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August 2018

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

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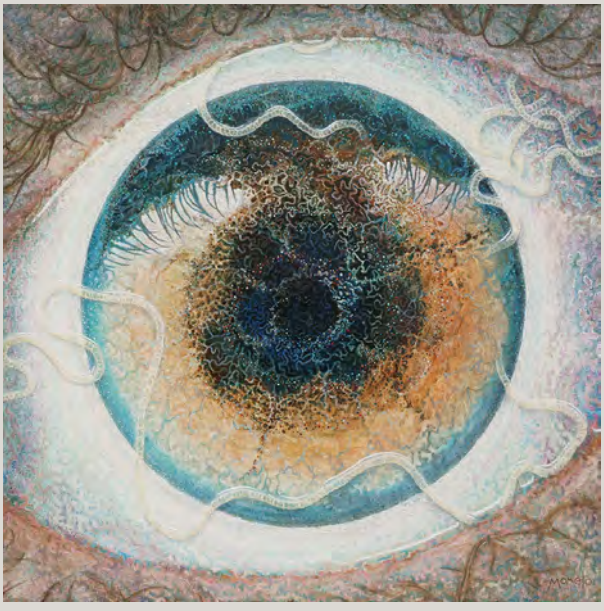

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Ben Taylor (aka Mometo) (1970–), *The Host* (2014). Oils and texture on canvas, 39.4 in × 39.4 in/100 cm × 100 cm). Image used by permission of the artist. Dartmoor, United Kingdom.

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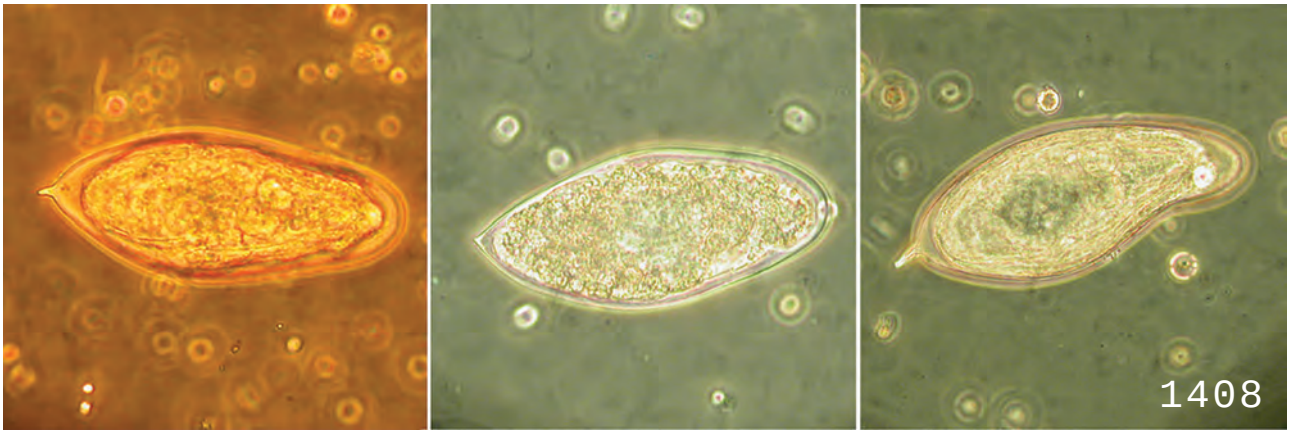

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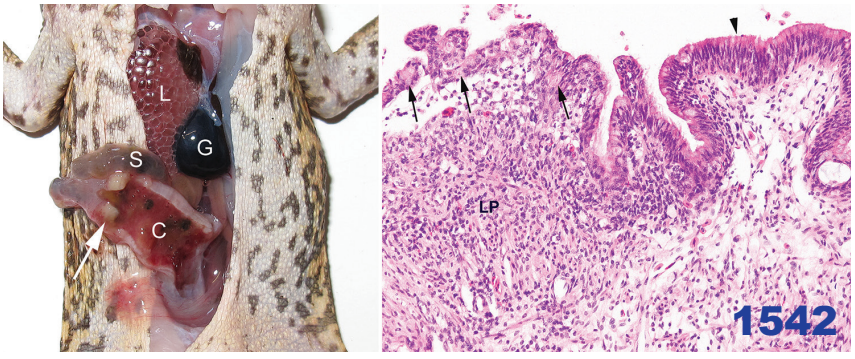

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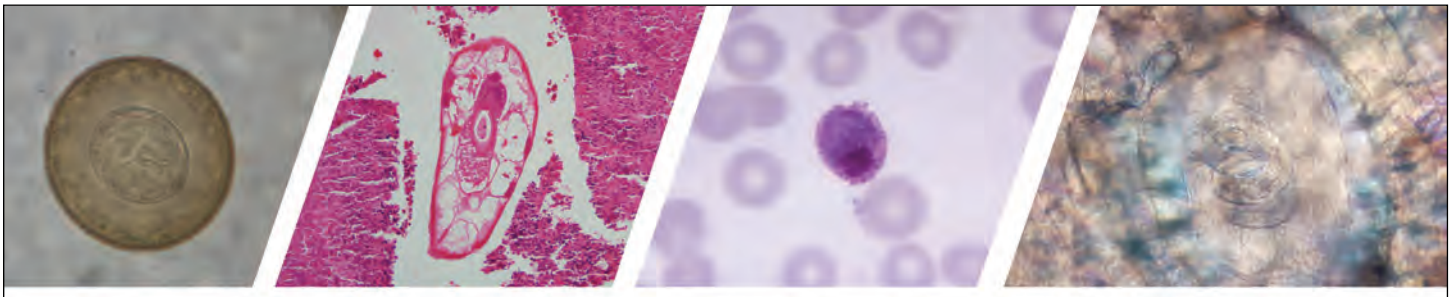

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Brucellosis in Dogs and Public Health Risk

Martha E. Hensel, Maria Negron, Angela M. Arenas-Gamboa

Brucella canis infects dogs and humans. In dogs, it can cause reproductive failure; in humans, it can cause fever, chills, malaise, peripheral lymphadenomegaly, and splenomegaly. *B. canis* infection in dogs is underrecognized. After evaluating serologic data, transmission patterns, and regulations in the context of brucellosis in dogs as an underrecognized zoonosis, we concluded that brucellosis in dogs remains endemic to many parts of the world and will probably remain a threat to human health and animal welfare unless stronger intervention measures are implemented. A first step for limiting disease spread would be implementation of mandatory testing of dogs before interstate or international movement.

Brucella canis is a gram-negative coccobacillary bacterium that primarily causes reproductive failure in dogs (*1*). The genus *Brucella* comprises 12 recognized species (*2*). Of these, *B. melitensis*, *B. abortus*, and *B. suis* are well-known causes of undulant fever and influenza-like symptoms in humans, but *B. canis* is less recognized as the cause of a zoonosis (*3*). In this review, we highlight information regarding occurrence of brucellosis in dogs, emphasizing *B. canis* as an underrecognized pathogen and describing current knowledge about its zoonotic potential.

Epidemiology

B. canis was initially characterized in 1966 after several outbreaks of abortion and infertility in dogs in multiple states (*1*). Since the discovery of *B. canis* as a cause of abortion, outbreaks in breeding and research kennels have been sporadically reported worldwide (*4*–*7*). The primary hosts are domesticated dogs; however, *B. canis* in wild canids and humans has also been reported (*8*,*9*).

Brucellosis in dogs occurs worldwide and is endemic to the Americas, Asia, and Africa (Figure) (*10*). In the 1970s and early 1980s, serologic surveys of dogs from multiple countries demonstrated a wide range of seropositivity, from 1% to 28%, depending on the country (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/17-1171-Techapp1.pdf). Within the past 30 years, few studies have been conducted to evaluate disease occurrence and distribution in the United States, so the current status is unknown. However, in the past 2 decades, serologic studies of dogs have been published from countries in Africa, Asia, and South America and have reported moderate to high seroprevalence, ranging from 6% to ≈35% (online Technical Appendix). This wide range of seroprevalence could be attributed to multiple factors, including but not limited to true disease prevalence in the region or country, sampling design and study sample, and diagnostic test algorithm used.

B. canis infection in dogs occurs predominantly through ingestion, inhalation, or contact with aborted fetuses or placenta, vaginal secretions, or semen (*11*,*12*). Like the rest of the *Brucella* species, *B. canis* exhibits tropism for reproductive tissue. Thus, infected dogs intermittently shed low concentrations of bacteria in seminal fluids and nonestrus vaginal secretions. Postabortion vaginal fluids contain a high level of bacteria and are a source of infection for other dogs and humans (*11*). Even after castration, dogs may still serve as a source of infection because the bacteria can persist in the prostate and lymphoid tissues (*13*,*14*). In addition to in reproductive secretions, dogs can shed the bacteria in the saliva, nasal secretions, and urine (*11*,*15*). Studies suggest that the concentration of *B. canis* in urine is higher in male than female dogs; this difference is attributed to urine contamination with seminal fluid (*11*). However, the role of urine as a source of infection is not fully understood.

Clinical Manifestations in Dogs

The clinical signs of *B. canis* infection are not pathognomonic. Dogs may be subclinically affected or may exhibit signs of reproductive failure. In male dogs, *B. canis* causes epididymitis, prostatitis, and orchitis (*15*); chronic testicular and epididymal inflammation can lead to unilateral or bilateral testicular atrophy and infertility (*13*).

The typical manifestation in females is mid- to late-term abortion (during days 45–59), followed by an odorless, brown-to-yellow vaginal discharge for 1–6 weeks (*1*). Another manifestation is embryonic death with resorption, which appears as conception failure after an apparently successful mating (*1*). It is possible for an

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DOI: https://doi.org/10.3201/eid2408.171171

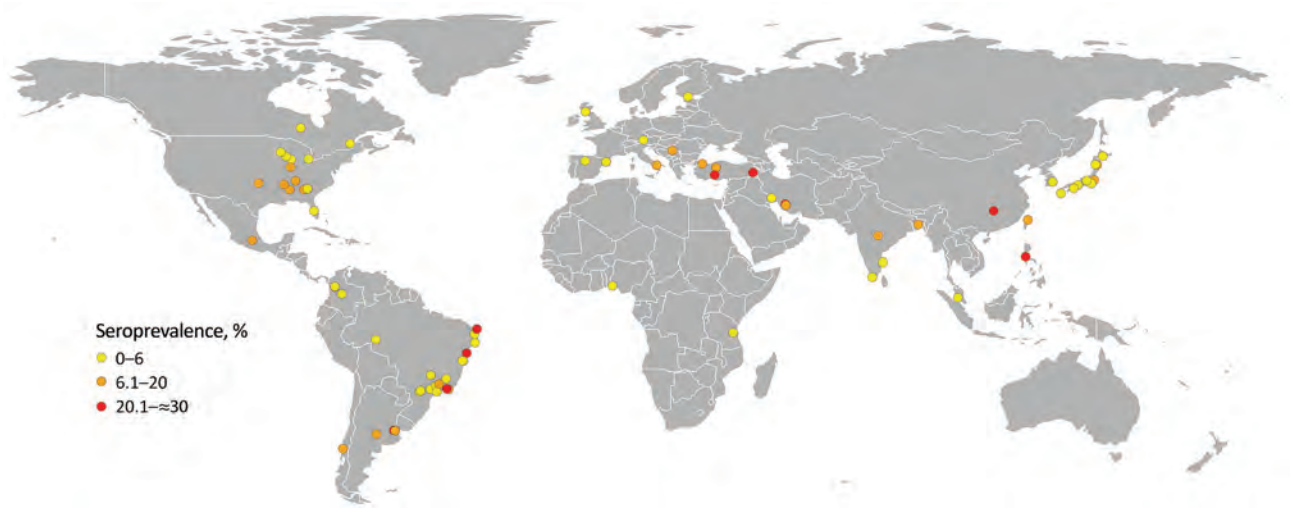

Figure. Locations of published *Brucella canis* serologic surveys of dogs (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/17-1171-Techapp1.pdf). Each dot represents 1 published study; colors represent seroprevalence determined in each study. Cartography: Cecilia Smith.

infected bitch to abort and subsequently have normal pregnancies or intermittently experience reproductive failure; these dogs may serve as reservoirs for infection in *B. canis*–naive dogs (*1*,*13*). Aborted pups have nonspecific lesions, such as subcutaneous edema, hemorrhage, or congestion (*1*). Pups from infected bitches that survive may be infected in utero or through nursing and can be bacteremic yet appear healthy (*13*). It is possible for seemingly healthy puppies from an infected bitch to disseminate the bacteria to other dogs and to humans (*16*). Because *B. canis* infection is the most common cause of reproductive failure in dogs, it should be ruled out before investigating other causes of infertility or abortion (*13*). However, if reproductive failure is not documented, canine brucellosis can be difficult to diagnose.

Another well-recognized manifestation of infection with *B. canis* is diskospondylitis, which can occur in otherwise healthy dogs or in those with a history of reproductive failure that was treated with antimicrobial drugs (*17*,*18*). Infected dogs have a history of lameness, spinal pain, neurologic dysfunction, muscle weakness, or any combination of these signs, caused by vertebral osteomyelitis and intervertebral disc infection (*18*). Incidence of diskospondylitis is higher in male than female dogs, perhaps because of a reservoir of bacteria in the prostate that results in intermittent bacteremia even in castrated males (*11*,*17*,*18*).

Antimicrobial drug treatment alone after signs of reproductive failure is usually unsuccessful because of the ability of the bacteria to sequester intracellularly for long periods and cause episodic bacteremia (*8*). The recommended course of treatment is multimodal and includes surgical sterilization and antimicrobial drugs.

Diagnostic Testing in Dogs

Serology

The initial diagnostic test for suspected brucellosis cases and the screening tool for evaluating breeding dogs is serologic testing (Table). Serologic tests evaluate antibody response against *Brucella* spp. cell wall antigens. *Brucella* spp. have 2 recognized cell wall morphologic appearances based on the structure of the *O*-polysaccharide subunit of lipopolysaccharide: smooth (considered more virulent; includes *B. abortus*, *B. suis*, and *B. melitensis*) and rough (*B. canis* and *B. ovis*) (*25*). These differences are noteworthy because serologic tests designed to detect infections with smooth *Brucella* spp. will not detect infection with *B. canis*.

The serologic methods most commonly used to screen for *B. canis* infections are the rapid slide agglutination test, 2-mercaptoethanol rapid slide agglutination test, agar-gel immunodiffusion, and ELISA (*8*). To confirm the results of these screening serologic methods, most diagnostic laboratories use the indirect fluorescent antibody test.

Use of serologic tests to diagnose *B. canis* infection has several pitfalls. The lack of a sensitive and specific screening test hampers the ability of veterinarians to diagnose the disease accurately. These tests are better at detecting early infections but have diminished sensitivity in chronically infected animals, which may be only intermittently bacteremic (*19*). Using *B. canis* M– antigen instead of *B. ovis* antigen reduces nonspecific reactions to the cell wall antigens of other gram-negative bacteria (e.g., *Pseudomonas* spp., *Actinobacillus equuli*, *Bordetella bronchiseptica*) and gram-positive bacteria (e.g., *Staphylococcus aureus*, *S. epidermidis*) and improves specificity (*14*,*26*). Furthermore, treating serum with 2-mercapthoethanol increases the

Table. Diagnostic tests for *Brucella canis* in dogs*

Test type	Antigen detected or target DNA	Sensitivity, %	Specificity, %	Reference
Serologic				
Rapid slide agglutination	Cell wall	50–75	83.34–99.7	(*19*)
2-mercaptoethanol rapid slide agglutination	Cell wall	31.76–70	100	(*19*)
Agar-gel immunodiffusion, cell wall antigen	LPS, outer membrane protein	27.98–52.94	100	(*19*)
ELISA	LPS or CPAg	88–97	94.3–96.7	(*20*)
Immunochromatographic	R-LPS with outer membrane proteins	89.58	100	(*21*,*22*)
Other				
PCR (ITS66 and ITS279)	16S-23S rRNA gene	100	86.45–100	(*23*)
PCR (JPF/JPR)	Outer membrane protein 2	16.67 (whole blood); 92.31 (vaginal swab sample)	100 (whole blood); 51.92 (vaginal swab sample)	(*24*)

*CPAg, cytoplasmic protein antigen; JPF, forward primer; JPR, reverse primer; LPS, lipopolysaccharide; R-LPS, rough LPS.

specificity of the test by destroying IgM pentamers that can interfere with evaluation of IgG but does not fully eliminate false positives because of heterologous cross-reactions (*14*,*27*). Treatment with antimicrobial drugs can affect testing by eliminating bacteremia (*8*).

Culture

The standard test for *B. canis* is culture (*8*). Commonly collected samples include blood, vaginal discharge, and semen. Of these, blood is the most commonly collected; however, because bacteremia can be intermittent, positive animals may be missed (*10*,*19*). The best time for culturing *Brucella* is 2–4 weeks after infection, after demonstration of reproductive failure, when bacteremia is the highest (*8*,*10*,*26*). Culture is not recommended if the dog has received antimicrobial drugs because they will clear the bacteremia regardless of the resolution of systemic disease (*8*). Culture requires up to 9 days, increasing the risk for exposure of laboratory personnel if the cultures are not handled appropriately (*28*).

