201/eid2604.190954

We detected West Nile virus (WNV) nucleic acid in crocodiles (*Crocodylus niloticus*) in Zambia. Phylogenetically, the virus belonged to lineage 1a, which is predominant in the Northern Hemisphere. These data provide evidence that WNV is circulating in crocodiles in Africa and increases the risk for animal and human transmission.

West Nile virus (WNV), the causative agent of West Nile fever (WNF), is an arbovirus of the genus *Flavivirus*, family *Flaviviridae*. WNV has been reported in a variety of species but is maintained mainly between birds and ornithophilic mosquitoes, with incidental transmission to end hosts, such as humans, horses, and other vertebrates (*1*). Recently, WNV was detected in snakes (*2*), and antibodies against WNV were found in farmed crocodiles (*Crocodylus niloticus*) in Israel and Mexico and in alligators (*Alligator mississippiensis*) in the United States (*3*). Moreover, a severe outbreak of WNV neurologic disease in farmed alligators was reported in Florida (*4*). Meanwhile, the role of reptiles in the epidemiology of WNV remains obscure.

Up to 9 genetic lineages of WNV have been proposed (*5*), but lineages 1 and 2 have been associated with most human outbreaks of neurologic disease. Lineage 1 is globally distributed, and major outbreaks involving this lineage have been reported in the Americas, Europe, Asia, Oceania, and North Africa (*5*). In contrast, lineage 2 was reported exclusively in southern Africa and Madagascar until the 2000s, when it emerged in Europe (*6*). Although lineage 2 is predominant in southern Africa, WNV lineage 1 was detected in a pregnant mare in South Africa in 2010 (*7*).

In March 2019, a crocodile farm in Southern Province, Zambia, reported that some yearlings had exhibited clinical signs including anorexia, weakness, swimming in circles, bloody diarrhea, and scoliosis and were euthanized. Postmortem examination revealed congestion of the lungs, hemorrhagic intestines and trachea, and hydropericardium. Clinical signs and postmortem findings led to the suspicion of WNF, coccidiosis, salmonellosis, or enterotoxemia. We collected 11 whole blood samples from the postoccipital sinus of the spinal vein of affected crocodiles for molecular detection of WNV.

We used a QIAamp Viral RNA Mini Kit (QIAGEN, https://www.qiagen.com) to extract total RNA and the OneStep RT-PCR Kit (QIAGEN) to detect part of the WNV genome by using the primer pair WNNY-514 (5′-CGG CGC CTT CAT ACA CW-3′) and WNNY-905 (5′-GCC TTT GAA CAG ACG CCA TA-3′). These primers amplified an ≈400 bp fragment in 2/11 samples tested, which we then used for direct Sanger sequencing. The 2 nucleotide sequences we obtained were 100% identical to each other, and a BLAST analysis (https://blast.ncbi.nlm.nih.gov) showed 99% sequence identity to WNV isolate ArD76986/1990/SN (GenBank accession no. KY703854), which was detected from *Culex poicilipes* mosquitoes in Senegal (*5*). To obtain the complete sequence of the polyprotein gene, we designed several overlapping primers (Appendix Table, https://wwwnc.cdc.gov/EID/article/26/4/19-0954-App1.pdf) to use in reverse transcription PCR (RT-PCR) assays and sequencing. We deposited the sequence, Croc110/2019/ZM, in GenBank (accession no. LC489409).

For phylogenetic analysis, we aligned complete polyprotein amino acid sequence of Croc110/2019/ZM and reference sequences from GenBank by using MUSCLE (http://www.drive5.com/muscle). We constructed the phylogenetic tree in MEGA6 (https://www.megasoftware.net) by using the maximum-likelihood method and the Jones-Taylor-Thornton matrix-based model with 1,000 bootstrap replicates. Phylogenetic analysis revealed that Croc110/2019/ZM belonged to lineage 1a (Figure) and was most closely related to a WNV isolate from a camel in the United Arab Emirates (GenBank accession no. KU588135) and isolate ArD76986/1990/SN from Senegal (accession no. KY703854).

Our study confirmed WNV infection in farmed crocodiles in Africa. Clinical signs and pathological changes in multiple organs correlated with those described for WNV infection in farmed American alligators in the United States (*4*). The source of the WNV in the outbreak remains unresolved. WNV might have been circulating on the farm because the farmer indicated that lymphohistiocytic proliferative cutaneous lesions were observed in the crocodiles for some time. Such lesions can be associated with WNV infection and cause considerable economic losses because of lowered skin quality (*8*). Transmission among the crocodiles could occur orally from cannibalism and by cloacal shedding of WNV from infected animals (*8*).