PCR

Several PCR primers have been designed to detect *B. canis* DNA in whole blood, vaginal secretions, and semen. PCR has the potential as a rapid, discriminatory test to screen dogs, or it can be a useful confirmatory test for seropositive dogs (*23*,*24*,*29*). However, use of PCR is not yet readily available in most diagnostic laboratories and remains an experimental test.

B. canis Infection in Humans

Humans acquire *B. canis* infection through direct contact with infected dogs or their reproductive or blood products (*30*–*32*). Clinical signs and symptoms include undulant fever, chills, malaise, splenomegaly, and peripheral lymphadenomegaly (*33*). In humans, diagnosis is often complicated because of the nonspecific signs and symptoms coupled with a low index of suspicion by many physicians. If the disease is part of the differential diagnosis, culture is the only test available for diagnosing *B. canis* infection in humans, and confirmation is problematic because of low-level and intermittent bacteremia (*34*). Even if physicians suspect brucellosis, diagnoses may be missed because the commercially available serologic tests screen for the smooth *Brucella* species and will not detect antibodies against *B. canis* (*35*). Canine serologic tests for *B. canis* infection have been adapted for use in humans, but test results should be interpreted with caution.

Laboratory personnel, veterinarians, and animal caretakers are at increased risk for exposure to *B. canis* (*3*,*32*,*36*). *Brucella* spp. are considered high-risk pathogens and require a specialized Biosafety Level 3 work space, which if not used can result in laboratory-acquired exposure from a variety of scenarios, such as working with unknown bacterial pathogens on the benchtop (*28*). Dentinger et al. described an incident in which 31 laboratory workers were exposed to *B. canis* after handling an unknown gram-negative bacterium on the benchtop (*16*). None became ill with clinical disease, even those characterized as having experienced high-risk exposures (according to Centers for Disease Control and Prevention guidelines) and who declined postexposure prophylaxis (5 of 21 at high risk) (*16*). One case of laboratory-acquired exposure was documented in a technician who used mouth-pipetting to resuspend the M– strain of *B. canis*; the technician experienced symptoms despite this particular strain being considered avirulent in dogs (*37*). Additionally, Krueger et al. applied available veterinary serologic diagnostic tests to 2 cohorts of persons with or without occupational exposure to dogs and found a seroprevalence of 3.6% among those exposed to dogs, which is higher than previously reported seroprevalence of 0.6% among those with occupational exposure (*3*,*38*). Identified risk factors included working as kennel staff, exposure to breeding bitches, and failure to wash hands after caring for a sick dog (*3*). Of note, in that study, only 2 of the 306 persons with occupational exposure to dogs reported any clinical signs or symptoms associated with brucellosis after contact with dogs who had confirmed brucellosis (*3*). Unfortunately, the temporality of the onset of clinical signs and symptoms and exposure could not be determined (*3*). Regardless, these

findings may suggest that healthy humans might be moderately resistant to clinical illness from *B. canis* infection.

Several case reports highlight pet ownership as a likely risk factor leading to infection in otherwise healthy persons (*9*,*16*,*32*,*33*,*39*). In particular, children and immunosuppressed persons might be at higher risk for acquiring the disease (*16*,*36*,*39*,*40*). Three cases in children <4 years of age have been reported (*16*,*36*,*39*). In 1 of the reports, Dentinger et al. described transmission of *B. canis* to a child from an infected puppy that had been purchased from a pet store and was deemed healthy during an initial veterinary visit (*16*). However, after the child became febrile and *B. canis* infection was diagnosed by blood culture, isolates from the child and puppy were submitted to the Centers for Disease Control and Prevention. The 2 isolates showed close genetic similarity, suggesting that the puppy was the source of infection. Clinical signs did not develop in 4 adults in the same household, all of whom had been exposed to the puppy. Several recent reports of *B. canis* in HIV-infected patients highlight the risk within this population (*31*,*40*,*41*). These cases of *B. canis* infection were linked to ownership of reproductively intact dogs that had a history of reproductive failure and a later diagnosis of *B. canis* infection according to serology and blood culture (*31*,*40*).

Public Health Implications

Brucellosis in dogs occurs worldwide (Figure), but many countries, regardless of their resource level, lack a cohesive plan to respond to cases of this infection in humans or dogs. Brucellosis in humans is notifiable in all 57 states and territories of the United States. Thus, cases must be reported to the National Notifiable Disease Surveillance System; reported in a case report to the Bacterial Special Pathogens Branch at the Centers for Disease Control and Prevention when identified by a health provider, hospital, or laboratory; or both. However, the causative *Brucella* species is not always reported. As a result, it is difficult to obtain accurate estimates of *B. canis* infections in humans. Despite the presence of this pathogen in geographically and politically diverse locations, few countries have *B. canis*–specific regulations. A lack of regulatory interest makes it likely that *B. canis* will continue to be an underrecognized pathogen of dogs and humans.

The public health relevance of *B. canis* infection in humans is unclear because much of the information comes from case reports. The perceived infrequency of human infection with *B. canis* and the lack of reliable diagnostic tools for disease detection has led to few serologic surveys in humans. Our current understanding of prevalence of *B. canis* infection in humans comes from a handful of serologic surveys that use diagnostic tests available for dogs and thus may not be truly representative (*3*,*38*,*42*–*44*).

In the United States, cross-sectional serologic surveys of military recruits and Florida residents and case–control surveys of animal caretakers with occupational exposure to canids documented an extremely low *B. canis* seropositivity (0.4%–0.6%) (*38*,*42*,*44*). Veterinarians from Florida with occupational exposure to dogs were also surveyed but were all negative according to serologic testing (*38*). In 1976, a serologic survey in Mexico City, Mexico, evaluated human blood samples from randomly selected patients for *B. canis* antibodies by using the plate agglutination test; documented seropositivity was 13.3% (*45*). More recently, in Brazil, convenience sampling of human blood samples for screening found that 4.6% of surveyed adults had a positive antibody titer (*46*). Most serologic studies have relied on random convenience sampling of human blood samples. In contrast, a case–control survey by Monroe et al. documented a high *B. canis* seropositivity (80.5%) in persons with fever of unknown origin, but these results were not confirmed by blood culture (*43*). Differences between these studies can be attributed to the test used (tube agglutination test vs. microtiter plate agglutination) and the study population.

When compared with owned dogs, stray dogs are more likely to be intact and have a higher documented level of *B. canis* seropositivity (*45*,*47*). A higher burden of canine brucellosis in the stray/roaming dog populations could lead to spillover into the human population in areas with a large number of intact, stray dogs because these dogs are taken into shelters or placed in foster homes pending adoption. In the United States, ≈30% of pet dogs are adopted from animal shelters, and testing for *B. canis* is not standard procedure before adoption (*48*). No definitive evidence demonstrates a direct link between the number of reproductively intact, stray dogs in an area and potential for human exposure. Studies that attempt to compare levels of *B. canis* antibodies in humans with results of serologic surveys of dogs may not correlate a positive antibody titer in humans to clinical signs of infection or may not correlate the findings with exposure to stray or owned dogs (*45*). In the absence of the full epidemiologic picture, it is difficult to draw conclusions between seropositive dogs and the potential for human exposure, but future research could clarify the risk potential.

Another potential source of *B. canis* dissemination is breeding kennels, given the nature of the disease, the fact that animals are housed in close contact, and the constant movement of dogs for breeding or sale (*49*). Recent outbreaks in kennels in the United States, Hungary, Sweden, and Colombia highlight the link between outbreaks and interregional/international movement of breeding dogs (*5*–*7*,*49*). Unrestricted movement of reproductively intact dogs or puppies is a known risk factor for the spread of infectious diseases and has led to human infection with *B. canis* (*16*,*49*). Quarantine periods and premovement health tests of dogs vary by region, but no region tests dogs for brucellosis before they are moved (*48*). Required testing of

breeding animals or their offspring before interstate or international movement would decrease the risk for *B. canis* transmission between dogs and from dogs to humans.

Practices to limit the number of intact stray animals include government- or private charity–sponsored sterilization or testing and euthanasia of *B. canis*–positive dogs. In resource-limited communities, the true risk associated with a large roaming population is unknown, but these dogs should be considered a possible zoonotic risk for humans until new data suggest otherwise. This population of dogs serves to keep brucellosis as an endemic zoonotic disease indefinitely.

The World Health Organization and the World Organisation for Animal Health do not have policies relating to brucellosis caused by *B. canis*. Perhaps because of a perceived low incidence, many countries also do not have response plans or routine surveillance for *B. canis* in dogs or humans (*5*,*46*). In the United States, where *B. canis* was first isolated, the response is piecemeal; however, published recommendations include requiring mandatory reporting of brucellosis in dogs to state health authorities, state health departments to enter into a memorandum of understanding with veterinary diagnostic laboratories to report positive cases to the state health department, and mandatory communication with veterinarians and dog owners to alert them of the zoonotic risk (*30*). Other measures to prevent zoonotic transmission include confirming the diagnosis with the veterinarian and providing educational materials about the zoonotic potential associated with interacting with a *B. canis*–positive dog (*30*). One aspect of reducing the zoonotic potential is educating owners about options for managing *B. canis*–positive dogs, such as sterilization, antimicrobial drug therapy, and repeat testing, or euthanasia if those measures cannot be applied (*30*). Anyone who has contact with an infected dog should maintain good hygiene standards when handling its urine, feces, or reproductive products (*30*).

Other methods to decrease the incidence of brucellosis in dogs include improving diagnostic tests and developing a vaccine. Improved diagnostic tests are needed for better evaluation of disease prevalence in at-risk communities and to help physicians and veterinarians more accurately identify cases of disease caused by *B. canis*. In addition to improved diagnostic tests, a *B. canis* vaccine, which is not currently available, could substantially decrease infection incidence in the dog population and thus reduce the risk for transmission to humans.

In conclusion, brucellosis in dogs remains endemic to many parts of the world and without stronger intervention measures will probably remain an underrecognized threat to human health and animal welfare. Future work is required to improve diagnostic assays for humans and animals and to generate policies to prevent the spread of disease. Implementation of mandatory testing before interstate or international movement of dogs would be a good first step.

Acknowledgments

We thank Cecilia Smith for her technical assistance with developing the map of the serologic data.

Financial support was provided by the American Kennel Club Canine Health Fund 02175-A (to A.M.A.-G.). Student stipend support was provided by National Institutes of Health T32 fellowship 5 T32 OD 11083-7 (to M.E.H.).

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Abnormal Helminth Egg Development, Strange Morphology, and the Identification of Intestinal Helminth Infections

Sarah G.H. Sapp, Michael J. Yabsley, Richard S. Bradbury

Occasionally, abnormal forms of parasitic helminth eggs are detected during routine diagnostics. This finding can prove problematic in diagnosis because morphologic analysis based on tightly defined measurements is the primary method used to identify the infecting species and molecular confirmation of species is not always feasible. We describe instances of malformed nematode eggs (primarily from members of the superfamily Ascaridoidea) from human clinical practice and experimental trials on animals. On the basis of our observations and historical literature, we propose that unusual development and morphology of nematode and trematode eggs are associated with early infection. Further observational studies and experimentation are needed to identify additional factors that might cause abnormalities in egg morphology and production. Abnormal egg morphology can be observed early in the course of infection and can confound accurate diagnosis of intestinal helminthiases.

Despite recent advances in molecular diagnostics, microscopic analysis of ova and larvae, and to a lesser degree adult worms, remains the mainstay for the diagnosis of intestinal helminths in humans and animals worldwide. In most cases, such morphologic diagnosis relies upon the identification of the helminth genera or species based on the characteristic morphology of eggs because adult parasites are rarely available. When unfamiliar egg morphologies are observed, parasitologists will often consult with atlases and textbooks that describe the morphology of eggs produced by various species of helminth infecting the host feces being examined to determine the species of helminth concerned. These references generally describe the standard presentation of eggs without consideration of potential abnormal forms. In addition, students are generally only provided the best specimens during practical classes in which they are taught to identify parasites.

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DOI: https://doi.org/10.3201/eid2408.180560

The authors of this article conduct disparate work in intestinal helminths of humans and animals. They routinely undertake classical morphologic diagnosis of parasites in their respective roles and have extensive experience in morphologic methodologies. Casual discussions between the authors revealed that they had each observed highly abnormal forms of helminth eggs from humans and animals during the course of their work.

One author (R.S.B.) had observed multiple highly abnormal forms of *Ascaris lumbricoides* roundworms being passed by humans during the course of human intestinal helminth surveillance studies in the eastern Solomon Islands. These forms were passed by different persons and included eggs with double morulae, giant eggs (size ranging up to 110 μm in length), and eggs not conforming to the traditional symmetric, ovoid morphology associated with those of *A. lumbricoides*. Nevertheless, these eggs demonstrated several distinct features that identified them as belonging to *A. lumbricoides*, and they were observed in association with other eggs of *A. lumbricoides* that demonstrated standard morphologic features (Figure 1). All of the eggs were observed in Kato Katz preparations, a method that is known to cause some malformation in helminth eggs, particularly those of schistosomes and hookworms, which will collapse or dissolve, respectively, if the smear is allowed to clear for too long (*1*). However, the degree of morphologic abnormality observed in these specimens was far beyond the relatively minor swelling and clearing of *A. lumbricoides* eggs common to Kato Katz preparations and only occurred occasionally, eliminating artifact of Kato Katz preparation as a cause. The observations of these highly abnormal egg morphologies were made in the context of a population with very high prevalence and intensity of ascariasis (prevalence 53%, with 28% having moderate- to heavy-intensity egg counts) and the possibility that this feature was caused by crowding of gravid female worms in the gut of the host was considered. This author had also previously observed abnormal variations in the eggs of *Schistosoma haematobium*, all recovered from the same urine specimen of a refugee

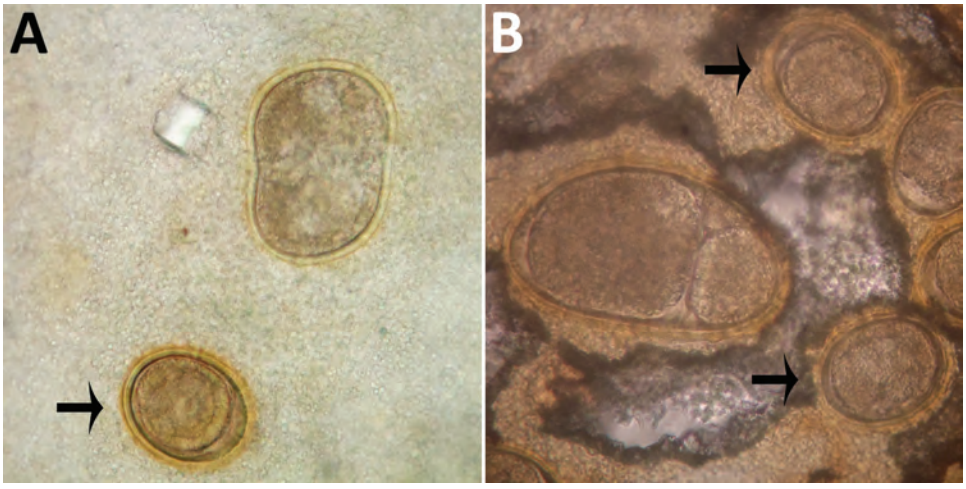

Figure 1. Abnormalities of *Ascaris lumbricoides* eggs from patients in the Solomon Islands, visualized on Kato-Katz. A) Giant egg with irregular indented shape. B) Giant egg with 2 morulae. Arrows indicate eggs of normal morphology. Original magnification ×400.

from Africa attending the Royal Hobart Hospital in Tasmania, Australia (Figure 2).