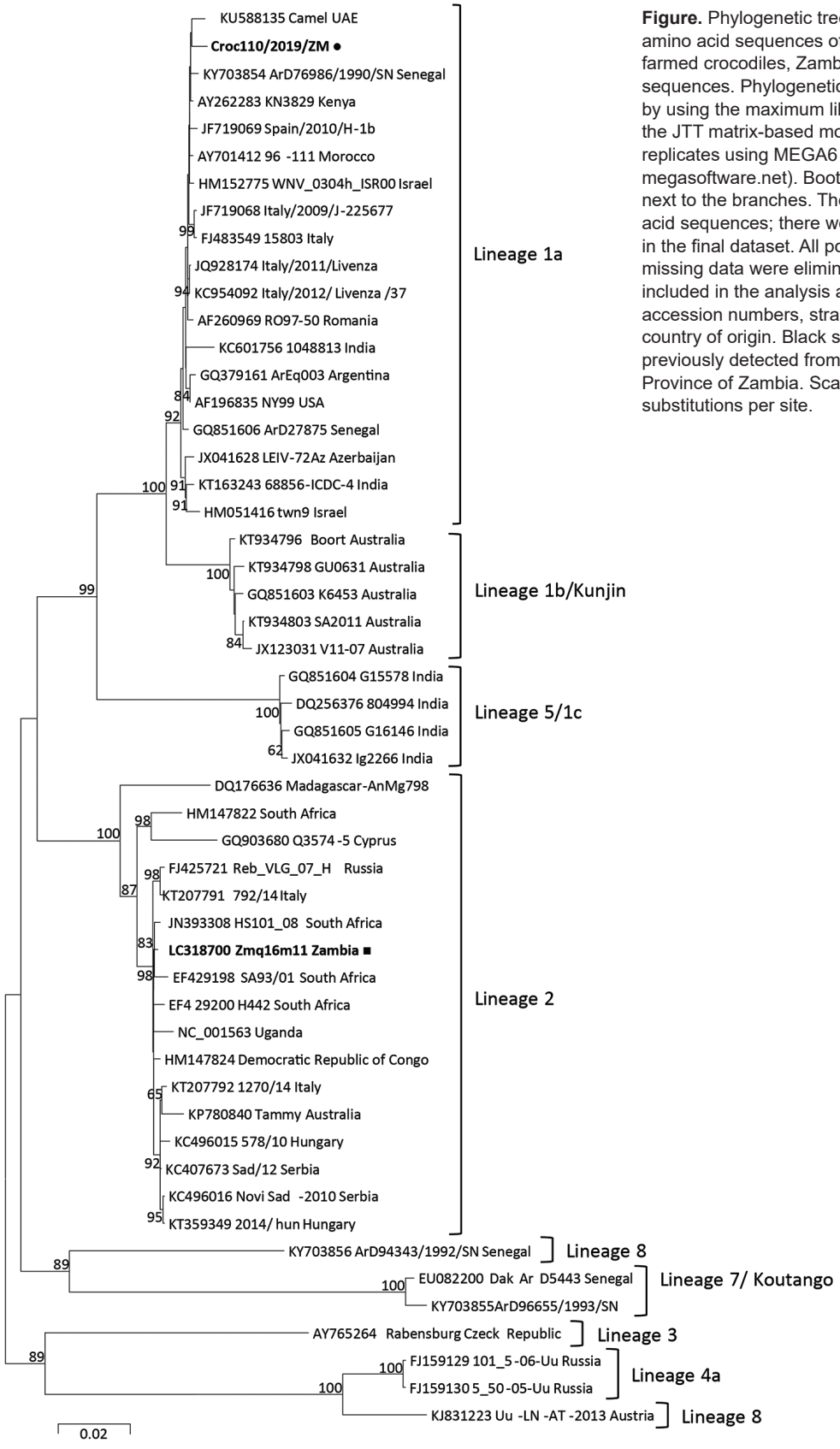

Figure. Phylogenetic tree of complete polyprotein amino acid sequences of West Nile virus (WNV) from farmed crocodiles, Zambia (black dot), and reference sequences. Phylogenetic analysis was conducted by using the maximum likelihood method based on the JTT matrix-based model with 1,000 bootstrap replicates using MEGA6 software (https://www.megasoftware.net). Bootstrap values ≥60% are shown next to the branches. The analysis involved 52 amino acid sequences; there were a total of 3,415 positions in the final dataset. All positions containing gaps and missing data were eliminated. Reference sequences included in the analysis are shown with their GenBank accession numbers, strain name or source, and country of origin. Black square indicates WNV previously detected from mosquito in the Western Province of Zambia. Scale bar indicates nucleotide substitutions per site.

Phylogenetic analysis of the complete polyprotein amino acid sequence of Croc110/2019/ZM grouped the virus in lineage 1a. Previous mosquito surveillance studies in Western Province, Zambia, identified WNV lineage 2 from *Culex quinquefasciatus* mosquitoes (*9*). Our findings suggest that multiple WNV lineages are co-circulating in Zambia and that multiple host species could be involved.

WNF outbreaks have not been reported in humans in Zambia, but 10.3% of 3,625 persons who participated in a serosurvey were seropositive for WNV in Western and North-Western provinces of the country (*10*), suggesting possible WNV infection. Detection of WNV in mosquitoes in Western Province and our finding of the virus in crocodiles in Southern Province suggest that WNV could be a neglected emerging infectious pathogen and might be associated with WNF in animals and humans in Zambia. Our study stresses the need for increased clinical awareness among veterinary and medical practitioners and continued monitoring of WNV in vectors and animals, including reptiles, to clarify the ecology and life cycle of this pathogen, particularly in regions where WNF is poorly understood.

Acknowledgments

We thank Chris Foggin and Jaelle Rowland for their scientific insights on West Nile virus.

This study was approved by the Department of National Parks and Wildlife (Act no. 14; 2015) and Crocodile Producers Consortium, Zimbabwe. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT), and Japan Society for the Promotion of Science (JSPS KAKENHI) (nos. JP16H05805JP16H06431, JP16H06429, and JP16K21723), and the Japan Initiative for Global Research Network of Infectious Diseases (J-GRID) from Japan Agency for Medical Research and Development (AMED) (no. JP19fm0108008); AMED and Japan International Cooperation Agency (JICA) within the framework of the Science and Technology Research Partnership for Sustainable Development (grant no. JP18jm0110019); and is part of the European & Developing Countries Clinical Trials Partnership program supported by the European Union (nos. RIA2016E-1609, PANDORA-ID-NET). K.N. was supported by CPC, Zimbabwe.

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Needlestick-Associated Rocky Mountain Spotted Fever, Brazil

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DOI: https://doi.org/10.3201/eid2604.191251

We report a fatal case of Rocky Mountain spotted fever (RMSF) in a man in Brazil without recent history of tick bites or environmental exposure. He received an accidental needlestick while working as a nurse. The nurse and his patient died. Both cases were confirmed as RMSF by molecular methods.

After viruses, bacteria are the most common infection risk in healthcare workers who have accidental exposure to blood or body fluids (*1*). Accidental exposures mainly occur from percutaneous injury or mucocutaneous contact (*1*).

Rickettsia rickettsii is the etiological agent of Rocky Mountain spotted fever (RMSF), a severe tickborne disease endemic to the Americas (*2*). In Brazil, RMSF is a notifiable disease, and 411 deaths were registered during 2007–2015 (*3*). Men from rural areas who were exposed to ticks in the environment around forests, rivers, and waterfalls accounted for >66% of cases (*3*). We report a fatal case of RMSF in a nurse who had no recent history of tick bite or environmental exposures.

In August 2018, two deaths in Minas Gerais state were classified as probable RMSF on the basis of clinical findings, including severe acute febrile syndrome. We retrospectively reviewed official report forms for the 2 cases (*4*). Case-patient A was a 74-year-old male farm worker from a rural area of Belo Horizonte municipality. On July 20, he began having symptoms of acute nonrash febrile syndrome, including myalgia, dysuria, and oliguria. He reported environmental exposure and an insect bite on his chest prior to onset of symptoms. He died on July 24 (Table).

Case-patient B was a 30-year-old man who had no history of recent travel, tick bites, or environmental exposures, nor did he own a dog. He was a nurse from the hospital where case-patient A was admitted. He reported an accidental percutaneous needlestick injury to his left thumb on July 23, after working with case-patient A in the hospital (Table). Following guidelines for biological hazards of healthcare workers in Brazil (*5*), clinicians collected blood from case-patient A and conducted serological tests for hepatitis B and C and HIV, all of which were negative. On July 30, case-patient B began having symptoms of acute febrile syndrome, including maculopapular rash, acute respiratory distress syndrome, shock, oliguria, thrombocytopenia, and leukopenia. Case-patient B died on August 5. Because RMSF was not suspected, neither case-patient received appropriate antimicrobial drugs.

After reviewing the official spotted fever case reports, we suspected *R. rickettsii* infection in both cases. We tested serum samples collected on July 22 from case-patient A and on August 2 from case-patient B. We used a *Rickettsia* genus–specific quantitative PCR to amplify rickettsial *gltA* gene from the patients' serum samples (*6*). Case-patient A had a cycle threshold value of 25.9 and case-patient B 35.3. We confirmed RMSF by using conventional heminested PCR protocol to amplify a 532-bp fragment of the rickettsial

Table. Information about confirmed fatal case of needlestick-associated Rocky Mountain spotted fever and related source case in Minas Gerais state, Brazil, 2018*

Case-patient	Age, y/sex	Clinical signs and symptoms	Exposure factors	Date			qPCR (*gltA*)	Conventional heminested PCR (*ompA*)†
				Symptom onset	Serum collected	Death		
A, patient	74/M	Fever, myalgia, dysuria, oliguria	Environmental exposure to woods, rivers, waterfalls; report of insect bite	Jul 20	Jul 22	Jul 24	+	+
B, nurse	30/M	Fever, maculopapular rash, acute respiratory distress syndrome, shock, oliguria	No reported tick or insect bites or environmental exposures; accidental percutaneous needlestick injury associated with case-patient A on July 23	Jul 30	Aug 2	Aug 5	+	+

*Clinical and epidemiological data were retrieved from official spotted fever–rickettsiosis case forms collected for each patient by the Ministry of Health, Brazil (*4*). *gltA*, rickettsial citrate synthase gene; *ompA*, rickettsial outer membrane protein A gene; qPCR, quantitative PCR; +, positive.
†All PCR amplicons were sequenced and confirmed a 100% identity with *Rickettsia rickettsii*.

ompA gene, as previously described (*6*). Rickettsial DNA from the samples generated sequences with 100% identity to the corresponding *ompA* gene fragment of *R. rickettsii* (GenBank accession no. CP003305).