Other authors (S.G.H.S. and M.J.Y.) made similar observations in regard to ascarid infections of raccoons (*Procyon lotor*) and domestic dogs (*Canis familiaris*). During experimental infections of naive, ascarid-free dogs and captive-bred raccoons with *Baylisascaris procyonis*, the raccoon roundworm, abnormal eggs were observed in fecal flotation exams conducted during the first few days and weeks of patency. Abnormalities observed included eggshell distortions resulting in irregular, crescent, budded, and triangular shapes, and twin eggs conjoined by an eggshell but with separate morulae and vitelline membranes (Figure 3). Many eggs during early patency were unusually oblong but remained within normal size variation. These abnormal eggs were detected in dogs that were infected through ingestion of tissues from infected mice (larvae) and in raccoons that were infected through

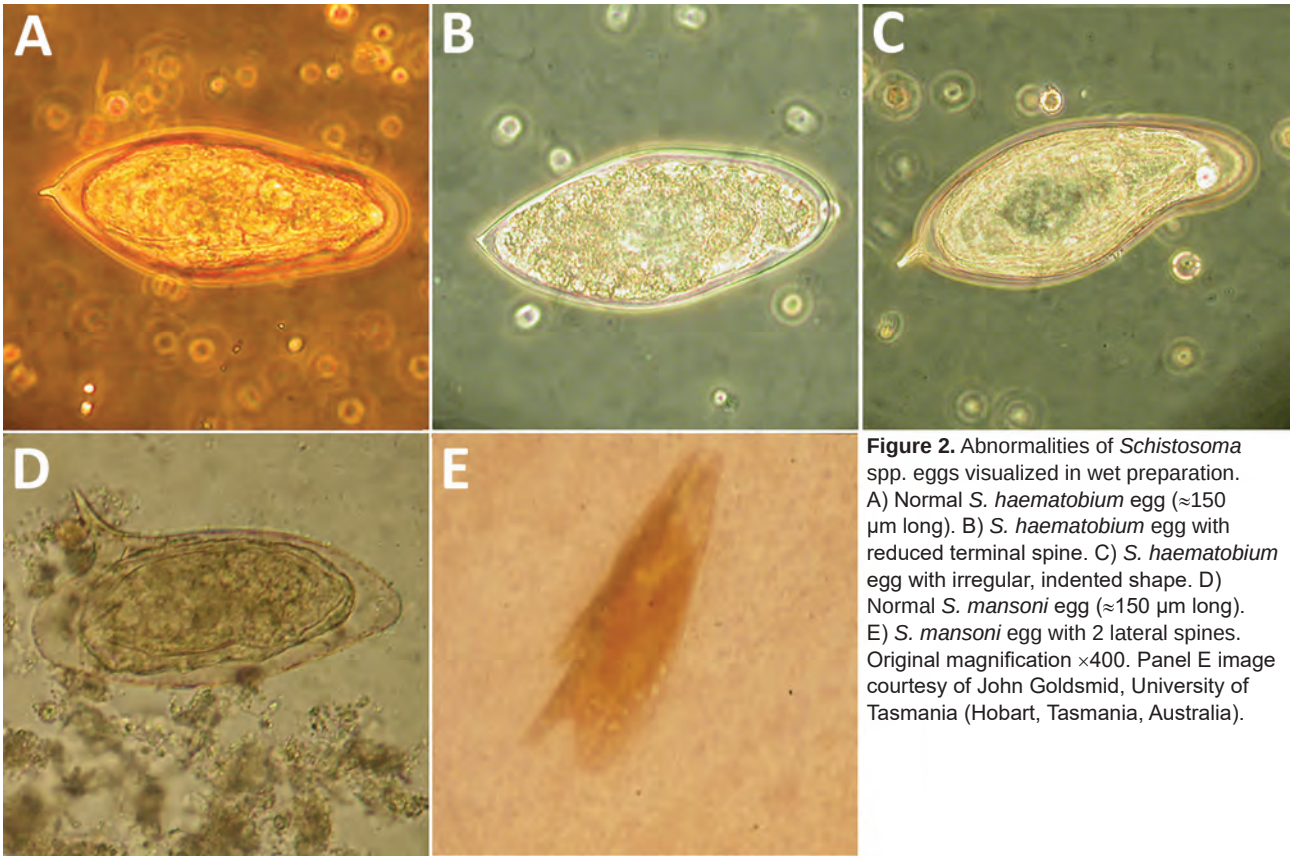

Figure 2. Abnormalities of *Schistosoma* spp. eggs visualized in wet preparation. A) Normal *S. haematobium* egg (≈150 μm long). B) *S. haematobium* egg with reduced terminal spine. C) *S. haematobium* egg with irregular, indented shape. D) Normal *S. mansoni* egg (≈150 μm long). E) *S. mansoni* egg with 2 lateral spines. Original magnification ×400. Panel E image courtesy of John Goldsmid, University of Tasmania (Hobart, Tasmania, Australia).

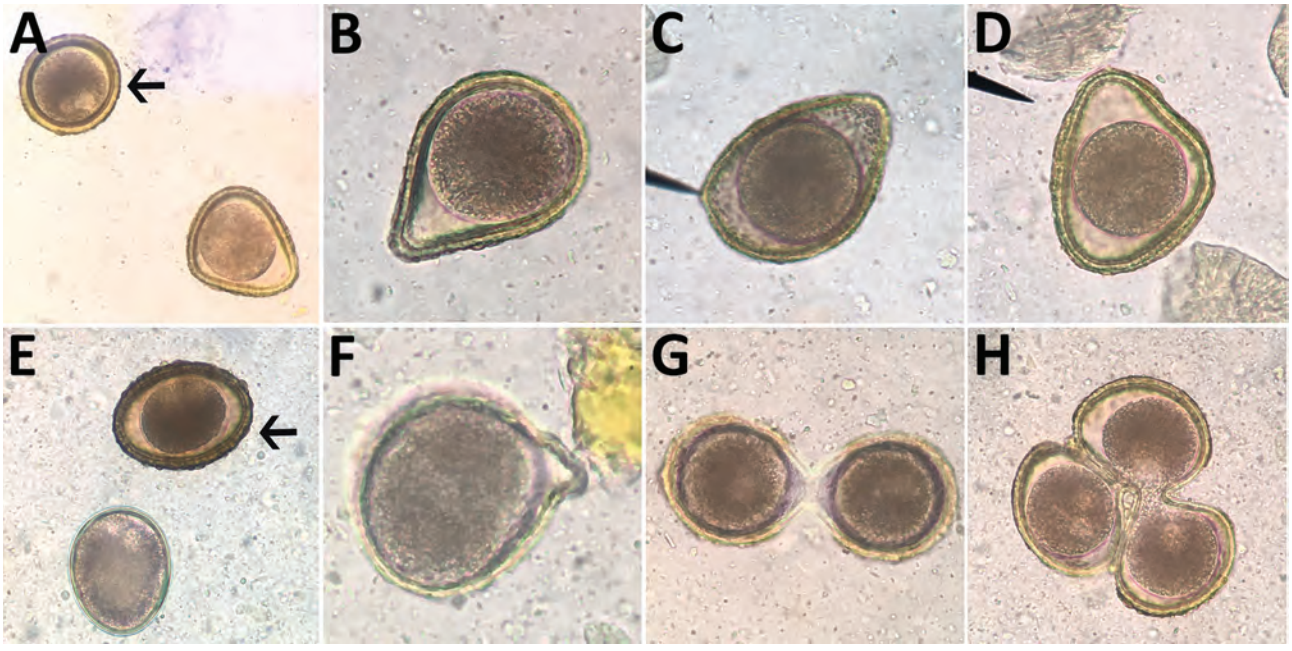

Figure 3. Abnormalities of *Baylisascaris procyonis* eggs shed by experimentally inoculated dogs and raccoons, visualized on fecal flotation. A) Triangular egg. B) Pear-shaped egg. C) Almond-shaped egg. D) Triangular egg with indented edge. E) "Immature" egg with underdeveloped morula and no cortex or proteinaceous coat. F) Budded egg. G) Twin conjoined eggs with separate morulae and vitelline membranes. H) Triplet conjoined eggs with distinct morulae; vitelline membrane might be shared between 2 eggs. Arrows indicate eggs of normal morphology (65–75 μm). Original magnification ×400.

ingestion of eggs or larvae. Among the 6 raccoons inoculated with larvae, all had a proportion of markedly malformed eggs early in patency; this malformation was also observed in 3 of 4 raccoon-inoculated eggs that became patent. Obviously malformed eggs represented ≈5% of eggs observed after fecal flotation of samples obtained within the first 2 weeks of patency, with limited variation (range 1.5%–7%). The frequency at which these malformed eggs were observed decreased with the length of infection; some animals ceased to pass any malformed eggs after ≈30 days postpatency.

Even for experienced morphologists, variability in size and shape of helminth eggs adds a layer of complexity to diagnosis. For example, unusually large *Trichuris* spp. eggs were observed in the stool of a child in the Bahamas (*2*). The size of these eggs was outside of the typically observed range for human-infecting *T. trichiura* whipworms (normal eggs also were observed in the patient) and instead were within the range of the canine *T. vulpis* and feline *T. campanula* (*felis*) worms. Otherwise, however, the eggs were not morphologically consistent with either of these animal whipworm species. Whether this case was an abnormal egg shedding by *T. trichium* worms, a zoonotic *Trichuris* species, or even human infection with a novel species is unknown. Modern molecular diagnosis would aid in species resolution, but this approach is not necessarily an option in underserved areas.

Morphologic deviation from typical ranges is also an important consideration and source of confounding in studies on natural infections of wildlife. Two authors of this article (S.G.H.S. and M.J.Y.) were consulted by a veterinarians who had received a diagnosis of *Baylisascaris* spp. infection for a captive bobcat (*Felis rufus*) kitten by a veterinary reference laboratory. The veterinarian requested confirmation because this case would have represented the first report of *Baylisascaris* spp. infecting a feline host. Upon examination, we found the eggs to be mostly morphologically consistent with *Toxocara cati* (size ≈75 × ≈80 μm, generally round to pear shaped, golden in color, and with a finely pitted shell), but some did resemble *Baylisascaris* spp. (smaller size, ≈68 × ≈60 μm, slightly ellipsoid, with a darker, amber color, and with a thicker shell). These eggs were allowed to embryonate for 3 weeks and were then artificially hatched to examine the morphology of larvae. The larvae from the normal and abnormal eggs were morphologically similar to each other and were identified as belonging to the species *T. cati* (≈350 μm long, ≈20 μm midbody diameter; slightly flared anterior end suggesting early development of cervical alae). Because this infection was identified in a kitten, it might represent an additional example of malformed eggs passed early in patency. No follow-up samples or recovered adult worms were available because the host had been treated for the infection.

We conducted a review of the literature to investigate previous descriptions of abnormalities in the morphology of intestinal helminth eggs, with particular reference to ascarids. Matuda summarized the findings of early investigators discussing abnormal forms of *A. lumbricoides* (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/18-0560-Techapp1.pdf) (*3*). These descriptions included several similar to those observed by 1 author (R.S.B.) in the Solomon Islands, which included double morulae in a single egg (categorized by Matuda as category B.I [online Technical Appendix]), enlarged eggs (B.II.a), and deformities of the egg shells (B.III). These observations in *A. lumbricoides* eggs were also similar to deformities observed in the related parasite *B. procyonis* by S.G.H.S. and M.J.Y., including a budded shell (B.III.a), triangular shape (B.III.c), almond to crescent shape (B.III.b), and fused eggs (B.I.a1, B.I.a2, and B.I.b.). No further work was completed on the mechanisms that caused the *Ascaris* egg abnormalities described by Matuda. Similar abnormalities, including united eggs, angular deformities, and unusual position or absence of polar globules, were later observed in structural studies on the poultry ascarids *Ascaridia galli* and *Heterakis gallinae* (*4*). Descriptions of abnormal egg morphology in the literature for other nematode taxa are scarce and perhaps underrecognized. One example found was that of conjoined *Trichuris vulpis* eggs from a routine dog fecal examination (Figure 4).

Leiper described variations in the morphology and position of spines of schistosome eggs in Egypt in the first decade of the 20th century (*5*). These abnormal findings led to controversy among investigators of the day regarding the number of species of schistosomes in Egypt. Much later, Goldsmid reported morphologic abnormalities in *Schistosoma mansoni* eggs in Zimbabwe, including a double-spined egg (Figure 2, panel E) (*6*).

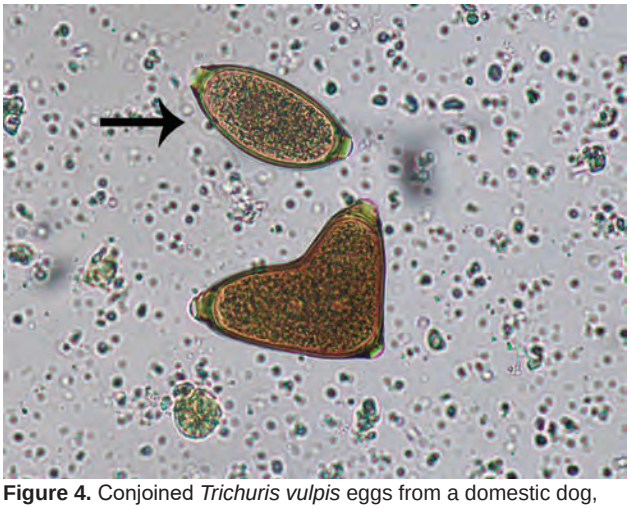

Figure 4. Conjoined *Trichuris vulpis* eggs from a domestic dog, visualized on fecal flotation. Arrow indicates a morphologically normal egg. Original magnification ×400. Photograph by Danielle E. Preston and courtesy of Mani Lejeune, both of the Cornell University College of Veterinary Medicine (Ithaca, NY, USA).

The etiology of malformation in these abnormally shaped eggs is unknown. During *B. procyonis* infection trials by S.G.H.S. and M.J.Y., malformed eggs were initially detected in infected dogs, suggesting that the passage of these eggs by dogs might be the result of an abnormal host–parasite relationship, host immunity, or both. Previous and current studies suggest that dogs are poor definitive hosts for *B. procyonis* (*7*; S.G.H. Sapp and M.J. Yabsley, unpub. data) compared with the natural raccoon host. However, the observation of deformed eggs in our subsequent trials with experimentally infected raccoons suggests a predominantly parasite-mediated (as opposed to host-mediated) process. Although not investigated mechanistically in nematodes to our knowledge, abnormalities in trematode (i.e., *Fasciola hepatica* and *Dicrocoelium dendriticum*) egg production have been attributed to differential vitelline gland activity in immature and senescent flukes (*8*). Also, Leiper attributed the observed malformations and variability in spines and shape in eggs produced by *S. haematobium* worms in Egypt to egg production by immature worms (*5*). In 1926, Manter also observed that eggs produced by immature specimens of the fish trematode *Otodistomum cestoides* were undersized and of unusual shape (*9*).

We also considered the possibility that malformed ascarid eggs might be caused by crowding stress on adult worms in high-intensity infections of the gut, given that the *A. lumbricoides* eggs were passed by persons in the Solomon Islands with high-intensity infections (i.e., passing unusually high numbers of eggs). However, the *B. procyonis* infections in the dogs and raccoons were not high-intensity infections; only a moderate egg burden was observed (maximum of ≈600 eggs/g feces in dogs and ≈9,000 eggs/g feces in raccoons). Given the high prevalence and intensity of infection in the Solomon Islands study, the abnormalities seen alongside normally formed eggs could be attributable to an immature female entering patency and producing abnormally shaped eggs alongside several mature females producing normal eggs. Moreover, anecdotal discussions with colleagues in the past have raised the possibility that these abnormalities are attributable to the effects of anthelmintic use on egg development, and sporadic reports exist of malformed eggs after unsuccessful or low-dose sublethal anthelmintic treatment (*10*,*11*). However, none of the hosts that were passing abnormal ascarid eggs during our observations had been treated with anthelmintics in the preceding 3 years. None of the raccoons given piperazine or ivermectin at the conclusion of the study passed abnormal eggs after treatment. Helminth taxa might respond differently to anthelmintics (e.g., ascarid eggs might be less likely to have abnormalities after exposure, whereas this development has been noted in *Trichuris* spp.) (*11*,*12*).

Furthermore, some classes of anthelmintics could feasibly interfere with egg development to a stronger degree than others, although data on the subject are limited.

The combined incidental findings described in this report demonstrate the potential for morphologically abnormal helminth egg morphologies to occur in certain host species. These abnormalities might confound or mislead inexperienced morphologists regarding the identity of such helminth eggs and thereby lead to incorrect or missed diagnoses. The current standard morphologic descriptions address only the commonly observed morphologies of intestinal helminth eggs, and the potential for substantial deformity is not generally considered by laboratory staff. In addition, numerous ecologic studies are conducted on parasites of wildlife, which frequently rely on nonlethal sampling through identification of eggs in fecal samples by researchers who might not have extensive training in morphologic identification of parasitic ova. Moreover, many parasite species of wildlife have poorly described or unknown egg morphology data available. The presence of abnormal eggs might lead to spurious descriptions of new species in such studies. Furthermore, many parasite species of wildlife have poorly described or unknown egg morphology data. Should abnormal eggs be observed, especially in an unusual host, molecular characterization might be necessary to obtain a definitive diagnosis.

Our findings and evidence from the historical literature suggest that immaturity of egg-producing helminths might be a major cause of the observed egg deformations we have described. Other etiologic factors such as host immunity cannot be discounted on the basis of such preliminary findings, and more work is needed to understand the causes and mechanisms leading to the passage of deformed eggs. This report serves to highlight the existence of such phenomena and the potentially underrecognized challenge that malformed helminth eggs represent in terms of correctly diagnosing helminth infections in humans and animals. We hope that more research will occur to determine the causes of these aberrations and the diversity of their occurrence across helminth taxa. Such research should consider the intensity of host infection, host or helminth age associations, and, ultimately, immunologic mechanisms. Our collaborative investigation also further highlights how even casual communication between scientists working on very different subjects can be unexpectedly enlightening, particularly in the highly interdisciplinary area of parasitology.

About the Author

Dr. Sapp is a recent graduate from the Department of Infectious Diseases, University of Georgia. Her research interests include the transmission and epidemiology of zoonotic helminths and the natural history of ascaridoid nematodes.