Besides the common transmission route through arthropod bite for infection, rare instances of *R. rickettsii* infection have been reported through accidental exposure in research laboratories or by percutaneous needlestick injuries in healthcare facilities. For instance, Johnson et al. described a series of 5 cases of laboratory-acquired RMSF cases in 1967, two of which occurred in workers who had accidental needlesticks involving a yolk-sac suspension of *R. rickettsii* (*7*). Both developed an acute febrile illness but were successfully treated with tetracycline (*7*). In another published case in a healthcare worker, a physician incurred a needlestick wound on his arm while assisting in the care of a patient with a presumptive diagnosis of RMSF. The patient died (*8*). The physician experienced sudden onset of a febrile illness 7 days after the puncture wound and a subsequent maculopapular rash. RMSF was confirmed by serological tests, and he was treated with oral tetracycline and recovered.

In accidental exposure, the risk of transmission varies according to the volume of blood inoculated and the number of infective agents in the inoculum (*1*). Median infective doses of rickettsiae are known to increase after endothelium destruction in severe cases of RMSF (*9*). In this case, when punctured with a needle, case-patient B probably was exposed to a high number of rickettsia released in the bloodstream of case-patient A just 1 day before his death.

Our report highlights the importance of considering RMSF in patients with symptoms compatible with the disease and in healthcare workers caring for patients with undifferentiated fever in RMSF-endemic areas. Administering doxycycline before a rash occurs and within 5 days of symptom onset is crucial to patient recovery. Patients with a history of an arthropod bite, sudden onset of fever, and exposure in an endemic area should prompt clinicians to provide immediate treatment. Primary, secondary, and tertiary healthcare facilities should educate and remind staff about RMSF and its associated signs and symptoms in patients.

Á.A.F.-M. was funded in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil (CAPES; Finance Code 001).

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Crimean-Congo Hemorrhagic Fever, Mauritania

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DOI: https://doi.org/10.3201/eid2604.191292

The distribution of Crimean-Congo hemorrhagic fever (CCHF), a tickborne arboviral disease, is not well known in West Africa. We report 2 recent human cases of CCHF with infectious syndrome and severe bleeding in Mauritania. CCHF was diagnosed by ELISA and real time reverse transcription PCR. No secondary CCHF cases were found.

Crimean-Congo hemorrhagic fever (CCHF) occurs in Europe, Africa, the Middle East, and Asia (*1*). The virus is transmitted to humans through tick bites or direct contact with blood, secretions, or infected tissue of a viremic animal or person. The incubation period in humans is usually ≈5–6 days and hemorrhaging often occurs on the fourth or fifth day after onset of illness; ≈30% of human case-patients die. In Mauritania, CCHF was first documented in 1983 (*2*). Although several cases have been reported since, its current distribution is not well known. We report 2 cases of CCHF in 2019 in southern Mauritania.

The first patient, a 51-year-old man, a cattle breeder who resided in Tintane, Hodh Elgharbi, was admitted to Kiffa Regional Hospital, Assaba, Mauritania, on June 17, 2019, for hemorrhagic syndrome. The patient began having symptoms, including abdominal pain, bloody diarrhea, and vomiting, 5 days prior. At admission, the patient was in a coma (Glasgow coma scale 8) and had a fever (temperature 41°C), epistaxis, gingivorrhagia, diffuse ecchymosis (Figure 1), pallor, rapid respiratory rate (20 breaths/min), and hypotension (60/40 mm Hg). Laboratory examinations showed severe anemia (3.5 mmol/L); leucocytosis (1.3 × 10^9 cells/L); severe thrombocytopenia (20 × 10^9/L); prolonged prothrombin time (61%); and elevated urea (35 mmol/L), creatinine (2,298 μmol/L), alanine aminotransaminase (1.2 μkat/L), and aspartate aminotransferase (1.8 μkat/L). Rapid diagnostic tests for malaria, hepatitis B antigen, and HIV were negative. An in-house ELISA developed by Institut Pasteur de Dakar (Dakar, Senegal) was positive for CCHF virus–specific IgM and negative for yellow fever, Rift Valley fever, West Nile virus, dengue, and chikungunya (*3*). Real time reverse transcription PCR (Liferiver Bio-Tech Corp., http://www.liferiverbiotech.com) further confirmed the diagnosis. The patient was treated with ribavirin for 10 days and received several blood transfusions. He recovered and was discharged without any sequelae after 14 days. During a follow-up 2 weeks later, he was well and remained asymptomatic.

The second patient, a 54-year-old man, also a cattle breeder, from Guerou, Assaba, was hospitalized in a private clinic with a presumptive diagnosis of malaria and treated with quinine despite a negative blood smear. On July 9, 2019, because of altered consciousness and diffuse hemorrhagic syndrome, he was transferred to Kiffa Regional Hospital. At admission, the patient was in a coma (Glasgow coma score 7) and had epistaxis, gingivorrhagia, hematemesis, diffuse ecchymosis, gross hematuria, fever (temperature 40.5°C), and hypotension (60/40 mm Hg). Laboratory examinations showed anemia (4.5 mmol/L); low leukocyte count (1.8 × 10^9 cells/L); severe thrombocytopenia (19 × 10^9/L); prolonged prothrombin time (51%); and elevated renal (urea 38.2 mmol/L, creatinine 3,270 μmol/L) and liver (alanine aminotransaminase 1.37 μkat/L, aspartate aminotransferase, 1.95 μkat/L) function tests. ELISA was positive for CCHF virus (IgM positive, IgG negative) and negative for other hemorrhagic fever viruses. Real time reverse transcription PCR also was positive for CCHF virus. As in the first case, the patient was isolated from other patients and treated with ribavirin, antipyretics, blood transfusions, rehydration, and tepid sponge baths. He

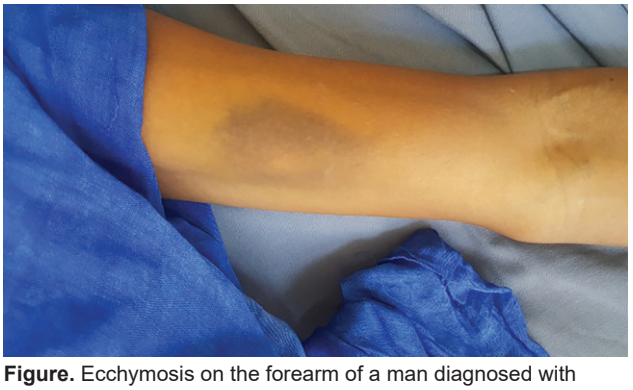

Figure. Ecchymosis on the forearm of a man diagnosed with Crimean-Congo hemorrhagic fever in Mauritania, 2019.

recovered favorably after 10 days of hospitalization. He was discharged and seen in the outpatient clinic 15 days later without any sequelae.

We did not observe ticks or tick bites during clinical examination of the patients. The most probable source of infection was close contact with infected animals. We identified all family members and hospital staff (n = 62) who came in direct or indirect contact with the 2 patients and followed them for possible secondary transmission for 3 weeks but did not observe any additional cases.