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Case Series of Severe Neurologic Sequelae of Ebola Virus Disease during Epidemic, Sierra Leone

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We describe a case series of 35 Ebola virus disease (EVD) survivors during the epidemic in West Africa who had neurologic and accompanying psychiatric sequelae. Survivors meeting neurologic criteria were invited from a cohort of 361 EVD survivors to attend a preliminary clinic. Those whose severe neurologic features were documented in the preliminary clinic were referred for specialist neurologic evaluation, ophthalmologic examination, and psychiatric assessment. Of 35 survivors with neurologic sequelae, 13 had migraine headache, 2 stroke, 2 peripheral sensory neuropathy, and 2 peripheral nerve lesions. Of brain computed tomography scans of 17 patients, 3 showed cerebral and/or cerebellar atrophy and 2 confirmed strokes. Sixteen patients required mental health follow-up; psychiatric disorders were diagnosed in 5. The 10 patients who experienced greatest disability had co-existing physical and mental health conditions. EVD survivors may have ongoing central and peripheral nervous system disorders, including previously unrecognized migraine headaches and stroke.

The 2014–2016 West Africa Ebola virus disease (EVD) epidemic resulted in an estimated 3,956 deaths and 10,168 survivors in Sierra Leone (*1*). The use of high-quality specialty services by Ebola survivors offers an opportunity to improve understanding of debilitating post-EVD sequelae.

Central nervous system (CNS) viral invasion by EVD had been suspected but unproven until the West Africa EVD epidemic. In this outbreak, individual case-patient reports describe clinical features of meningoencephalitis or meningitis during and after acute Ebola virus (EBOV) infection, accompanied by EBOV PCR results in nonbloodstained cerebrospinal fluid samples (CSF) (*2*–*6*). Cranial imaging of 3 encephalitic patients documented changes consistent with cerebral atrophy (*3*), meningoencephalitis (*4*), and areas of diffusion restriction suggesting ischemia (*4*,*5*). Nonhuman primate EVD models and human Marburg neuropathology found EBOV-immunoreactive glial nodules and perivascular infiltrates (*7*–*9*) and evidence of choriomeningioencephalitis (*10*). In addition, a novel retinal lesion in Ebola survivors that appears to follow ganglion cell axons as they exit the optic nerve has been described (*11*). Combined with the observation that human CSF can be EBOV PCR–positive after plasma testing shows negative results (*3*,*4*), these observations raise the possibility that infected CNS cells may have a role in persistent or recurrent neurologic disease.

Observational studies of survivors report a broad range of neuropsychiatric symptoms (*12*–*14*), including increased fatigue, diminished work capacity, and sleep disturbance (*15*,*16*). Psychosocial distress caused by bereavement, stress, and stigma and formal psychiatric diagnoses of depression, anxiety, and adjustment disorder have been reported (*17*–*21*).

To define the full spectrum of characteristics and severity of neurologic and psychiatric disease, we investigated neurologic sequelae in patients with neurologic symptoms by providing specialist neurologic evaluation, psychiatric and disability assessment, and brain computed tomography (CT) imaging and retinal imaging to an EVD survivor cohort. Our additional objective was to describe psychiatric, disability, and ophthalmic outcomes for survivors with neurologic sequelae.

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DOI: https://doi.org/10.3201/eid2408.171367

Materials and Methods

We completed this prospective observational study during February 4–May 10, 2016. Patients eligible for inclusion were ≥12 years of age, had complete clinical records, and attended the 34 Military Hospital (34MH) Ebola Survivors Clinic, Freetown, Sierra Leone. All patients provided Ebola survivor discharge certificates as proof of identity at initial enrolment in the 34MH cohort and on attending the preliminary clinic. Furthermore, staff at the 34MH clinic had provided care in the 34MH emergency treatment unit (ETU) and could certify the validity of survivors. The preliminary clinic took place at the 34MH Ebola Survivors Clinic and the specialist clinics at Connaught Hospital, Freetown, Sierra Leone.

Patients were invited to the preliminary clinic on the basis of having reported ≥1 major or ≥2 minor criteria (Table 1). These criteria were selected to maximize sensitivity for neurologic and psychiatric conditions. In addition, clinic staff invited additional patients suspected of having neurologic symptoms.

In the preliminary clinic, an intern physician, supported by trained nursing staff, obtained informed written consent to publish clinical data and images and administered an initial questionnaire. Further history and examination, including full neurologic examination, were accomplished by 2 physicians who used structured data recording forms. Patients with prominent or disabling symptoms of neurologic origin that required referral to the joint neurologic and psychiatric clinic were defined as having severe neurologic features. Patients with neurologic sequelae who did not warrant referral became a no severe neurologic features group. Laboratory tests, including lumbar puncture and brain CT, were available according to clinical need. Patients who had ≥2 psychiatric symptoms were referred for psychiatric assessment.

In the specialist clinic, full neurologic history and examination were performed individually or jointly by 2 consultant neurologists. Psychiatric assessment was performed onsite by 2 higher-level psychiatry trainees. Psychiatric assessment included Mini International Neuropsychiatric Interview (MINI-plus) and Mini Mental State Examination (MMSE; Mapi Research Trust PROVIDE, Lyon, France) and the World Health Organization Disability Assessment Schedule 2.0 (WHO-DAS 2.0; http://www.who.int/classifications/icf/whodasii/en/). The WHO-DAS 2.0 is a cross-cultural and validated tool providing a score that is compared to population percentile values (*22*). Although no cognitive or psychiatric assessment tools have been validated for the Sierra Leone population, the MMSE is frequently used by staff in the Connaught mental health clinic. Patient follow-up occurred at a second neurology clinic, in their local mental health clinic, and by telephone.

Patients underwent enhanced axial CT imaging of the brain, and scans were reviewed by a consultant neuroradiologist by using Mango software (http://ric.uthsc-sa.edu/mango/). All patients reviewed by specialists were invited for ophthalmologic examination, including retinal imaging. Images were reported by ophthalmologists.

Table 1. Criteria used to select patients for assessment in study of severe neurologic sequelae among Ebola virus disease survivors, Sierre Leone*

Major selection criteria	Minor selection criteria
Focal weakness	Headache
Tremor	Insomnia
Altered sensation	Weakness
Vision loss	Loss of appetite
Deafness	Blurred vision
Anxiety	Dizziness
Confusion	
Depression	
Psychosis	
Inability to balance	
Auditory disturbance	
Tinnitus	
Double vision	

*Patients were selected for inclusion in a preliminary clinic examination if they exhibited ≥1 major or ≥2 minor criteria.

Statistical Analysis

We collected data on paper forms structured for clinical use, entered it into Microsoft Excel 2011 (Microsoft, Redmond, WA, USA), and edited it for missing information. We analyzed data by using Stata version 14.0 (StataCorp LLC, College Station, TX, USA). For sample sizes ≥35, we calculated 95% CIs for proportions by using an exact binomial method. Unadjusted odds ratios were calculated for binary and ordinal variables. We used the Wilcoxon rank sum test for comparison of continuous data and the Fisher exact test for categorical data. For multivariable logistic regression of factors associated with attending or not attending the preliminary clinic, we used a predetermined model with age (linear term), sex, and presence of major or minor criteria as explanatory variables. EBOV PCR cycle threshold (C_t) (a figure inversely representative of plasma viral load, with >40 cycles used as a negative cutoff value) was not included in the regression models because different laboratories used different thresholds.

This study was reviewed in accordance with University of Liverpool human subjects review procedures and determined to be a nonresearch public health response activity. Ethics approval was confirmed in writing from the Sierra Leone Ethics and Scientific Review Committee. All data collection instruments were stored in a secured location, accessible only by study staff. Personal identifiers were removed from the database before analysis.

Results

Of 361 patients, 5 patients were excluded because clinical data were incomplete and 22 because they were <12 years of age. Of the 334 included patients, 161 (49.7%,

95% CI 44.1%–55.3%) were female and 163 (50.3%, 95% CI 44.7%–55.9%) male; sex was not recorded for 10 patients. Median patient age was 28 (IQR 23.0–37.0) years. A total of 111 (33.2%, 95% CI 28.2%–38.6%) patients were eligible for the preliminary clinic; 32 (9.6%, 95% CI 6.6%–13.3%) patients had 1 major criteria, 74 (22.2%, CI 95% 17.8%–27.0%) had ≥2 minor criteria, and 12 (3.3%, 95% CI 1.7%–5.8%) were referred by clinic staff. A total of 40 (12.0%, 95% CI 8.7%–15.9%) patients attended the clinic (Figure 1). Among the 334 patients evaluated, the most common symptoms were headache (167, 50.0%, 95% CI 44.5%–55.5%), loss of appetite (33, 9.9%, 95% CI 6.9%–13.6%), and generalized weakness (22, 6.6%, 95% CI 4.2%–9.8%) (Figure 2). Female patients were more likely to be invited to the preliminary clinic than were male patients (OR 2.01, 95% CI 1.22–3.32; p = 0.03) (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/8/17-1367-Techapp1.pdf). In those invited to the preliminary clinic, on multivariable analysis, the presence of minor criteria was associated with nonattendance (OR 0.10, 95% CI 0.03–0.56; p = 0.005) (online Technical Appendix Table 2).

Of the 40 patients attending the preliminary clinic, 26 (65%, 95% CI 48.3%–79.3%) were female, and the median age was 32 (IQR 25–43) years. Patients were seen in the clinic a median of 430 (IQR 401–473) days after the first positive diagnostic results. At the time of preliminary clinic, 35 (87.5%, 95% CI 73.2%–95.8%) had neurologic or psychiatric symptoms (Table 2). None reported any substantial medical history of neurologic or mental health disorder. Of the 40 patients, 19 (47.5%, 95% CI 31.5%–63.9%) were defined as having severe neurologic signs and symptoms and were offered referral to the joint neurologic and psychiatric clinic, brain CT, and retinal imaging. An additional 5 patients were referred for psychiatric review only. We found no significant difference in demographic or acute EVD features between patients with and without severe neurologic features (Table 3). A greater proportion of patients with severe

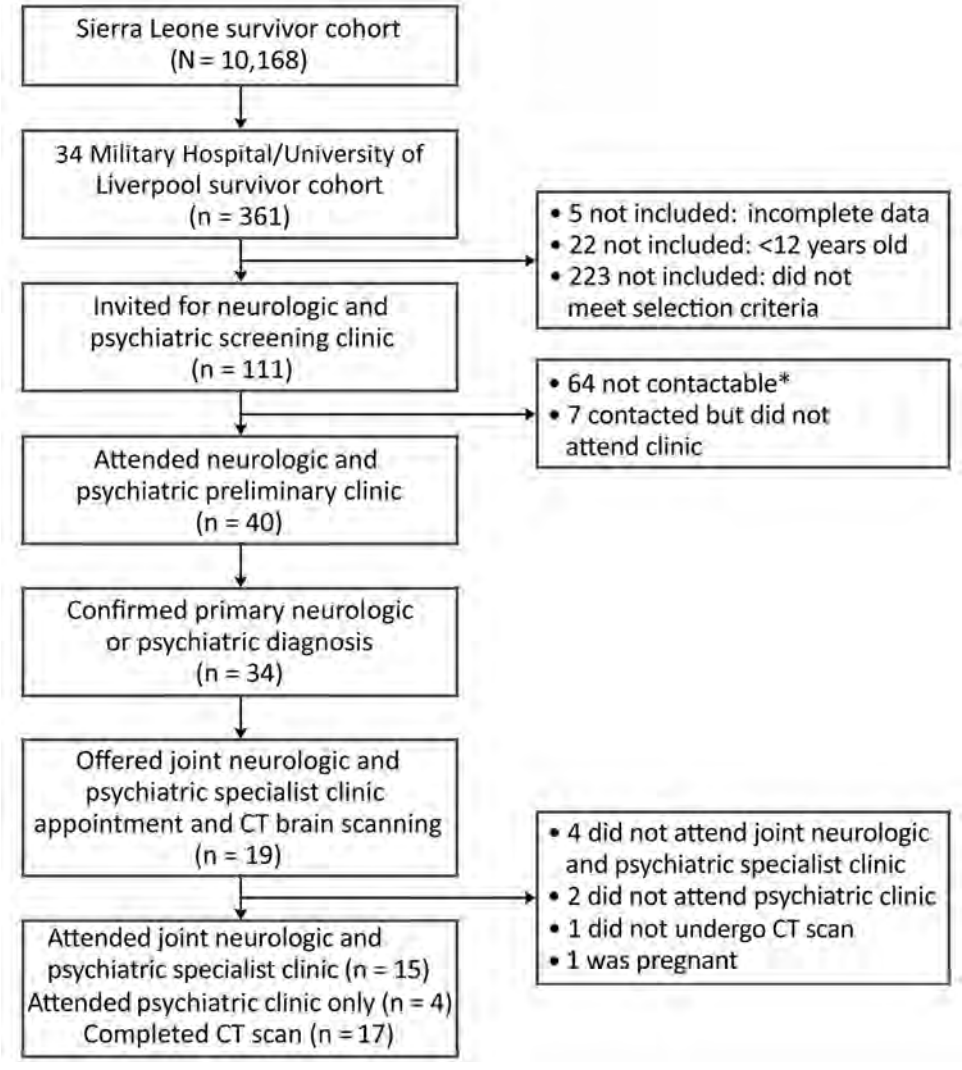

Figure 1. Flowchart showing clinic referral process from initial patient cohort to preliminary clinic and then specialist clinics in study of severe neurologic sequelae among Ebola virus disease survivors, Sierre Leone. Criteria for selection for preliminary clinic assessment from the 34 Military Hospital/University of Liverpool cohort were presence of ≥1 major or ≥2 minor criteria (see Table 1) or nurse-led selection on the basis of symptoms. CT, computed tomography. *Indicates telephone number was not available or telephone was repeatedly switched off.

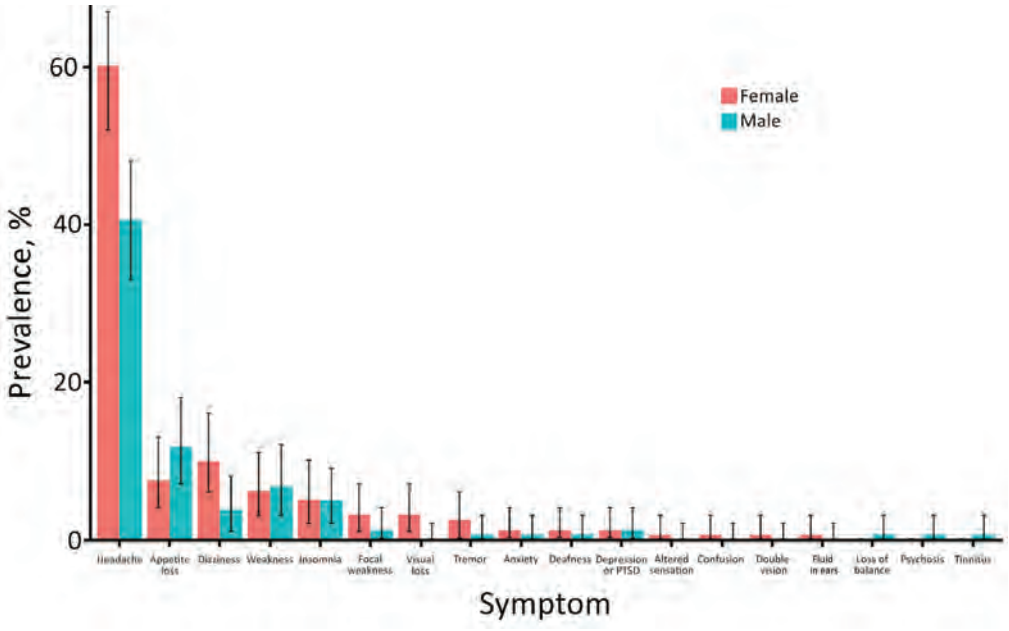

Figure 2. Prevalence of neurological symptoms by sex in study of severe neurologic sequelae among Ebola virus disease survivors, Sierre Leone. Cohort consisted of 24 survivors attending the 34 Military Hospital/University of Liverpool survivors clinic. Error bars indicate 95% CI. PTSD, Posttraumatic stress disorder.

neurologic symptoms were unconscious during any point in admission to the ETU, but this association was weak (OR 3.32, 95% CI 0.79–15.40; p = 0.11). Due to data sparsity, multivariable analysis was not performed.

Clinical Features

In the preliminary clinic, a new or different headache since acute EVD admission was reported by 30 (75.0%, 95% CI 58.8%–87.3%) patients; female:male ratio was 2:1. Of those with headache, 14 (46.6%, 95% CI 38.3%–65.7%) had undifferentiated headache, 13 (43.3%, 95% CI 25.5%–62.6%) migraine, and 3 (10.0%, 95% CI 2.1%–26.5%) tension-type headaches (online Technical Appendix Table 3). Five patients who had migraine headaches were prescribed oral propranolol (20 mg 1×/d), in keeping with WHO guidance on survivor care (*23*); 4 returned for follow-up 1 month after treatment and reported symptomatic improvement.

One male and 1 female survivor, both 42 years of age, had evidence of stroke; symptom onset occurred at the time of acute EVD. These patients had the highest disability scores (WHO Disability Assessment Schedule 2.0 scores 89.58 and 33.33, respectively) and met criteria for a mental health disorder (see Case Study 1). Given the major vessel territory distribution on CT, these strokes are suspected to be mature ischemic infarcts.

Two survivors had peripheral sensory neuropathy and 2 focal peripheral nerve lesions. Brachial plexopathy was diagnosed in a 27-year-old woman during acute EVD. Neuropathy screening of the patient for treatable causes was negative, and she was referred for physiotherapy. Asymmetric glove and stocking peripheral sensory neuropathy was diagnosed in a 35-year-old man, occurring since ETU discharge. Diabetes and major depressive disorder were diagnosed, and he was referred to the diabetes and mental health clinic. Other reported neurologic symptoms in the cohort included 3 cases of tinnitus, 2 cases of tremor, and 1 case of asymmetric lower limb atrophy with weakness of unknown etiology. Of the 19 patients who attended the specialist clinic, 12 were reviewed 1 year later, in June 2017; 10 reported improvement of symptoms, 1 reported no changes, and 1 reported a new headache. After this, case-study patient 1 died.

Psychiatric symptoms were common among 21 (52.5%, 95% CI 36.1%–68.4%) survivors describing difficulty sleeping; 12 (30.0%, 95% CI 16.5%–46.5%) described depressive symptoms and 11/40 (27.5%, 95% CI 14.6%–43.9%) anxiety symptoms (online Technical Appendix Table 4). Of 24 (60.0%, 95% CI 43.3%–75.1%) survivors referred for psychiatric review, 19 (47.5%, 95% CI 31.5–63.8%) attended the clinic. Of those, 16 (63.3%) required referral for local mental health follow-up, of whom 5 met criteria for mental disorder (2 generalized anxiety disorder; and 3 major depressive disorder). The most common reasons for mental health referral were stigma, grief, and loss of employment. Of the 19 patients who attended the psychiatric clinic, median MMSE score was 93.3% (IQR 87.7%–96.3%). No patient reported suicidal ideation.

Among 19 survivors assessed for disability, the median WHO-DAS 2.0 score was 8.3% (IQR 3.1%–13.5%) corresponding to the 69th percentile of the normative population. The 9 patients who had a disability score >10 (corresponding to scores found in <27.65% of the normative population) included all survivors affected by mental health disorders, stroke, and peripheral neuropathies for which disabilities were assessed. The most severe case of disability is described in Case Study 2.

Table 2. Demographics, diagnoses, and management and outcome of 35 Ebola virus disease case-patients in whom neurologic and psychiatric conditions were diagnosed at preliminary and specialist neurologic and psychiatric clinics, Sierre Leone*

Patient no.	Age, y/sex	Diagnoses	Management and outcome
1	21/M	Migraine headache, psychosocial issues	MH follow-up
2	47/M	Resolved migraine headache, left retinal detachment	Review at 1 y: no change in symptoms
4	33/M	Migraine headache	DNA specialist clinic
5	54/F	Psychosocial issues, undifferentiated headache	Referred to psychiatry for assessment but did not attend
6	18/F	Undifferentiated headache	Referred return to general survivor's clinic
7	21/F	Tension-type headache, major depressive disorder	Local MH follow-up
8	29/F	Undifferentiated headache	Referred return to general survivor's clinic
9	26/F	Migraine headache	Referred to MH for assessment but did not attend Review at 1 y: improvement in symptoms
10	27/F	Right brachial plexus neuropathy	Physiotherapy. Review at 1 y: substantial improvement in weakness
11	42/F	Right striatocapsular infarct, generalized anxiety disorder	Physiotherapy, MH follow-up
13	58/F	Undifferentiated headache	Referred return to general survivor's clinic for nonneurologic and other symptoms
14	38/M	Possible anterior uveitis, undifferentiated headache	Ophthalmology referral
15	49/F	Tension-type headache	Referred return to general survivor's clinic for nonneurologic symptoms
16	31/F	Migraine headache	Propranolol 20 mg/d; symptoms improved (unable to quantify)
17	51/F	Undifferentiated headache, peripheral sensory neuropathy	Referred return to general survivor's clinic for nonneurologic symptoms
18	32/F	Tinnitus, anterior uveitis	Ophthalmology referral. MH follow-up. Review at 1 y: improvement in tinnitus, now occasional
19	38/M	Undifferentiated headache	Local MH follow-up
20	30/F	Resolved migraine headache	Review at 1 y: new onset headache with cluster-type features
21	32/F	Migraine headache, right eye cataract, tinnitus	Ophthalmology referral
22	21/F	Migraine headache, tinnitus	Propranolol 20 mg/d. Headache improved from 8/10 to 4/10. Review at 1 y: no further headache
23	46/M	Essential tremor, undifferentiated headache	DNA specialist clinic
24	43/F	Migraine headache	Propranolol 20 mg/d, initially 10/10 headache pain now better (not able to quantify). Review at 1 y: decreased frequency of headaches, now occasional
25	42/M	Extensive right MCA infarct, major depressive disorder	Physiotherapy, MH follow-up. Review at 1 y: improvement in symptoms. Patient subsequently died.
26	25/F	Ulnar nerve palsy	DNA specialist clinic
27	25/M	Migraine headache, asymmetric lower limb muscle wasting	MH follow-up. Review at 1 y: decreased frequency of headaches, now occasional
28	21/F	Tension-type headache	Review at 1 y: decreased frequency of headaches; now occasional. Fever/rash during pregnancy; miscarriage
29	61/F	Migraine headache, bilateral cataract	Local MH follow-up
30	19/F	Anterior uveitis, undifferentiated headache	Urgent referral to local ophthalmology clinic
31	33/F	Migraine headache, generalized anxiety disorder	Propranolol 20 m/d; improved headache from 10/10 to 6/10. MH follow-up
32	43/F	Undifferentiated headache, arthralgia	Referred to local ophthalmology clinic
33	41/F	Migraine headache, anxiety	MH follow-up, simple analgesia. Review at 1 y: decreased frequency of headaches, now occasional
34	25/F	Undifferentiated headache	Referred to general survivor's clinic
35	35/M	Migraine headache, asymmetric sensory peripheral neuropathy, major depressive disorder	MH follow-up, propranolol 20 mg/d, gabapentin 300 mg each night; diet advice and review in diabetic clinic referral. Headache improved (unable to quantify); pain in feet improved. Review at 1 y: decreased frequency of headaches, now occasional; improvement in neuropathy
37	12/F	Severe neurocognitive impairment, postviral encephalitis	Referral to orphanage for 24-h care
38	21/M	Undifferentiated headache, arthralgia	ND

*MH, mental health; MCA, middle cerebral artery; ND, no data.

Of 17 patients who underwent brain CT, abnormalities were shown for 7. Three scans showed evidence of cerebral or cerebellar atrophy that was atypical for patient age (Figure 3, panel A), 2 confirmed the clinical assessment of stroke (Figure 3, panel B), and 2 showed evidence of calcification, differentials of which include previous focal hemorrhage occurring ≥1 year before the scan.

Of the 40 survivors evaluated at the prelimnary clinic, 12 described eye pain (30.0%, 95% CI 16.6%–46.5%) and 8 (20.0%, 95% CI 9.1%–35.6%) described partial

Table 3. Demographics, clinical characteristics during acute admission, and cycle threshold of preliminary clinic group in study of severe neurologic sequelae among Ebola virus disease survivors, by those who had severe and those who had no severe neurologic conditions, Sierre Leone*

Characteristic	No severe neurologic features, n = 21	Severe neurologic features, n = 19	Crude odds ratio† (95% CI)
Age, y, median (IQR)	28 (23–60)	32 (25–42)	0.01 (0.00–0.036)/y
Female sex, % (95% CI)	48 (43–54)	68 43–87	2.3 (0.79–7.60)
Length of stay, d, median (IQR)	18 (14–28)	25 (13–29)	0.02/d
Seizures during admission, % (95% CI)	19 (5–42)	21 (6–46)	1.13 (0.18–7.23)
Unconscious during admission, % (95% CI)	33 (15–57)	63 (38–83)	3.32 (0.79–15.4)
Bleeding during admission, % (95% CI)	19 (5–42)	5 (0.1–26)	0.24 (0.00–2.80)
Cycle threshold, median (IQR)	22.8 (22.1–24.1), n = 9	27.2 (22.5–30.1), n = 10	0.22 (0.7–1.3) for each increment

*Severe conditions were those requiring specialist referral.
†Odds ratio of patients having severe neurologic features compared with those who did not.

visual loss. Of 17 patients who attended the ophthalmology specialist clinic for examination, and wide field-scanning laser ophthalmoscope imaging, 3 (17.6%) had Ebola retinal lesions (Figure 3, panels C, D) (*11*). One survivor had unilateral retinal detachment, 1 intermediate uveitis, and 1 posterior subcapsular cataract suggestive of previous uveitis.

Case Studies

Case Study 1—Patient No. 25

Patient no. 25 was a previously fit and well 41-year-old male soldier who had an uncomplicated 8-day acute admission to a hospital for treatment of EVD; 3 days after discharge, he had sudden onset of left-sided weakness and dysphasia. In the neurology clinic, 545 days after his admission for acute illness, examination was consistent with a right upper motor neuron lesion. His MMSE was 26/27 and WHO-DAS 2.0 score 89.58, conforming to significant disability. He exhibited a pervasive low mood, anhedonia, feelings of worthlessness, guilt, frustration, and hopelessness regarding the future because of disability. His CT results showed extensive gliosis within the left middle cerebral artery territory, in keeping with mature infarct (Figure 3, panel B.). Retinal imaging showed bilateral Ebola retinal lesions (Figure 3, panels C, D). Stroke and major depressive disorder were diagnosed. He was referred for physiotherapy, which resulted in marked improvement in symptoms, and received mental health clinic follow-up. Approximately 1 year after the intial clinic visit, the patient had an undifferentiated fever; serum from a blood sample tested EBOV PCR negative, but he died several days later.

Case Study 2—Patient No. 37

A 12-year-old girl who had a normal developmental history had a C_t of 27.9 at hospital admission for EVD; she improved with treatment and became serum EBOV PCR negative on days 15 and 17. On day 20, her consciousness level gradually declined and fever recurred; she then had recurrent seizures for 48 hours that were partially controlled by administration of phenytoin and diazepam. Her consciousness level gradually improved over the next 4 weeks to spontaneously alert but confused. At the preliminary clinic, 454 days after acute admission, she was blind and had substantial hearing loss and severe cognitive impairment. She was doubly incontinent and required 24-hour care for all activities of daily living. Her CT results showed disproportionate parietal and temporal lobe atrophy (Figure 3, panel C). CSF test results were EBOV negative; results of a specialist's ophthalmology review were unremarkable. Planning for her complex care needs required multiagency and multidisciplinary coordination to find an orphanage and provide resources and training to that facility to help manage her needs. She was unable to attend the specialist neurology clinic because of the remote location of her orphanage. Follow-up visits to the orphanage from the medical, psychiatric, and therapies team found no major functional improvements.

Discussion

Previous studies have outlined the frequency of a variety of neurologic symptoms in EVD survivors (*13*). Our specialist case series from the 34MH survivor's cohort confirms the presence of central and peripheral nervous system disorders and found these to be associated with a broad range of disability. The most frequent neurologic diagnosis was migraine headaches; the next most common, respectively, were stroke, peripheral sensory neuropathy, and focal peripheral nerve lesions. Most survivors had co-occurring mental health problems, the most frequent psychiatric diagnoses being major depressive disorder and generalized anxiety disorder. The most severely affected patients had symptoms of blindness, deafness, focal weakness, and cognitive dysfunction associated with disability and mental illness.

The diagnosis of migraine headache found in 13 case-patients was characterized by intermittent, throbbing headaches associated with photophobia, phonophobia, and, in some cases, vomiting. These symptoms were either new or substantially worse after acute EVD. In a small group, treatment with propranolol according to WHO guidelines (*23*) led to subjective improvement. To date, headaches in the EVD survivor population have not been well

described; a small group of survivors was noted to have unilateral and throbbing headaches (*19*), although frequency from the 2014–2016 West Africa Ebola disease outbreak ranges 22%–68% (*14*,*19*,*20*,*25*), In the only case–control study in which 90% of survivors reported headache, a high prevalence of 75% in the control population meant this finding was not significant (*16*). A recent meta-analysis reported a community migraine prevalence of 5.6% (95% CI 4.6%–6.7%) in community-based studies in Africa (*26*). Because our preliminary clinic selection criteria required patients with headache to have ≥1 associated symptom, our headache findings and prevalence may not be representative of the survivor population. Potential mechanisms for migraine headache in EVD survivors may include autonomic dysregulation (*27*), changes in tryptophan-serotonin levels after infection (*28*), or ongoing neuroinflammation, as seen in HIV infection (*29*). With limited diagnostic methods, we are unable to determine specific etiologies of all neuropathy or suspected myopathy cases; however, diabetic neuropathy, entrapment neuropathy, or critical illness polyneuropathy with slow recovery are potential causes.

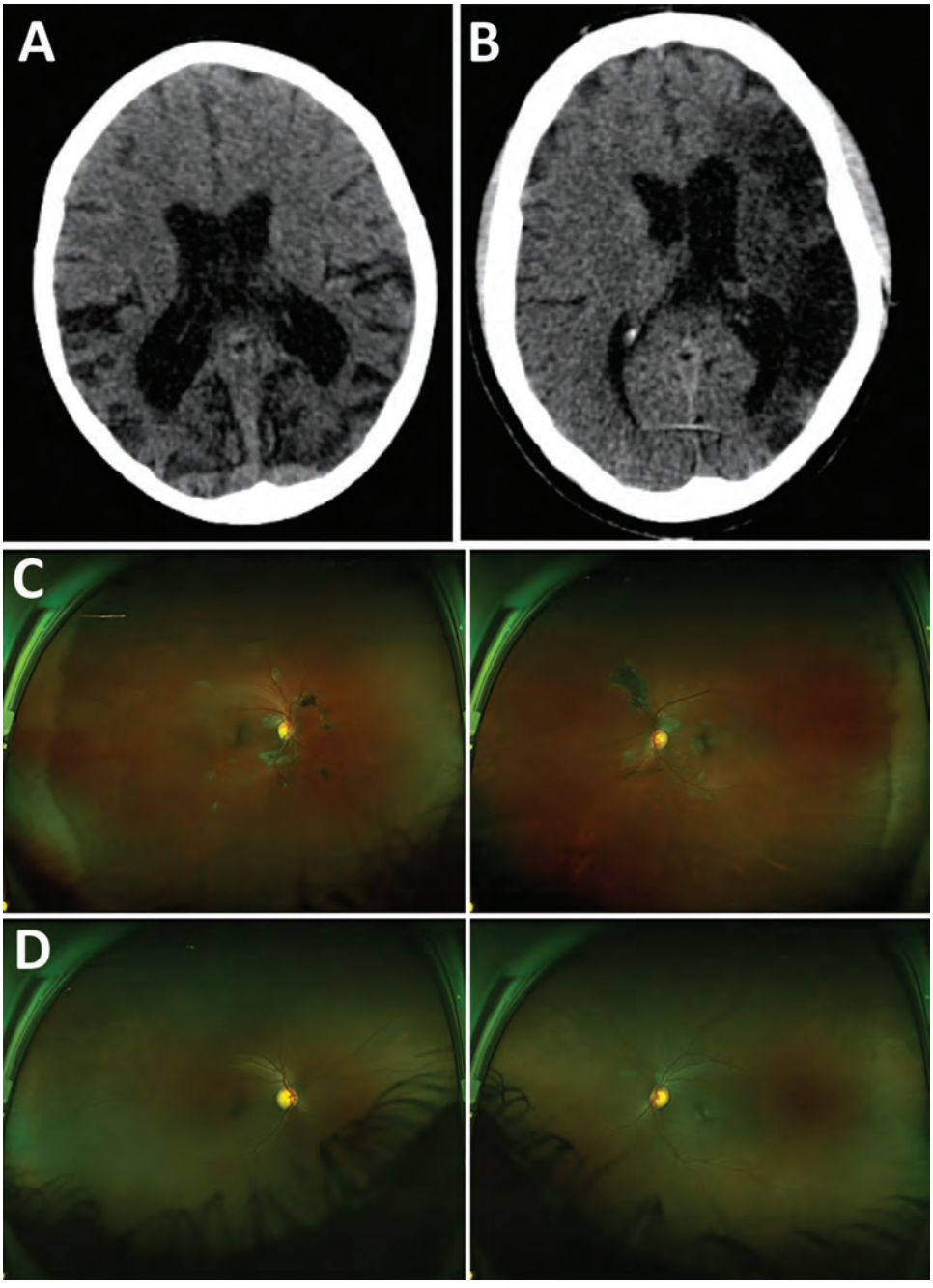

Figure 3. Representative nonenhanced computed tomography (CT) brain scans and composite scanning laser ophthalmoscope fundus images of 2 Ebola virus disease survivors attending a joint neurologic and psychiatric clinic in Sierre Leone. A) Patient no. 37, female, age 12. CT of brain shows disproportionate parietal and temporal lobe atrophy. B) Patient no. 25, male, age 42. CT of brain shows extensive gliosis within the left middle cerebral artery territory reflecting an old infarct with ex-vacuo dilatation of left lateral ventricle due to hemispheric volume loss. C) Patient no. 12, age 40. Retinal imaging shows right and left eye, with extensive bilateral peripapillary pale retinal lesions and pigmentation of larger lesions. Lesions appear to spare the fovea. Visual acuity was 20/25 (right) and 20/20 (left) (*24*). D) Patient no. 25, male, age 42. Retinal imaging shows right and left eye, with peripapillary pale retinal lesions. Visual acuity was 20/25 in both eyes (*24*).