Most known human cases of CCHF in West Africa have been reported from southern Mauritania. The first documented case in West Africa occurred in 1983 in a camel and cattle breeder in Selibaby, Guidimakha region, southern Mauritania, probably after close contacts with infected camels or cattle (*2*,*4*). Subsequent reports on 6 CCHF virus–infected patients came from Rosso in the Trarza region and suggested that sheep could be a major source of transmission to humans (*5*). These sporadic cases were followed by an outbreak involving 35 persons in Nouakchott, the capital of Mauritania, including secondary infections among hospital staff, and 3 isolated cases in Brakna and Hodh Elgharbi regions in 2003 (*3*). A decade later, an unusual case of human CCHF with subdural hematoma was reported from Nouakchott (*6*). In 2015, an analysis of blood samples from cases of hemorrhagic fever during a Rift Valley fever outbreak in Mauritania showed that 6/184 (3.3%) samples, mostly from the southern part of the country, were positive for CCHF viral RNA (*7*). Most CCHF reported in humans in the region occurred during the long dry season, December–July. Animal studies also have demonstrated a high seroprevalence in cattle in Mauritania (*8*). These reports from Mauritania, together with recent findings in the neighboring countries (*9*,*10*), strongly suggest that CCHF is both enzootic and endemic in West Africa and highlight the need for better diagnostic capacity, increased awareness and knowledge of CCHF epidemiology among health providers, and regional surveillance.

Acknowledgments

We thank the medical staff of Kiffa Regional Hospital, Assaba, Mauritania, for supportive care of the patients.

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Trombiculiasis in a Dog with Severe Neurologic Disorders, Spain

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DOI: https://doi.org/10.3201/eid2604.191313

Chiggers, the larvae of trombiculid mites, parasitize a wide variety of terrestrial vertebrates worldwide. Their bites cause seasonal trombiculiasis in humans and animals. Affected canines can have a variety of digestive and systemic clinical signs. We describe a case of canine trombiculiasis in a dog exhibiting severe neurologic symptoms.

Larvae of trombiculid mites (Acari: Trombiculidae), commonly known as chiggers, are widespread ectoparasites of vertebrates. More than 3,000 trombiculid species are known, and small mammals and birds are their main hosts (*1*). In Asia, chiggers transmit scrub typhus, a life-threatening human infection caused by *Orientia tsutsugamushi* (*2*). In Europe, chiggers are associated with a seasonal trombiculiasis, a dermatitis that affects humans and animals, mostly dogs and cats (*3*). Infected animals can have signs of skin lesions, pruritus, asthenia, fatigue, pyrexia, digestive disorders, or neurologic dysfunction (*3*–*5*). We describe a case of a dog in Spain heavily infested by chiggers and exhibiting severe neurologic symptoms.

On October 14, 2013, a 2-year-old male basset hound in poor health was admitted to Vetersalud Asís Hospital in Logroño, Spain. The owner reported that the dog had been vomiting and having trouble breathing for 12 hours before he was brought to the veterinary hospital. The dog was drooling, torpid and confused, vocalizing, and barely able to stand. His symptoms started the evening before with a lack of appetite, lethargy, and licking and biting his paws. The symptoms progressed to convulsions and ataxia so severe that the dog could no longer scratch itself. At initial evaluation, he had ataxia and loss of balance. Further clinical examination revealed an elevated body temperature of 39.6°C (normal 38°C–39°C), tachypnea with hyperventilation, and mucosal congestion. Skin examination revealed focal areas of erythema and papules on the abdomen, above the eyes, and in the interdigital areas. We performed skin scrapings on several lesions and needed tweezers to remove many mites that were attached to the dog's skin (Figure, panel A). Microscopic examination of skin scrapings revealed numerous live mites, which we morphologically identified as *Neotrombicula inopinata* (*6*,*7*) (Figure, panel B).

We treated the dog with phenylpyrazole, a broad-spectrum topical insecticide, and most of the mites detached within 12 hours. We treated the dog's allergic reaction with methyl prednisolone at an initial dose of 10 mg/kg/day, which was gradually tapered to 0.5 mg/kg/day over 15 days. Complete blood count and chemical tests at admission, 24 h, and 48 h showed no changes except an increase in the number of neutrophils from 21.2 × 10^3 cells/μL at admission to 35.4 × 10^3 cells/μL at 48 h (upper limit 16.9 × 10^3 cells/μL). The dog was hospitalized for 4 days, but ataxia was still evident after discharge (Video, https://wwwnc.cdc.gov/EID/article/26/4/19-1314-V1.htm). Clinical signs went into remission 7 days after treatment began.

The dog had been walking and playing with its owners through grassy and brushy areas in a pine

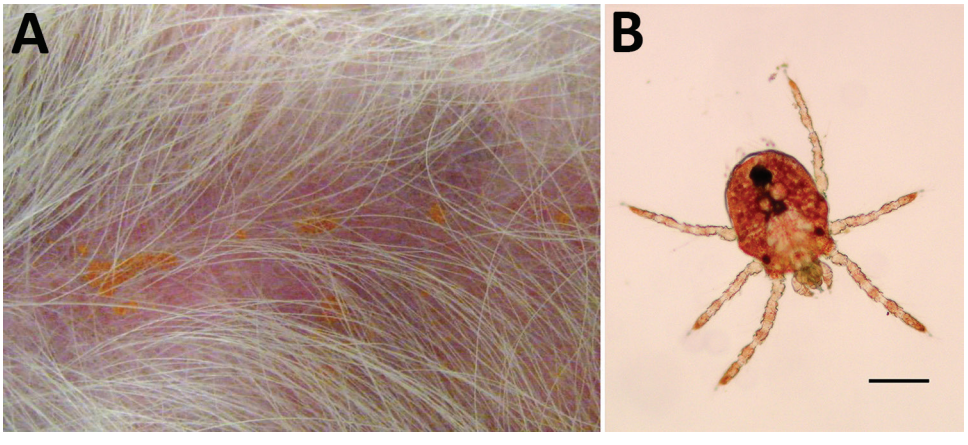

Figure. Larval *Neotrombicula inopinata* mites on a dog with severe neurologic symptoms, Spain. A) *N. inopinata* mites attached to the abdomen of the dog. B) Microscopic image of *N. inopinata* larva. Scale bar indicates 100 μm.

forest in Sierra Cebollera National Park, La Rioja, Spain (42°6′N, 2°33′E), ≈8 hours before the onset of symptoms. One owner had an itchy dermatitis that was diagnosed as trombiculiasis and treated at the Infectious Diseases Department at the Hospital Universitario San Pedro, Logroño, 4 days after the outdoor activities (*8*). We previously reported on trombiculid mites in the vegetation of certain areas of Sierra Cebollera National Park, their causality in human cases of seasonal dermatitis, and co-occurrence with canine cases (*4*,*6*,*8*–*10*). However, canine trombiculiasis associated with severe neurologic signs had not been described in Spain.

In dogs, massive infestations with chiggers have been related to death, especially when left untreated (*10*). Orange spots, especially on the lacrimal areas, can assist in the diagnosis of suspected cases of trombiculiasis and should prompt owners to seek immediate veterinary advice. A severe allergic host response, hypersensitivity to mites or their products, or pathogen transmission have been speculated causes of clinical signs in canids (*3*). The role of *N. inopinata* mites collected in La Rioja as vectors of arthropodborne bacteria has not been demonstrated (*10*), and the clinical signs do not suggest an infectious disease process. We hypothesize that severe cases are attributed to the inflammatory response secondary to infestation, but the mechanism is unknown. However, we cannot disregard the implication of a neurotoxic process.

Successful management of symptoms is dependent on early treatment to remove chiggers. Topical insecticides, especially pyrethroids, are considered effective against chigger infestations in canids (*3*). Our experience has shown efficacy of isoxazolines, although not label indicated, at eliminating chiggers on dogs within 6–8 hours. As noted in this case, a short course of glucocorticoids at an antiinflammatory dose might be necessary to relieve pruritus and to reduce inflammation (*3*). Currently, no products are specifically licensed for preventing chigger bites. Sprays containing phenylpyrazole, which is licensed for use in dogs and cats against fleas and ticks, also are thought to be effective against mites. Because trombiculiasis is a seasonal threat, the most useful approach to prevent infestations, if feasible, consists of keeping pets away from areas where exposure can occur whenever chiggers are known to be active. Local veterinarians should be aware of the occurrence of canine trombiculiasis and its clinical signs to properly diagnose and manage this potentially fatal condition.