Diagnostic imaging showed sequelae of focal or generalized atrophy or stroke in some patients. As previously reported (*5*,*12*), we found substantial cerebral atrophy in 2 patients and isolated cerebellar atrophy in 1 other survivor. One patient had a reported case of late onset encephalitis (*3*), and 1 patient's imaging correlated with substantial cognitive deficit, cortical blindness, and hearing impairment (see Case Study 2). Although it is possible the atrophy was related to birth complications, nutritional deficiency, or childhood illness, the prominent parietal and temporal lobe atrophy of this adolescent case-patient resembles radiologic findings in subacute sclerosing panencephalitis, a chronic CNS infection caused by defective measles virus, raising the possibility of similar CNS mechanisms of EVD and measles or persistent CNS infection (*30*). Cerebral CT images of 2 stroke case-patients, whose neurologic symptom onset occurred during acute EVD, were consistent with ischemic stroke. Suspected stroke during acute EVD has been reported (*31*), and thromboelastography, a measurement of thrombotic tendency, done during and after acute EVD illness, suggests a prothrombotic period in the immediate aftermath of EVD (*32*).

In 3 (15.8%) of 19 patients in the severe neurologic features group, we observed the novel Ebola peripapillary retinal lesion, recently reported by Steptoe et al. (*11*), who described a similar prevalence (14.6%) among a wider survivor population. Although the most likely mechanism of CNS viral entry is from circulating infected cells, the presence of retinal peripapillary lesions, thought to represent virus spread along the retinal nerve fiber or ganglion cell axon layers, raises the possibility of CNS viral entry by neuronal spread.

The group of patients who had severe neurologic features generally had good results from adapted MMSE testing. For a patient who had a confirmed case of late-stage EVD encephalitis and initial neurocognitive impairment (*3*), assessment 1 year later showed good long-term recovery. This finding is encouraging and in keeping with 2 case reports of recovery from neurocognitive impairment (*33*). Despite onset being 1 year after acute disease and many patients having been initially referred to counselors, 5 of 19 patients met criteria for psychiatric disorder, all 19 had concurrent physical symptoms, and 16 required mental health follow-up. As previously reported, survivors cited stigma, grief, and loss of employment as major stressors impeding recovery (*17*,*34*).

A recent case–control study found survivors had major limitations of vision, cognition, affect, and, most markedly, mobility (*35*). In our study, we found 10 participants who reported high levels of disability and also had physical symptoms and co-occurring mental health issues. This clustering of physical and psychiatric sequelae and disability suggests a subset of patients most affected after acute EVD and with the greatest care needs. In the small number of self-selecting case-patients on whom we followed up 18 months after the first neurologic/psychiatric clinic, patients generally reported symptomatic improvement; however, improvement was not uniform. One case-patient subsequently died (patient no. 25; see Case Study 1) and another remains dependent for all activities of daily living (patient no. 37; see Case Study 2).

Our study observed no association between severe neurologic conditions and admission C_t. To the contrary, among the 2 patients who had both prolonged periods of unconsciousness and cerebral atrophy on CT (patients no. 2 and 16), the neurologic episodes occurred late in the acute disease period, not at the time of peak viral load. Similarly, 2 case reports describe a prolonged meningoencephalitic stage of disease or meningoencephalitis occurring months after recovery (*4*,*5*). Of note, we found no cases of CNS infection recurrence. Unconsciousness during acute admission was more common among those who had severe neurologic symptoms on follow-up, although not to a significant degree, possibly caused by limited sample size (OR 3.32, CI 0.79–15.4; $p = 0.11$). Our preliminary group was selected on the basis of existing neurologic symptoms, which precludes a conclusion of causation and generalization to the wider EVD survivor population.

A major limitation of our case series is that we cannot firmly determine causation between our findings and the diagnosis of EVD beyond the temporal association. Furthermore, in keeping with other observational studies, a lack of reliable countrywide denominator data on conditions such as headache or stroke means we cannot assess the representativeness of our results. Validating our findings would require a large case–control study, in which our data could be used as a basis for study design. Retrospectively asking about acute symptoms incurs the possibility of recall bias; however, as acute records of the EBV outbreak clinics are sparse and linkage-challenging, this represented the most viable option. Despite our multiple attempts, the outcomes of 71/111 patients who were invited to but did not attend the preliminary clinic remain unknown. Although our analysis shows those with minor selection criteria were among those less likely to attend ($p = 0.005$), it is still possible we underrepresented patients who had more disabling conditions and were unable to access the service, as exemplified by the patient in Case Study 2. Further research should focus on a complete characterization of pathways of sequelae and persistent infection (*36*).

Our case series, supported by brain CT imaging, confirms there are long-term neurologic sequelae in EVD survivors and a substantial proportion of these patients have ongoing mental health problems and disability. Often, these issues cluster together, and services should therefore seek out and support patients with a high burden of illness. If we wish to expand specialist services to the remaining EVD survivors and broader population, the only credible and sustainable option is to greatly increase support for in-country specialist training of doctors.

Acknowledgments

We thank the study participants, the Sierra Leone Association of Ebola Survivors; the staff at 34 Military Hospital; the Main Outpatients Department, Radiology Department, and administration, Connaught Hospital; and the Sierra Leone Research Ethics Committee for their guidance.

This research was supported by the Wellcome Enhancing Research Activity in Epidemic Situations Grants (reference: 107779/Z/15/B) and the National Institute for Health Research (NIHR) Health Protection Research Unit in Emerging and Zoonotic Infections at University of Liverpool in partnership with Public Health England, in collaboration with Liverpool School of Tropical Medicine. Follow-up neurological assessments were completed as part of the Ministry of Health and Sanitation's Comprehensive Package for Ebola Survivors program, funded from USAID through JSI Research & Training Institute, Inc. The funding source had no direct involvement in investigational design, conduct, data collection, data analysis, data interpretation, and writing of the report. The corresponding author had full access to the study data. All authors share the responsibility for the submission for publication. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, the Department of Health, or Public Health England.

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Unilateral Phrenic Nerve Palsy in Infants with Congenital Zika Syndrome

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Release date: July 11, 2018; Expiration date: July 11, 2019

Learning Objectives

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

- Evaluate clinical features and course in 4 infants with congenital Zika syndrome and diaphragmatic paralysis, based on a case series
- Assess the significance of diaphragmatic paralysis in these infants with congenital Zika syndrome, based on a case series
- Determine the significance of arthrogryposis in these infants with congenital Zika syndrome, based on a case series.

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Since the first identification of neonatal microcephaly cases associated with congenital Zika virus infection in Brazil in 2015, a distinctive constellation of clinical features of congenital Zika syndrome has been described. Fetal brain disruption sequence is hypothesized to underlie the devastating effects of the virus on the central nervous system. However, little is known about the effects of congenital Zika virus infection on the peripheral nervous system. We describe a series of 4 cases of right unilateral diaphragmatic paralysis in infants with congenital Zika syndrome suggesting peripheral nervous system involvement and Zika virus as a unique congenital infectious cause of this finding. All the patients described also had arthrogryposis (including talipes equinovarus) and died from complications related to progressive respiratory failure.

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DOI: https://doi.org/10.3201/eid2408.180057

Zika virus is a neurotropic flavivirus transmitted primarily by *Aedes* spp. mosquitoes (*1*,*2*). Vertical transmission results in congenital infection, the full spectrum of effects of which are yet to be completely defined. The devastating central nervous system (CNS) effects of congenital Zika virus infection have been described in multiple publications since the onset of the recent outbreak in Brazil in 2015 (*3*–*5*). Neural progenitor cells have been identified as a primary viral target in the CNS (*6*,*7*). Defects in neuronal proliferation, migration, and differentiation have also been shown to play a major role in the pathogenesis of congenital Zika syndrome (CZS) (*6*,*7*). Aside from a known association with Guillain-Barré syndrome, the effects of Zika virus infection on the peripheral nervous system (PNS) remains poorly understood, especially in the context of congenital infection (*8*). We describe 4 cases of right unilateral diaphragmatic paralysis in infants with CZS.

Methods

We obtained clinical, laboratory, and radiologic data from a chart review of patients with diagnoses of CZS who were noted to have unilateral elevation of the diaphragm on chest radiograph. We defined cases of CZS by both the presence of clinical features suggestive of CZS and the detection of Zika virus RNA, Zika virus neutralizing antibodies, or both in serum samples, cerebrospinal fluid (CSF) samples, or both. We tested for Zika virus RNA using the Zika Virus RNA Qualitative Real-Time RT-PCR test (Focus Diagnostics, San Juan Capistrano, CA, USA) on serum and urine in patient 1 and by the CDC Trioplex RT-PCR assay in patients 2–4 (*2*). We screened all patients for Zika virus IgM by using an IgM antibody capture ELISA (MAC-ELISA) that had received US Food and Drug Administration emergency use authorization; we tested samples from patient 1 by the InBios ZIKV Detect MAC-ELISA (InBios International Inc., Seattle, WA, USA) and tested samples from patients 2–4 by the CDC Zika MAC-ELISA (*9*,*10*). Following US CDC recommendations, all specimens that screened positive for IgM to Zika virus were confirmed by PRNT (Table). We classified the HC of all patients according to the International Fetal and Newborn Growth Consortium (INTERGROWTH-21st) to determine z-scores (*11*). We defined severe microcephaly as HC z-score <–3 for sex and gestational age. We defined arthrogryposis as ≥2 joint contractures involving the upper and/or lower limbs.

Cases

Patient 1

A female infant was delivered at 38 weeks' gestation via cesarean section for breech presentation to a 27-year-old previously healthy primigravida woman. The mother reported having an illness characterized by mild fever, myalgia, arthralgia, and a generalized pruritic maculopapular rash at 4–6 weeks' gestation, while the mother was living in rural Guatemala (Table). The illness lasted 5–7 days and was self-limited. The mother did not seek medical attention during that time, and no diagnostic testing was performed.

At 30 weeks' gestation, the mother immigrated to the United States. Fetal ultrasound at 34 weeks' gestation revealed severe fetal growth restriction (below the second percentile), severe microcephaly (>5 SD below the norm, estimated to be 12 weeks delayed), and diffuse intracranial calcifications. The mother declined amniocentesis; noninvasive prenatal screening for aneuploidy was negative. Maternal serologic testing was negative for dengue virus (DENV) and Zika virus IgM and negative for evidence of recent infection with cytomegalovirus, rubella, varicella-zoster virus, syphilis, *Toxoplasma gondii*, HIV, and parvovirus B19.

The infant had a birthweight of 2,020 g and a head circumference of 27.5 cm (z-score –4.3), in keeping with severe microcephaly. Arthrogryposis involving the hips, knees, ankles, and elbows was observed, along with bilateral talipes equinovarus. The infant was intubated shortly after birth because of poor respiratory effort. A computed tomography (CT) scan of the head showed severe microcephaly with intracranial volume loss, including thinning of the cortical mantle, and callosal and pontocerebellar hypoplasia with ex vacuo ventriculomegaly. Multiple dystrophic bandlike calcifications were seen along the corticomedullary junction and periventricular white matter, as well as within the basal ganglia and brainstem. Optic nerves were diminutive. Overriding cranial sutures, prominent occipital shelf, and scalp rugae were confirmatory for fetal brain disruption sequence. Chest radiography revealed a markedly and persistently elevated right hemidiaphragm (Figure, panel A). Molecular testing for Zika virus RNA on infant serum and urine specimens obtained shortly after birth were negative. Lumbar puncture could not be performed safely. Serum Zika virus IgM was positive. Plaque reduction neutralization testing (PRNT) was positive for both Zika virus (titer ≥1:1,280) and DENV-1 (at the cutoff titer of 1:10) but negative for DENV-2. Molecular testing of multiple formalin-fixed paraffin-embedded placental tissue samples for Zika virus RNA was performed by the US Centers for Disease Control and Prevention (CDC), and results were negative. The infant was extubated on day of life 2 and died from progressive respiratory failure on day of life 13. The family declined autopsy.

Patient 2

A female infant was born at 40 weeks' gestation to a 21-year-old primigravida mother from Brazil via cesarean section for breech presentation. The mother reported

Table. Summary of maternal and infant characteristics in 4 cases of congenital Zika syndrome in infants who had unilateral elevation of the diaphragm*

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4
Maternal characteristics				
Maternal age, y	27	21	17	21
Prenatal care	Yes	Yes	Yes	Yes
Zika virus symptoms, trimester	1st	1st	1st	1st
Fever	+	–	+	–
Rash	+	+	–	+
Arthralgia	+	–	–	–
Abnormal fetal ultrasound, trimester	3rd	3rd	2nd	3rd
Infant characteristics				
Delivery type	Cesarean	Cesarean	Vaginal	Cesarean
Sex	F	F	F	F
Gestational age, wk	38	40	39	41
Birthweight, g	2,020	2,025	2,565	2,075
Length, cm	40	42	46	41
HC at birth, cm	27.5	28.5	28	28
z-score	–4.3	–4.3	–4.2	–4.9
Arthrogryposis	Hips, knees, ankles, elbows	Hips, ankles, wrists	Hips, ankles, wrists	Hips, ankles, wrists
Talipes equinovarus	+	+	+	+
Head imaging findings	V, C, H	V	V, C, H	V, C
Elevated right hemidiaphragm	+	+	+	+
Cause of death	Respiratory failure	Respiratory failure	Respiratory failure	Respiratory failure
Day of life	13	10	86	4
Maternal testing for Zika virus				
RT-PCR				
Amniotic fluid	NA	NA	+	NA
Serum	NA	NA	–	–
IgM, serum	–	NA	–	–
Infant testing				
RT-PCR for Zika virus				
Serum	–	–	–	–
CSF	NA	–	–	+
Urine	–	NA	NA	NA
Placenta	–	NA	NA	NA
Zika virus IgM				
Serum	+	+	+	+
CSF	NA	+	+	+
PRNT titer				
Zika virus	>1:1,280	180†	897†	270†
DENV-1	1:10	<20	<20	<20
DENV-2	<1:10	<20	<20	<20

*C, calcifications; CSF, cerebrospinal fluid; DENV, dengue virus; H, cerebral hypoplasia; HC, head circumference; NA, not available; PRNT, plaque reduction neutralization test; RT-PCR, reverse transcription PCR; V, ventriculomegaly; +, positive finding/test result; –, negative finding/test result.
†Indicates 50% plaque reduction neutralization test titer.

having an illness characterized by rash but no fever during the first trimester of pregnancy (Table). Fetal ultrasound performed during the third trimester indicated microcephaly, ventriculomegaly, cerebral hypoplasia, and intracranial calcifications. Amniocentesis and maternal serologic testing were not performed. At delivery, the infant had a birthweight of 2,025 g and head circumference of 28.5 cm (z-score –4.3) and was noted to have arthrogryposis involving the hips, ankles, and wrists, as well as bilateral talipes equinovarus. Chest radiograph revealed a persistently elevated right hemidiaphragm (Figure, panel B); a head ultrasound confirmed the prior fetal intracranial findings. Infant serum and CSF samples were positive for Zika virus IgM (Table), but Zika virus RNA was not detected in serum or CSF samples. The infant died from respiratory failure on day of life 10.

Patient 3

A female infant was born at 39 weeks' gestation to a 17-year-old primigravida mother from Brazil via spontaneous vaginal delivery (*12*). The mother reported having a febrile illness without rash during the first trimester of pregnancy (Table). Fetal ultrasound performed during the second trimester showed microcephaly, ventriculomegaly, cerebral hypoplasia, and intracranial calcifications. An amniocentesis was performed at 29 weeks' gestation, and Zika virus RNA was detected in the amniotic fluid. The infant's birthweight was 2,565 g, and head circumference at birth was 28.2 cm (z-score –4.2). The neonate was noted to have arthrogryposis involving the hips, wrists, and ankles, as well as bilateral talipes equinovarus. A head ultrasound confirmed the prior fetal intracranial findings, and chest radiograph revealed an elevated right hemidiaphragm

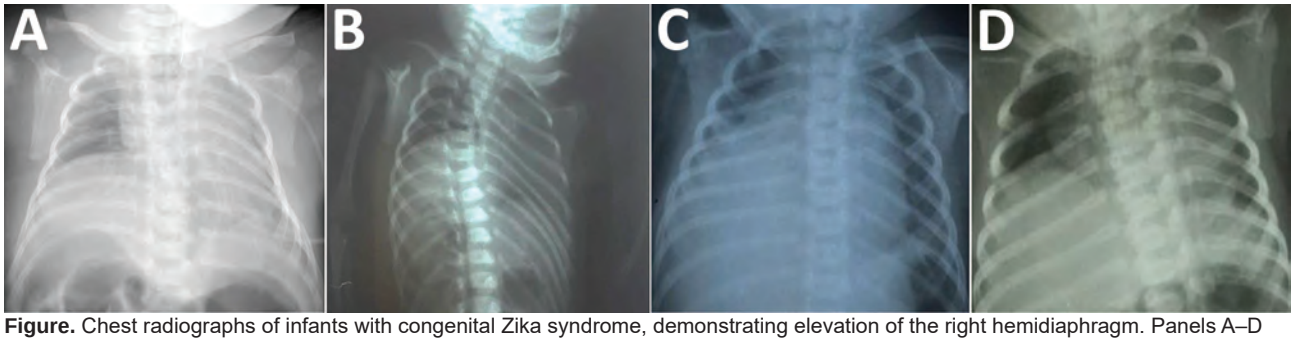

Figure. Chest radiographs of infants with congenital Zika syndrome, demonstrating elevation of the right hemidiaphragm. Panels A–D represent patients 1–4, respectively. In each instance, only the right hemidiaphragm was noticeably elevated. All patients also had arthrogryposis (including talipes equinovarus) and died from complications related to progressive respiratory failure.

(Figure, panel C). Zika virus IgM was detected in infant serum and CSF samples (Table), but Zika virus RNA was not detected in serum or CSF samples. The infant died from respiratory failure on day of life 86.

Patient 4

A female infant was born at 41 weeks' gestation to a 21-year-old primigravida mother from Brazil via cesarean section for breech presentation. The mother reported having an afebrile illness with rash during the first trimester of pregnancy (Table). Fetal ultrasound performed during the third trimester showed microcephaly, ventriculomegaly, and intracranial calcifications. No amniocentesis or maternal serologic testing was performed. The infant had a birthweight of 2,075 g and head circumference of 28 cm (z-score –4.9); arthrogryposis involving the hips, wrists, and ankles; and bilateral talipes equinovarus. Chest radiograph revealed an elevated right hemidiaphragm (Figure, panel D), and the infant required noninvasive respiratory support. Zika virus IgM as detected in both serum and CSF samples (Table). Molecular testing for Zika virus RNA on a serum sample was negative, but Zika virus RNA was detected in a CSF sample. No head imaging could be performed before the infant's death from respiratory failure on day of life 4.

Discussion

We report a series of 4 patients with CZS and right unilateral diaphragmatic paralysis suggesting PNS involvement in CZS and Zika virus as a unique congenital infectious cause of this finding. All the patients were female infants born at term to primiparous mothers who reported symptoms suggestive of Zika virus infection during the first trimester of pregnancy. All infants in this case series had severe microcephaly and arthrogryposis and died from progressive respiratory failure.

A recent study of Zika virus–infected macaques reported evidence of viral tropism to the peripheral nerves (*13*). Cases of acute Zika virus infection associated with peripheral sensory neuropathy in a child and an adult also have been published (*14*,*15*). Guillain-Barré syndrome, which is typically a postinfectious phenomenon, is known to involve the phrenic nerve, causing diaphragmatic paralysis in adults, but has not been described in neonates (*8*). Unilateral diaphragmatic paralysis in adults resulting from presumed postviral phrenic neuropathy has been described after infection with varicella zoster virus, poliovirus, West Nile virus, HIV, and DENV but has not been described in newborns or in association with any known congenital infections (*16*–*21*).

Arthrogryposis observed in CZS is thought to be of neurogenic origin, with involvement of both upper and lower motor neurons, resulting in restricted fetal movement and the consequent development of joint contractures (*22*). A recent case series demonstrated thinning of the entire spinal cord and brainstem hypoplasia on magnetic resonance imaging in infants with CZS and arthrogryposis (*23*). These findings were also associated with more severely reduced conus medullaris anterior roots and more frequent periventricular calcifications when compared with infants with CZS without arthrogryposis (*23*). Recent postmortem examinations of 2 neonates with CZS and arthrogryposis also established the presence of Zika virus and associated tissue injury in the spinal cord (*24*). Taken collectively, these results suggest that infants with CZS and arthrogryposis may represent a more severely affected subgroup reflecting earlier fetal infection and possibly more severe interruption in neuronal migration, cortical organization, or both.

Our results indicated that congenital Zika virus infection appears to be an infectious cause of congenital unilateral diaphragmatic paralysis (*9*,*12*,*25*). All patients in this series had arthrogryposis and severe microcephaly, suggesting an association of unilateral diaphragmatic paralysis with severe manifestations of CZS. The precise mechanism(s) by which diaphragmatic paralysis occurs, and why it is consistently unilateral and right-sided in these cases, remains unknown. Potential mechanisms include abnormal diaphragmatic innervation secondary to early interruption of neuronal migration; direct, viral-mediated phrenic nerve or spinal cord injury; or, less likely, a demyelinating neuropathy or other immune-mediated process.

Respiratory insufficiency and subsequent failure have not been commonly reported in CZS; the prominence of respiratory difficulties in the cases presented here is likely secondary to impaired diaphragmatic function. Unilateral diaphragmatic paralysis may represent a unique risk factor for death in infants with CZS, considering that all patients in this case series died within the first 3 months of life (3 of 4 within the first 2 weeks of life). In a previous report of a large cohort (n = 87) of neonates with CZS, only infants with unilateral diaphragmatic paralysis (n = 3; patients 2–4 in this report) died before hospital discharge, and all required respiratory support following birth (*9*).

The long-term prognosis of infants with CZS and persistent diaphragmatic paralysis who survive the immediate postnatal period is unknown. The true prevalence of this finding is also unknown, because chest radiography has not been routinely recommended for all infants with CZS (*26*,*27*). However, recent guidelines recommend considering diaphragmatic paralysis in infants with CZS who develop respiratory distress or failure or are unable to be weaned from ventilator support (*27*).

Limitations of this study include that the chest radiograph findings could not be confirmed by dynamic imaging studies of the diaphragm (e.g., ultrasound or fluoroscopy) because of patient instability and the risks associated with patient transport to a radiology suite in all the cases presented. Electrodiagnostic studies (electromyography/nerve conduction studies) and nerve biopsies to further investigate the underlying mechanism of the diaphragmatic paralysis also could not be obtained, for similar reasons. The differential diagnosis of right unilateral hemidiaphragm elevation includes diaphragmatic eventration, a hepatic mass, or decreased lung volume (pulmonary hypoplasia, atelectasis); however, there was no evidence of these abnormalities on examination or other imaging studies and, aside from some reports of pulmonary hypoplasia, they have not been previously reported in patients with CZS (*28*,*29*). Bilateral diaphragmatic involvement is also a possibility but may not be obvious on radiographic studies given the presence of the cardiac silhouette on the left side. Autopsies could not be completed in any of the cases presented to exclude these possibilities because of the lack of parental consent.

This case series of infants with CZS, arthrogryposis, and unilateral diaphragmatic paralysis describes a unique constellation of clinical findings that has not been described with other congenital infections. The presence of both arthrogryposis and unilateral diaphragmatic paralysis suggests likely involvement of the PNS, spinal cord, or both in these severely affected infants. The presence of diaphragmatic paralysis may also represent a risk factor for early death in infants born with CZS. Further studies to elucidate the precise mechanism leading to diaphragmatic paralysis in these patients are required.

About the Author

Dr. Rajapakse is a senior associate consultant in pediatric infectious diseases at the Mayo Clinic in Rochester, Minnesota, USA. Her research interests include antimicrobial stewardship, antimicrobial resistance, and emerging infectious diseases.

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Epidemiology of *Diphyllobothrium nihonkaiense* Diphyllobothriasis, Japan, 2001–2016

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Release date: July 13, 2018; Expiration date: July 13, 2019

Learning Objectives

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

- Assess the parasitology of diphyllobothriosis
- Analyze the epidemiology of diphyllobothriosis
- Evaluate the clinical presentation of diphyllobothriosis
- Distinguish the most common treatment for diphyllobothriosis

CME Editor

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Authors

Disclosures: ***Hiroshi Ikuno, BS; Shinkichi Akao, PhD;*** *and* ***Hiroshi Yamasaki, PhD****, have disclosed no relevant financial relationships.*

We report 958 cases of cestodiasis occurring in Japan during 2001–2016. The predominant pathogen was *Diphyllobothrium nihonkaiense* tapeworm (n = 825), which caused 86.1% of all cases. The other cestode species involved were *Taenia* spp. (10.3%), *Diplogonoporus balaenopterae* (3.3%), and *Spirometra* spp. (0.2%). We estimated *D. nihonkaiense* diphyllobothriasis incidence as 52 cases/year. We observed a predominance of cases during March–July, coinciding with the cherry salmon and immature chum salmon fishing season, but cases were present year-round, suggesting that other fish could be involved in transmission to humans. Because of increased salmon trade, increased tourism in Japan, and lack of awareness of the risks associated with eating raw fish, cases of *D. nihonkaiense* diphyllobothriasis are expected to rise. Therefore, information regarding these concerning parasitic infections and warnings of the potential risks associated with these infections must be disseminated to consumers, food producers, restaurant owners, physicians, and travelers.

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DOI: https://doi.org/10.3201/eid2408.171454

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In Japan, the occurrence of soil-transmitted helminthiases declined sharply in 1949 (*1*). However, foodborne parasitic infections, which are closely associated with the Japanese food custom of eating raw fish, have remained. Diphyllobothriasis caused by the adult tapeworm *Diphyllobothrium nihonkaiense* (proposed as *Dibothriocephalus nihonkaiensis* in 2017) (*2*), an infection closely associated with the consumption of raw Pacific salmon, is the most frequently occurring foodborne parasitic infection in Japan. Paleoparasitologic studies have revealed that diphyllobothriasis has existed in Japan for ≈1,000 years (*3*).

Adult *D. nihonkaiense* tapeworms are ribbon-like and composed of a slender and spatulated scolex (2.4–2.8-mm long and 1.2–1.5-mm wide) with paired slit-like bothria, neck (14.4–16.8-mm long and 1.16–1.28-mm wide), and strobila comprising numerous proglottids (*4*) (Figure 1). The *D. nihonkaiense* tapeworm is parasitic in mammals; brown bear, domestic dog, and humans are their definitive hosts (*5*,*6*). Inside humans, the parasite can grow ≥10 m in length. Adult worms lay millions of eggs, and these eggs are excreted in feces. The *D. nihonkaiense* tapeworm, as well as other diphyllobothriid species, uses 2 intermediate hosts to complete its life cycle (*4–6*). The first intermediate host (species in which the procercoid develops) is probably brackish zooplanktonic copepods (*7*). The first intermediate host is consumed by the second intermediate host, Pacific salmonids, namely cherry salmon (*Oncorhynchus masou*), chum salmon (*O. keta*), and pink salmon (*O. gorbuscha*) (*8–10*). In the second intermediate host, procercoids develop into plerocercoids, the larval form needed to infect the definitive host (e.g., humans). *D. nihonkaiense* infections are generally asymptomatic or induce relatively mild symptoms, such as mild diarrhea and abdominal pain (*5*,*6*,*11*).

In Japan, the causative agent of diphyllobothriasis has long been considered to be the tapeworm *Diphyllobothrium latum* (proposed as *Dibothriocephalus latus* in 2017) (*2*), ever since the first case of diphyllobothriasis reported in 1889 (*12*). This belief has caused confusion over diagnostics; whether the cases of diphyllobothriasis reported in Japan in the past were caused by *D. latum* tapeworm or another species was debatable (*13*). However, in 1986, Yamane et al. (*4*) identified the causative agent of diphyllobothriasis as the *D. nihonkaiense* tapeworm from Japan, which is morphologically and ecologically distinct from the *D. latum* tapeworm from Finland. This finding was further verified by DNA analyses (*14–16*).

In Japan, all infections, including parasitic infections, linked to the consumption of food should be reported to health authorities as food poisoning, in accordance with the Ordinance for Enforcement of the Food Sanitation Act of 2012. However, despite diphyllobothriasis being the most frequent parasitic infection in Japan, no cases have been duly reported. Thus, diphyllobothriasis epidemiology has been estimated by using only case reports published in journals and the number of outpatients in hospitals (*5*,*17*).

The Department of Bacteriology of BML Inc. (Kawagoe, Saitama, Japan) routinely identifies parasites and diagnoses parasitic infections as requested by physicians from the medical institutions of Japan. During 2001–2016, we examined 632 proglottid samples and 326 egg samples from 958 patients with cestodiasis (Table). In this article, we report the etiologic agents associated with cestodiasis, focusing on diphyllobothriasis, the predominant type of cestodiasis in Japan. We describe the geographic distribution of *D. nihonkaiense* diphyllobothriasis cases and demographic characteristics of patients with this infection. Perspectives of diphyllobothriasis are also discussed.

Identification of Etiologic Agents of Tapeworm Infections

Proglottid and egg samples were collected from patients with diphyllobothriasis and taeniasis in hospitals in Japan.

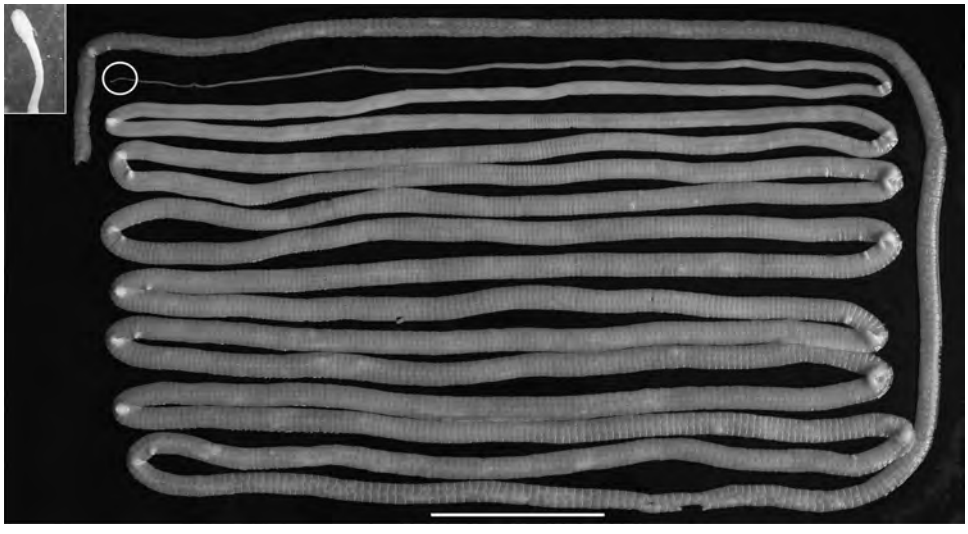

Figure 1. Adult *Diphyllobothrium nihonkaiense* tapeworm expelled from patient, Japan, 2008. Circle indicates the scolex (enlarged in the inset). Scale bar indicates 10 cm.

Table. Cestode species identified in patient fecal samples, Japan, 2001–2016

Species	No. samples		Total, no. (%)†
	Proglottid (no.)*	Egg	
Diphyllobothrium nihonkaiense	526 (153)	299	825 (86.1)
Diplogonoporus balaenopterae	32 (8)	0	32 (3.3)
Spirometra spp.	2	0	2 (0.2)
Taenia spp.	72 (18)	27	99 (10.3)
Total	632 (179)	326	958 (100)

*Number in parentheses indicates the number examined by molecular analysis during 2012–2016.
†Numbers do not add up to 100% because of rounding.

Proglottids were fixed in formalin solution by hospital staff and sent to BML Inc.'s general laboratory for species identification. Almost all proglottids were not attached to a scolex; only 26 (20 diphyllobothriids and 6 taeniids) proglottids had a scolex attached. Fecal samples containing eggs were also collected by hospital staff and sent to BML Inc. These samples were not fixed with formalin; the eggs in the fecal samples were concentrated, and the species were identified on the basis of morphology and egg size. Two *Spirometra* plerocercoids removed surgically from a subcutaneous nodule in the abdomen of 1 patient and a subcutaneous nodule in the ankle of another patient were also received as formalin-fixed samples. We identified proglottids by their morphologic and morphometric markers, such as length and width of mature proglottids and ratio, number, shape, and position of the hermaphrodite genitalia; we also noted the shape and size of the scolex (if available) and the eggs in the uterus (*4*).

During 2012–2016, we identified 179 proglottid samples (161 diphyllobothriids and 18 taeniids) using molecular methods (Table); restriction fragment length polymorphism analysis with PCR-amplified cytochrome *c* oxidase subunit 1 (*cox1*) gene fragment (249-bp long corresponding to base pairs 880–1128) was introduced to confirm diphyllobothriid species (*18–20*) and PCR-amplified *cox1* sequencing (145-bp long corresponding to base pairs 641–785) was used for identification of taeniid species (*20*). To amplify the *cox1* gene fragments, we used paired primers 5′-ACAGTGGGTTTAGATGTAAAGACGGC-3′ (forward) and 5′-AGCTACAACAAACCAAGTATCATG-3′ (reverse) for diphyllobothriids (*19*) and 5′-AATTTAGTTCTGCGTTTTTTTGATCC-3′ (forward) and 5′-CTTATWCTRAAACATATATGACTAAT-3′ (reverse) for taeniids (*20*).