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Geographic Distribution of Raccoon Roundworm, *Baylisascaris procyonis*, Germany and Luxembourg

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DOI: https://doi.org/10.3201/eid2604.191670

Infestation with *Baylisascaris procyonis*, a gastrointestinal nematode of the raccoon, can cause fatal disease in humans. We found that the parasite is widespread in central Germany and can pose a public health risk. The spread of *B. procyonis* roundworms into nematode-free raccoon populations needs to be monitored.

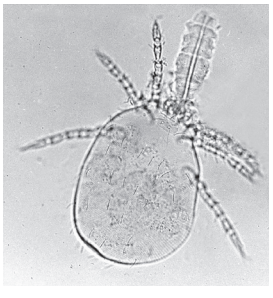
The raccoon roundworm (*Baylisascaris procyonis*) is a gastrointestinal parasitic nematode of the raccoon (*Procyon lotor*). It is common in its native range in North America, where its prevalence in raccoons can reach 82% (*1*). Through their feces, infested raccoons can shed millions of *B. procyonis* eggs, which may remain infective in the environment for years (*2*). Paratenic hosts can acquire the parasite when ingesting nematode eggs from raccoon latrines (*3*).

etymologia

Trombiculiasis *[trom-bik"u-li'ə-sis]*

Ronnie Henry

Infestation with mites of the family *Trombiculidae* (from the Greek *tromein*, "tremble," and Latin *culex*, "gnat") in their larval form (chiggers, from the Carib *chico*). A wide variety of livestock and wild animals, as well as humans, can become infested with chiggers. Trombiculid mites are vectors of *Orientia tsutsugamushi*, which causes scrub typhus. References to these mites appear as early as the sixth century in China. Linnaeus described the species *Trombicula batatas* in 1758.



Photograph of a parasitic mite of domestic animals. Wikimedia Commons, Alan R. Walker, 2014.

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B. procyonis infestations are usually benign in the raccoon but can be fatal in paratenic hosts, including humans (*1*). Since 1980, several fatal cases of neural larva migrans have occurred in humans in the United States (*3*); infants have been frequently affected because of fecal–oral transmission (*4*). Increasing raccoon densities in close proximity to humans has increased public health concern about *B. procyonis* roundworms (*2*).

As a result of joint translocation with raccoons, *B. procyonis* roundworms have increased their geographic range (*5*). Raccoons are common in Germany and Luxembourg (Figure, panel A). All raccoons in Germany are assumed to have stemmed from a small number of founders and 2 separate introduction events in western Germany (Hesse) during the 1930s and eastern Germany (Brandenburg) during the 1940s (*8*). However, genetic analysis has inferred a minimum of 5 founder events (*6*). In addition to 2 genetic populations clustered around the known introduction sites (referred to as the Hesse and Brandenburg populations), distinct raccoon populations were identified in Saxony (eastern Germany), around the Harz Mountains in central Germany, and in Luxembourg and neighboring regions (Figure, panel B).

B. procyonis roundworms occur in the Hesse and Harz populations (*5*) but are absent from Brandenburg (*9*). No information is available about the remaining 2 populations in Luxembourg and Saxony. Although only a few human cases of baylisascariasis have been reported from Germany (*9*), a detailed overview of the parasite's geographic distribution is needed to identify potential risk areas.

During 2008–2018, we collected 8,184 legally harvested or road-killed raccoons from Germany and Luxembourg (Figure, panel A), focusing on different regions every year or every few years, and investigated their intestines for the presence of *B. procyonis* roundworms. We plotted the presence of the parasite onto the 10 × 10–km ETRS89-LAEA5210 EEA reference grid, a base map provided by the European Environment Agency (https://www.eea.europa.eu/data-and-maps/data/eea-reference-grids-2). We calculated the proportion of infested raccoons for 69 of Germany's 294 administrative districts where *B. procyonis* roundworms were present and >25 raccoons had been sampled. We generated maps by using ArcMap v.10.3 (ESRI Inc. https://www.esri.com).

B. procyonis roundworms were widespread in central Germany, their distribution probably corresponding to the geographic extent of the Hesse and Harz genetic populations (Figure, panels B, C). However, we did not detect the parasite in Luxembourg and western areas of Germany or in a northern/eastern region that included the federal states of Brandenburg, Mecklenburg-Western Pomerania,

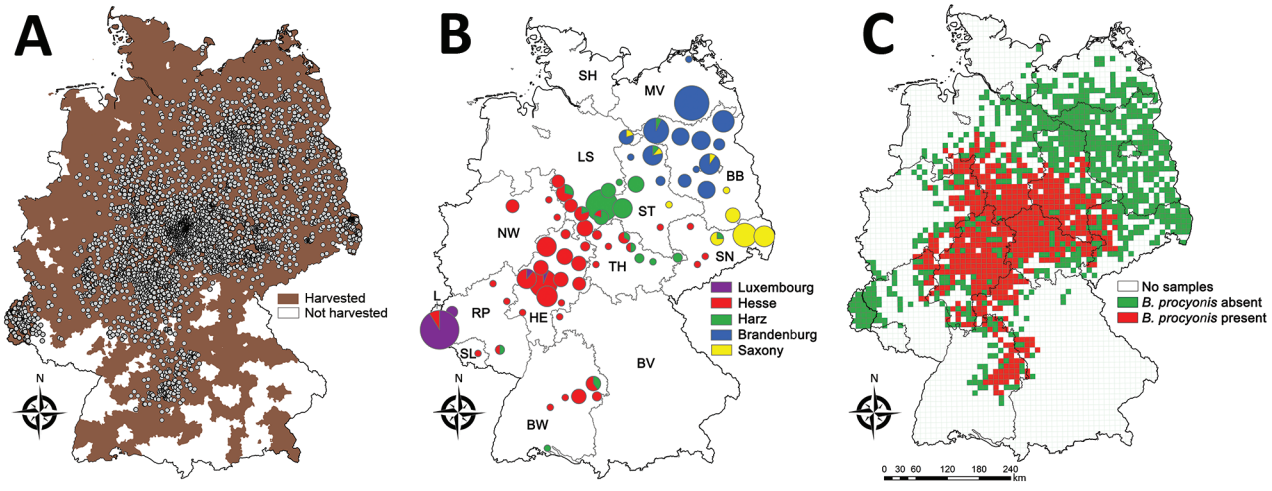

Figure: Characteristics of the geographic distribution of the raccoon roundworm (*Baylisascaris procyonis*). A) Geographic origin of 8,184 dissected raccoons and the German administrative districts (Landkreise) in which raccoons were harvested during 2017–2018. Dots indicate sampling sites. B) Population genetic structure of raccoons in Germany and Luxembourg. Reanalysis of the dataset by (*5*) but including 26 raccoons from Luxembourg (genotyped following [*5*]) and omitting animals from the city of Kassel (no distinct introduction [*6*]). The genetic data were analyzed by using the clustering of individuals algorithm implemented in BAPS v.6.0 (*7*). Different colors represent different genetic populations. Pie charts represent the genetic populations of origin of all the raccoons in an administrative district, and chart size indicates the number of samples included. BB, Brandenburg; BV, Bavaria; BW, Baden-Württemberg; HE, Hesse; L, Luxembourg; LS, Lower Saxony; MV, Mecklenburg-Western Pomerania; NW, North Rhine-Westphalia; RP, Rhineland-Palatinate; SH, Schleswig-Holstein; SL, Saarland; SN, Saxony; ST, Saxony-Anhalt; TH, Thuringia; C) Geographic distribution of *B. procyonis* roundworms, plotted onto the10 × 10–km ETRS89-LAEA5210 EEA reference grid.

Schleswig-Holstein, northern parts of Lower Saxony, Saxony-Anhalt, and eastern Saxony. In other words, the parasite was not detected in the areas covered by the Luxembourg, Brandenburg, and Saxony genetic populations (Figure, panels B, C). A median of 43.6% (interquartile range 34.4%–49.7%) of raccoons were infested in the 69 administrative districts where the parasite was present and >25 raccoons had been sampled.

Identification of risk areas for human *B. procyonis* roundworm infestation is necessary because of the frequent proximity of raccoons to human populations. Our results show that the nematode is widespread and prevalent in central Germany. Given that *B. procyonis* eggs remain infective for years, the nematode is likely to pose a public health risk in its distribution area (*10*). To reduce the risk for *B. procyonis* infestation, protective measures (procedure masks, gloves, handwashing) should always be applied when raccoons or their feces in the risk area are handled. In this context, educational material should be made available to schools and daycare centers and to persons who have occupational contact with raccoons.

The match between the distribution of the roundworm and the extent of the different genetic populations of the raccoon suggests that the absence of the parasite results from the founder animals' parasite-free status. However, we cannot exclude the possibility that ecologic or geographic differences between the introduction sites also contributed to the lack of parasites in some populations. Further research and monitoring are needed, especially in view of a possible spread of the parasite into nematode-free raccoon populations. Also, because of the rapid spread of raccoons, assessment of the status of the parasite in northwestern and southwestern Germany and at the periphery of its current distribution more generally should be considered.

Acknowledgments

We thank all regional and district hunting associations who provided raccoons for the study. We thank Louise Nicht for her help with dissection work.

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Tick-Borne Relapsing Fever Caused by *Borrelia persica* in Traveler to Central Asia, 2019

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DOI: https://doi.org/10.3201/eid2604.191771

We report a case of tick-borne relapsing fever caused by *Borrelia persica* in a traveler returning to Switzerland from central Asia. After the disease was diagnosed by blood smear microscopy, the causative *Borrelia* species was confirmed by shotgun metagenomics sequencing. PCR and sequencing techniques provide highly sensitive diagnostic tools superior to microscopy.

We report a case of tick-borne relapsing fever (TBRF) in a 21-year-old male tourist who returned from Kyrgyzstan in July 2019 after having traveled for 5 months to Mexico, Taiwan, and central Asia (Uzbekistan, Tajikistan, and Kyrgyzstan). While in Tajikistan, he experienced acute fever of 39.5°C, chills, and generalized aches on June 11, which lasted 3 days. He experienced identical episodes around June 17 and 25.

After returning to Switzerland, he sought care on June 28 from his general practitioner, who referred him to the regional hospital, where malaria test results were negative. After the patient experienced 2 more episodes of fever (July 2 and 14), the general practitioner referred him to a tropical medicine specialist on July 15. Anamnesis revealed that the patient had consumed unpasteurized milk and had been bitten by insects nightly while trekking in Tajikistan. Other than fever of 38.5°C and pain on palpation of the liver, physical examination revealed no pathologic findings. Abdominal sonography showed a borderline enlarged spleen but was otherwise unremarkable. Chest radiography indicated no abnormalities. Laboratory results are shown in the Appendix (https://wwwnc.cdc.gov/EID/article/26/4/19-1771-App1.pdf).

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Detection of spirochetes in blood films (Figure) confirmed the diagnosis of a relapsing fever borreliosis, already suspected from the classical presentation of recurrent fever episodes separated by asymptomatic intervals of ≈1 week. Shortly after starting doxycycline, the patient experienced a self-limiting crisis with chills and fever of 41°C, which we interpret as Jarisch-Herxheimer reaction. Subsequently, the patient's condition rapidly improved.

To determine the *Borrelia* species, we performed 16S rRNA gene sequencing from the blood sample. Analysis of traces of capillary-sequenced amplified DNA after broad-range 16S rRNA gene PCR (660bp), performed by using RipSeqMixed (Pathogenomix, https://www.pathogenomix.com), could not differentiate between *Borrelia recurrentis* and *B. persica* within the 5′ end of the 16S rRNA gene. Therefore, we used a short-read shotgun metagenomic sequencing approach on DNA on an Illumina NextSeq500 platform (https://www.illumina.com).

Of the 7.8 million sequencing reads, 692 (0.009% of the sequence data) mapped (by CLC Genomics Workbench v.12.0.3 [QIAGEN, https://www.qiagen.com] with a length fraction of 0.8 and a similarity fraction of 0.95) to a derived database of *Borrelia* genomes comprising reference genomes of *B. recurrentis* (GenBank accession nos. CP000999–CP001000), *B. persica* (Assembly accession AYOT), *B. duttonii* (Assembly accession AZIT), *B. hispanica* (Assembly accession AYOU), and *B. crocidurae* (GenBank accession no. LN609267). The top hit was to *B. persica*, with 684 (98.8%) mapped reads, followed by *B. duttoni* with 6 reads and *B. recurrentis* with 2 reads. Across the *B. persica* reference genome, reads from the isolate in this case mapped across the whole genome, representing sections of 101 of the 245 assembly scaffolds. We submitted the *Borrelia* reads to the European Nucleotide Archive (https://www.ebi.ac.uk/ena) under project PRJEB35490. We did not submit the 16S RNA gene sequence to GenBank because of the low quality of the sequence (multiple undetermined nucleotides).

These results strongly suggest that *B. persica* was the infectious agent of TBRF. Pending microscopic confirmation, we ordered several serologic studies, including assays to detect antibodies against the *Borrelia* species that cause Lyme disease and against rickettsial pathogens (Appendix Table 1). Whether the mildly elevated serologic titer for spotted fever *Rickettsia* resulted from cross-reactivity or co-infection with a tick-borne *Rickettsia* remains unclear.

TBRF occurs in temperate and tropical countries and is caused by several species of *Borrelia* maintained in enzootic cycles in which small mammals serve as

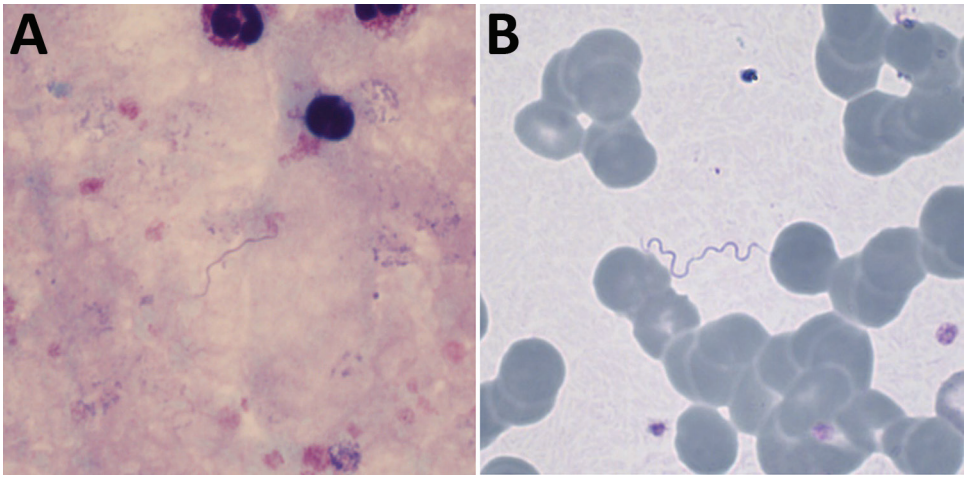

Figure. Giemsa-stained thick (A) and thin (B) blood films, demonstrating extracellular spirochetes. Original magnifications ×1,000.

animal reservoirs and *Ornithodoros* soft ticks as vectors. Humans are accidental hosts (except for *B. duttonii* in Africa, which seems strictly limited to humans with no identified animal reservoir), usually exposed to tick bites when sleeping in rustic cabins or caves (*1*). The disease is characterized by recurrent fever episodes separated by afebrile periods and constitutional symptoms. Complications include meningoencephalitis and treatment-induced Jarisch-Herxheimer reaction. Diagnosis can be made by microscopic examination of blood smears collected during fever episodes or by molecular methods.

TBRF in international travelers is rare. The GeoSentinel Surveillance Network reported only 4 cases of relapsing fever among 24,920 returning febrile travelers during 1997–2006 (*2*), and we found only 40 other travel-related cases published since 1982 (Appendix Table 2). Most TBRF infections in travelers are caused by *B. crocidurae* and almost exclusively acquired in Senegal. Recently, a new species, *Candidatus* Borrelia kalaharica, was found in 2 travelers to southern Africa (*3*,*4*).

Reports on *B. persica* infections are few and largely restricted to Iran and Israel. Only 2 other cases of *B. persica* infections in travelers returning from Uzbekistan/Tajikistan have been reported (*5*,*6*). Considering the wide geographic distribution of the transmitting tick, *Ornithodoros tholozani* (India, Pakistan, Afghanistan, western China, Kazakhstan, Kyrgyzstan, Tajikistan, Turkmenistan, Uzbekistan, Iran, Iraq, Turkey, Cyprus, Syria, Jordan, Israel, Egypt, and Libya [*7*,*8*]), considerable underreporting and underrecognition is likely. Although apparently rare, central nervous system involvement and acute respiratory distress syndrome may complicate TBRF caused by *B. persica* (*9*).

For patients with periodic fever and supporting exposure risk, clinicians should consider a differential diagnosis of TBRF and carefully examine blood smears by microscopy. Increasingly available PCR and sequencing techniques provide highly sensitive diagnostic tools superior to microscopy.

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Imported Human Babesiosis, Singapore, 2018

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DOI: https://doi.org/10.3201/eid2604.200025

In 2018, *Babesia microti* infection was diagnosed for a 37-year-old man in Singapore who acquired the infection in the United States. This case highlights the recent rise of tickborne infections in the United States and the risk for their spread, because of increasing global interconnectivity, to regions where they are not endemic.

Babesia spp. are intra-erythrocytic protozoal organisms that can infect mammals and birds. Human babesiosis is an emerging tickborne zoonosis, caused mainly by *Babesia microti* and transmitted by ixodid ticks. It is endemic to the United States (*1*–*3*) and, to a lesser extent, China (*3*,*4*). Recently, sporadic cases of human babesiosis caused by several species of *Babesia* have been reported in other countries: *B. microti* (Germany, Australia, South Korea), *B. microti*–like (Japan, Taiwan, China), *B. duncani* (United States, Canada), *B. divergens* (Europe), *B. venatorum* (Europe, China), *B. crassa*–like (China), *B. motasi*–like (South Korea), and other cases elsewhere (*1*–*4*).

In humans, babesiosis can cause mild influenza-like signs and symptoms, but it can also cause hemolytic anemia and severe infections, especially in asplenic or immunocompromised persons (*1*,*3*). Cases of congenital and transfusion-related transmission have been reported (*1*–*4*). Since 2011–2015, babesiosis incidence in the United States has risen (*2*,*5*). Travel-related tickborne infections in general (*6*) and cases acquired from North America have been reported (*3*,*6*,*7*). To our knowledge, no case of human babesiosis has been reported in Singapore, but cases of *Babesia* infection in canids and birds have been recorded (*8*), suggesting presence of potentially receptive ticks.

On July 23, 2018, a 37-year-old man from the United States sought care at Tan Tock Seng Hospital, Singapore, reporting fever and other influenza-like signs and symptoms that had started on July 5. The patient had resided in Singapore since 2012, working as a finance professional, but he had traveled to multiple places in the year before his illness. In 2017, he vacationed in Vietnam (Ho Chi Minh City, Danang), Thailand (Bangkok, Pattaya), Indonesia (Lombok, Anambas Islands), and Cambodia (Phnom Penh), all without having received pretravel typhoid vaccine or malaria prophylaxis. In 2018, he traveled to Indonesia (Bali) in January and March, then to the United States during June 14–25, where he visited friends and relatives in Boston (MA), Nantucket (MA), and New York (NY).

The patient did not recall any tick bites but on June 17 noticed a right ankle papule, which lasted 3 weeks. He sought consultation at a travel clinic because of high fever (104°F), rigors, and headaches, which had persisted and worsened over 18 days. His fever had not resolved with amoxicillin, which he had started taking a week after symptoms onset. He had no relevant medical history or allergies and was taking no other medication. Physical examination findings were unremarkable, including absence of jaundice, hepatosplenomegaly, or eschars.

Laboratory test results revealed moderate thrombocytopenia and anemia, and malaria blood films revealed trophozoites forming in erythrocytes, suggestive of *Babesia*. The National Public Health Laboratory in Singapore differentiated

between malaria and babesiosis by performing microscopy and PCR for both parasites, confirming the presence of *B. microti* and excluding *Plasmodium*. Because of the risk for concomitant tickborne infections, the reference laboratory at the Mayo Clinic (Rochester, MN) conducted PCR testing for *Babesia*, *Ehrlichia*, *Anaplasma*, and *Borrelia burgdorferi* and serologic testing for *Rickettsia rickettsii*. Results confirmed *B. microti* infection and excluded those concomitant tickborne infections. The National Public Health Laboratory further characterized the parasites by using PCR and sequencing according to (*8*) for the *18S ssrRNA* (GenBank accession no. MK609547) and the mitochondrial *cox1* (GenBank accession no. MK609548) genes and genotyping based on the internal transcribed spacer region (GenBank accession no. MK609547), which identified the *Babesia* strain as the type most commonly found in the United States (Figure).

After diagnosis, the patient received outpatient treatment with quinine and clindamycin and recovered uneventfully. Consistent with US clinical guidelines, he was advised to not donate blood indefinitely.

This case of travel-acquired human babesiosis diagnosed in Singapore highlights the clinical importance of considering tickborne infections in any traveler with compatible clinical signs and symptoms returning from summertime travel in the United States. It also highlights the challenges of differentiating between malaria and babesiosis in patients who have traveled to areas where both infections are endemic. Even in countries with good access to diagnostic testing, babesiosis may be missed or misdiagnosed as malaria (*7*).

Vectors for babesiosis in canids and bovids have been reported among tick fauna in Singapore (*9*), but to our knowledge, no such vectors for human babesiosis have been reported, which limits the risk for introduction and subsequent autochthonous transmission. However, babesiosis is the most common serious infectious pathogen transmitted by blood transfusions in the United States, and the US Food and Drug Administration has issued screening recommendations to reduce the risk for transfusion-transmitted babesiosis (*10*).

Given the increased incidence of babesiosis and other tickborne bacterial diseases in the United States and the high volumes of international travel, the risk of persons with travel-acquired babesiosis subsequently causing transfusion-transmitted infections is real, albeit small. In many countries, the

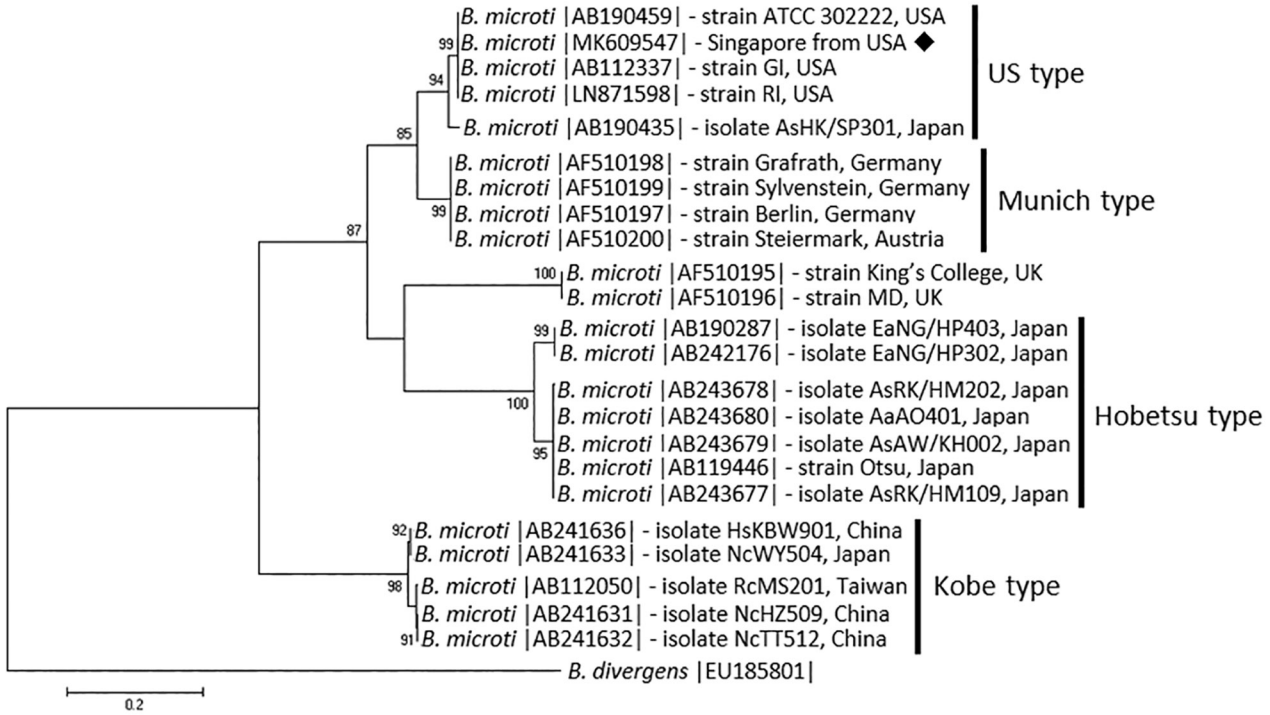

Figure. Molecular phylogeny of *Babesia microti* types based on the internal transcribed spacer region. Analysis inferred by maximum-likelihood using the general time reversible plus gamma model showing sequence MK609547 from a human patient in Singapore, 2018 (black diamond) and 22 sequences of *B. microti* isolates from diverse geographic areas, retrieved from GenBank (accession numbers provided). Bootstrap values were 10,000 replicates, >85% shown. Scale bar indicates nucleotide substitution rate per site.

blood supply is not screened for nonendemic, rare, or geographically limited pathogens, such as *Trypanosoma cruzi* (Chagas disease). Although screening for babesiosis in blood supplies outside the United States may not be financially or logistically feasible, mitigating risk by raising clinician and public health awareness of this emerging problem may be possible.

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Dr. Lim is the director of the High-Level Isolation Unit and senior consultant at the National Centre for Infectious Diseases, Singapore. Current research interests include travel and tropical medicine, emerging infection outbreaks, and public health preparedness for biosecurity threats.

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Mosaic of Justinianus I (detail), Basilica of San Vitale, San Vitale, Ravenna. Photo: Petar Milošević / CC BY-SA

Different Angles, Changing Perspectives

Byron Breedlove and Reginald Tucker

This month's cover image is a detail from the mosaic of the Emperor Justinian and his court in the Basilica of San Vitale in Ravenna, Italy. Ancient Roman mosaics such as these, typically created by unknown artisans, may be found in private villas and public buildings and provide durable, vivid documentation of ancient Roman life. According to the Getty Museum, many of these intricate, detailed works served as floors in numerous villas and were "designed to be viewed from different angles and to change as your perspective moves."

The artisans who assembled these mosaics combined thousands of mostly square tiles made from limestone, marble, glass, ceramic, and sometimes precious stones. They arranged these tiles like a complex jigsaw puzzle and affixed them into position with mortar.

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DOI: https://doi.org/10.3201/eid2604.AC2604

This particular mosaic, viewed as a whole (Figure), depicts the emperor in a ceremonial purple robe with a golden halo, a traditional rendering that symbolizes the importance of the Roman emperor in the Christian church and sets him apart from the more plainly dressed figures surrounding him, further emphasizing the authority of the emperor and his reign. The soldiers to his right and clergy on his left affirm his stature as the center of church and state. The mosaic, which imparts no sense of motion or depth, most likely documents a ceremonial gathering or formal event, perhaps in the same manner that a modern "photo op" might.

Justinian saw himself as the "defender of the faith," with a mandate to spread that faith throughout the empire. That power, however, did not allow him to escape what historians have called the Plague of Justinian, an outbreak now thought to be due to *Yersinia pestis*, that left him at the brink of death for several weeks, though he did survive. In

modern times, scientific progress has enabled clinicians to diagnose suspected cases of plague sooner and administer life-saving treatments with antimicrobial drugs.

Figure. Artist Unknown. Mosaic of Justinianus I, Basilica of San Vitale, San Vitale, Ravenna, Italy (ca. 547 A.D.) Photograph: José Luiz Bernardes Ribeiro / CC BY-SA 4.0

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Article Title

Ecology and Epidemiology of Tickborne Pathogens, Washington, USA, 2011–2016

CME Questions

1. You are advising a large infectious disease practice in Washington state about anticipated needs for management of tickborne diseases (TBDs). According to the analysis of surveillance data by Dykstra and colleagues, which of the following statements about TBD cases reported in Washington state residents during 2011 to 2016 is correct?

A. Three-quarters of TBD cases were autochthonous (indigenous or native)
B. Diseases with autochthonous cases included Lyme disease, Rocky Mountain spotted fever (RMSF), tickborne relapsing fever (TBRF), and tularemia
C. RMSF was the most reported autochthonous tickborne disease
D. TBRF was the most reported imported TBD

2. According to the analysis of surveillance data by Dykstra and colleagues, which of the following statements about detection of pathogens in 977 field-collected, unfed, host-seeking ticks in Washington state during 2011 to 2016 is correct?

A. In *Ixodes pacificus* ticks, the prevalence of *Borrelia burgdorferi* sensu stricto was 4%, and of *B. burgdorferi* sensu lato was 3.8%
B. No larval ticks were found
C. The primary vector species were *I. auritulus* and *Dermacentor variabilis*
D. The primary vector species were predominantly active during the fall

3. According to the analysis of surveillance data by Dykstra and colleagues, which of the following statements about clinical and public health implications of detection of tickborne pathogens and diseases in Washington state would be correct?

A. This study has thoroughly described the true underlying morbidity of TBDs in Washington
B. Clinicians in Washington state are likely to have a high index of suspicion for TBDs, facilitating early diagnosis
C. Ongoing surveillance of human cases and vector ticks is needed to determine the true burden of disease and to improve public health prevention messaging to clinicians and the public
D. The prevalence of TBDs reported in this study is likely to be accurate