Of the 958 cestode samples we examined, 825 (526 proglottid and 299 egg, 86.1%) were *D. nihonkaiense*, 32 (3.3%) were *Diplogonoporus balaenopterae* (proposed as *Diphyllobothrium balaenopterae* in 2017) (*2*), 2 (0.2%) were *Spirometra* spp., and 99 (10.3%) were *Taenia* spp. (Table). Of the 179 diphyllobothriid proglottids with which we performed restriction fragment length polymorphism, 153 were confirmed as *D. nihonkaiense* (Figure 2) and 8 as *Dip. balaenopterae*. Of the 18 taeniid proglottids we tested by *cox1* sequencing, 16 were *Taenia saginata*, 1 was *T. solium*, and 1 was *T. asiatica*.

Regarding *Spirometra* plerocercoids, 2 species (*S. erinaceieuropaei* and *S. decipiens*) have been found to be responsible for human sparganosis in Japan (*21*). However, these 2 species were not identified by DNA analysis in this study.

D. nihonkaiense Diphyllobothriasis

Annual and Seasonal Occurrence

We analyzed the 825 diphyllobothriasis cases attributed to *D. nihonkaiense* infection for their annual and seasonal occurrence. *D. nihonkaiense* diphyllobothriasis occurred persistently, although the frequency varied over the years of the study (Figure 3). Using our data, we estimated that 52 *D. nihonkaiense* diphyllobothriasis cases occurred per year in Japan. The rate of *D. nihonkaiense* diphyllobothriasis cases estimated by examining reports in the literature was ≈40 cases/year (*17*). However, the actual rate is probably much higher and has been estimated to be 100–200 cases/year (*17*).

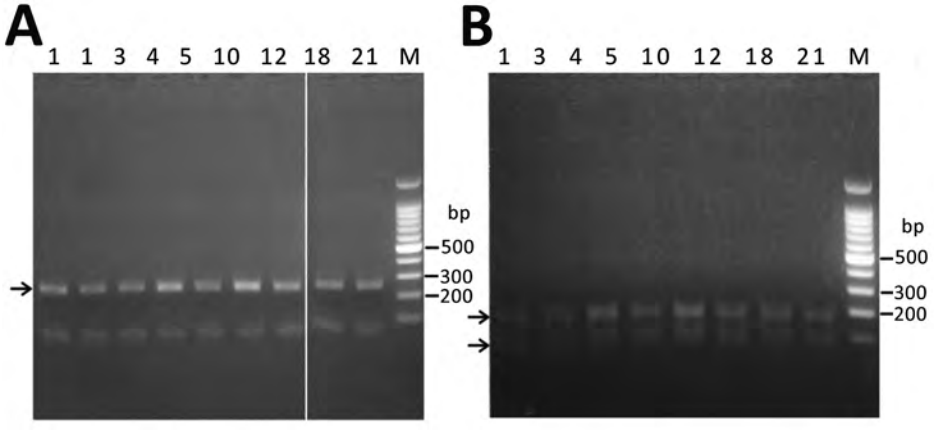

Figure 2. Molecular identification of *Diphyllobothrium nihonkaiense* species by restriction fragment length polymorphism analysis of PCR-amplified *cox1* gene fragments, Japan, 2012–2016. Number above each lane indicates the number of proglottids in the sample. A) Digestion of *cox1* gene fragments (249 bp, arrow) with *Age*I. The leftmost lane is a mock digested sample. *D. nihonkaiense cox1* gene did not get cut by the *Age*I enzyme. B) Digestion of *cox1* gene fragments with *BspH*I. The 2 arrows indicate the DNA fragments (164 bp and 85 bp) resulting from the digestion. M, marker.

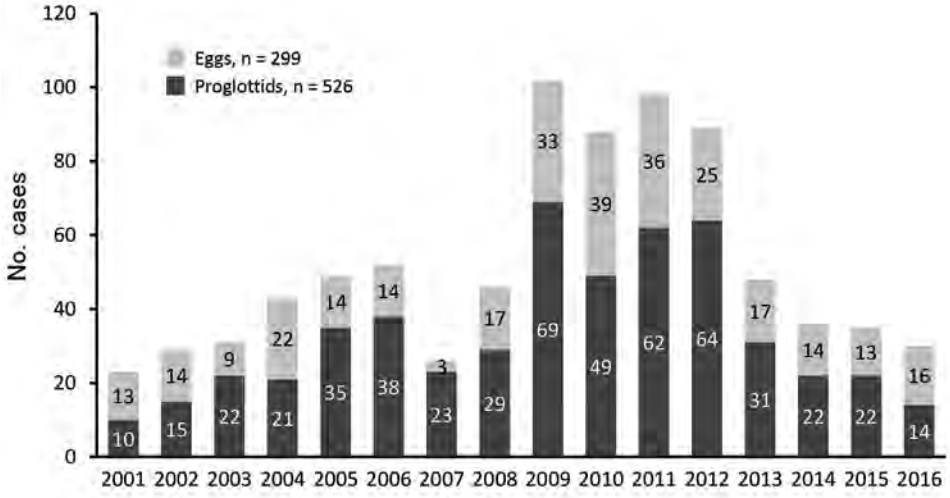

Figure 3. Number of cases of *Diphyllobothrium nihonkaiense* infection, by year, Japan, 2001–2016.

Although *D. nihonkaiense* diphyllobothriasis occurred throughout the year, the incidence was remarkably higher during March–July, showing a seasonal pattern of occurrence (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/24/8/17-1454-Techapp1.pdf). Considering that the prepatent period (time from start of infection to time infection is discovered, e.g., person notices strobila excreted in feces) is 2–4 weeks, patients probably acquired infective plerocercoids during February–June. This timing coincides with the season when cherry salmon and immature chum salmon are usually caught and sold. Although specifying the sources of infection is difficult, Pacific salmon are clearly implicated; cherry salmon are caught during March–May, and tokishirazu (i.e., immature chum salmon), which originate from the Amur River in Russia, are caught during May–July (*9*). *D. nihonkaiense* plerocercoids have not been found in akizake (i.e., mature chum salmon), which are not prevalent in fishing waters during February–June because they return to their natal rivers for spawning in autumn (*9*). However, considering that *D. nihonkaiense* diphyllobothriasis occurs throughout the year, akizake and other fish that salmonids eat might also be associated with the occurrence of diphyllobothriasis. Further study is necessary for elucidating this possibility.

Geographic Distribution of *D. nihonkaiense* Diphyllobothriasis

D. nihonkaiense diphyllobothriasis occurred widely (40/47 prefectures) throughout Japan, from Hokkaido Prefecture to Okinawa Prefecture. The regions where *D. nihonkaiense* diphyllobothriasis occurred most often were the populous cities of Tokyo and Saitama in the Kanto region (Figure 4), owing to their high consumption of raw Pacific salmon, followed by the Hokkaido Prefecture, Chubu region along the Sea of Japan, Tohoku region (where salmon are caught and consumed locally), and Kinki region (with populous prefectures, e.g., Osaka Prefecture). The incidence of *D. nihonkaiense* diphyllobothriasis was lower in the southern regions than in the northern regions.

From 1979 through the 1990s, diphyllobothriasis occurred mainly in Hokkaido Prefecture, Tohoku region, and along the coastal regions of the Sea of Japan, where salmon are caught and consumed locally (*22*). However, with the rapid advancement of food transportation systems and techniques to retain freshness, diphyllobothriasis spread from the northern parts of Japan to its big cities, such as Tokyo and Osaka, in which salmon consumption has been on the rise since the 1990s.

Demographic Analysis of Patients and Clinical Signs

During 2012–2015, we conducted a survey to investigate patient demographics. Of the 139 patients who participated, 136 indicated their sex: 85 (61%) were male and 51 (37%) were female. In total, 114 patients indicated their age; age ranged from 2 years to >90 years, but most patients (of either sex) were in the 20–60-year age range (online Technical Appendix Figure 2).

Most patients noticed they expelled strobilae when they defecated; for 8 patients, the strobilae were incidentally detected during colonoscopy. Of the 78 patients indicating clinical symptoms, 29 (37.1%) were asymptomatic. Light diarrhea occurred in 28 (34.0%) patients; abdominal pain in 18 (22.0%) patients; abdominal discomfort in 4 (4.9%) patients; and constipation, vomiting, and weight loss in 1 patient each. Most patients experienced mental distress over defecating and discharging proglottids.

Possible Sources and Locality of Infection

Regarding questions on the consumption of raw fish in the 2012–2015 patient survey, 12 of 15 patients replied that

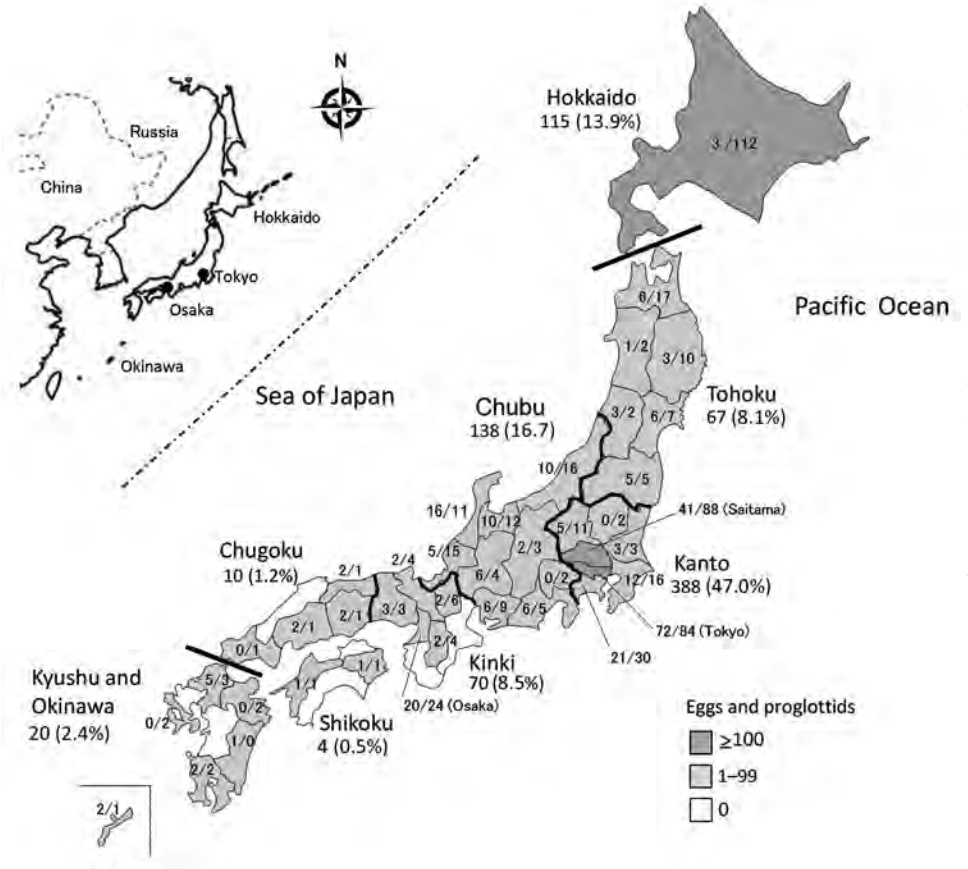

Figure 4. Geographic distribution of patients infected with *Diphyllobothrium nihonkaiense* tapeworm, by administrative region, Japan, 2001–2016. Thick lines indicate divisions between the 8 regions of Japan (Hokkaido, Tohoku, Kanto, Chubu, Kinki, Chugoku, Shikoku, and Kyushu and Okinawa). The total numbers of egg and proglottid samples and percentage of *D. nihonkaiense* infections are given per region. The percentages do not add up to 100% because of rounding. The numbers of egg/proglottid samples are given per prefecture. The prefectures of 13 (1.6%) of 825 patients were unknown; these patients were, therefore, not included. Inset map shows location of Japan in East Asia.

they had eaten dishes containing raw salmon, such as sushi and sashimi. However, the salmon species consumed could not be specified in all cases.

Seven patients had traveled abroad, some to multiple countries: 4 patients went to the United States; 2 patients to South Korea; and 1 patient each to Vietnam, Myanmar, the Netherlands, Belgium, and Italy. However, all 7 patients were considered to have been infected with *D. nihonkaiense* tapeworm in Japan because they had not consumed any kind of raw fish during travel.

The patients with diplogonoporiasis caused by *Dip. balaenopterae* infection were also all infected in Japan. The 17 cases of *T. saginata* and *T. solium* infection that occurred during 2012–2016 were all imported cases, but 1 case of *T. asiatica* infection was acquired in Japan through the consumption of raw pork liver.

Treatment and Prevention

Praziquantel is recommended as the first-choice anthelminthic drug for diphyllobothriasis (*23*), and this drug was used in all the cases in this study. To prevent recurrence, excretion of the scolex in the feces must be confirmed. If excretion is not detected, further observation of the feces for discharged proglottids or eggs is needed for 2–3 more months.

The most effective prevention method for diphyllobothriasis is to avoid the consumption of raw and undercooked Pacific salmon. If the salmon is cooked at 55°C or frozen at either –8°C for 12 hours or -10°C for 6 hours, plerocercoids in the salmon are killed, and their infectivity is lost (*24*). The US Food and Drug Administration recommends that fish be frozen at –35°C for 15 hours or -20°C for 7 days before consumption of raw or poorly cooked fish (*25*). However, this standard is difficult to achieve in Japan, considering the preference for and, thus, high consumption of traditional raw fish dishes.

In Japan, deep freezing has become a legal obligation to prevent infection with *Kudoa septempunctata* myxozoan parasite in flounder and *Sarcocystis fayeri* protozoon parasite in horse meat. However, this practice has not been implemented for the fishborne parasites *Diphyllobothrium* spp., *Dibothriocephalus* spp., and *Anisakis* spp.

Perspectives of Diphyllobothriasis

The number of diphyllobothriasis cases attributable to *D. nihonkaiense* infection is expected to rise in Japan, considering this pathogen's association with Japanese food customs. Also on the rise in Japan is tourism; in 2016, ≈24 million international travelers came to Japan, a 21.8% increase from the year before (http://www.jnto.go.jp/jpn/statistics/

visitor_trends/index.html). With the increase in numbers of persons traveling to Japan for sightseeing and business purposes, international travelers acquiring infections with *D. nihonkaiense* tapeworm via the consumption of Japanese foods made with raw salmon, such as sushi and sashimi, is of great concern. In fact, 1 case was reported in a visitor from China (*26*).

Infection with *D. nihonkaiense* tapeworm is no longer a public health problem limited to East Asia and the North Pacific coast of North America; this pathogen is spreading due to the globalization of trade and increased commerce with salmon. Several cases of infection with *D. nihonkaiense* tapeworm have been reported in Europe (*27*) and New Zealand (*28*), where this pathogen was previously absent. The sources of infection for these cases are suspected to be the salmon imported from North America (*27*). Furthermore, regardless of immunity, anyone can get infected with *D. nihonkaiense* tapeworm in the countries where the pathogen exists, such as Korea (*29*,*30*), China (*26*,*31*), the United States (*32*), Canada (*33*,*34*), and eastern Russia (*35*).

Besides infections with *D. nihonkaiense* tapeworm, the following rare and autochthonous cestodes have been sporadically reported in humans in Japan: *Diphyllobothrium stemmacephalum* (*36*), *Adenocephalus pacificus* (*37*), *Dip. balaenopterae* (*38*), and *Spirometra* spp. (*39*). In contrast, human taeniasis has been exclusively reported as imported cases, but *T. asiatica* infections in Japan have been confirmed to be autochthonous infections through the consumption of raw pork liver (*17*,*40*).

From the public health point of view, most of the population in Japan are still unaware of the risk for *D. nihonkaiense* infection associated with the consumption of raw salmon or the risks for infections with other cestodes. Therefore, information regarding parasitic infections and warnings of the potential risks associated with these infections must be disseminated to consumers, food producers, restaurant owners, physicians, and visitors.

Conclusions

D. nihonkaiense diphyllobothriasis is no longer a public health issue limited to only East Asia, including Japan, and North America but is becoming a global threat due to the increasing consumption of raw salmon worldwide. Since 2005, *D. nihonkaiense* diphyllobothriasis has been reported in Europe and New Zealand, where the disease has no endemic foci. Considering these global occurrences, anyone consuming salmon is at risk for *D. nihonkaiense* diphyllobothriasis, not only in Japan and along the North Pacific coast of North America, where local salmon is consumed, but also in Europe, where imported salmon is consumed. The effects of globalization, such as the expansion of the salmon market, the increase in travel to and from diphyllobothriasis-endemic countries, and the global change in eating habits, might cause an increase in the incidence of *D. nihonkaiense* infections worldwide, in places where diphyllobothriasis was previously present and in places where it was not.

Acknowledgments

The authors thank the staff members of the domestic medical institutions who participated in the survey.

This study was partially supported by grants-in-aid from the Ministry of Health, Labour and Welfare, Japan (H24-H26 Shinkosaiko-Ippan-016, to H.Y.), and the Research Program on Emerging and Re-emerging Infectious Diseases from the Japan Agency for Medical Research and Development (H27-H28 Kansensho-Jitsuyouka-Ippan, 40100703, to H.Y.).

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Hypoglycemic Toxins and Enteroviruses as Causes of Outbreaks of Acute Encephalitis-Like Syndrome in Children, Bac Giang Province, Northern Vietnam

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We investigated the cause of seasonal outbreaks of pediatric acute encephalitis-like syndrome associated with litchi harvests (May–July) in northern Vietnam since 2008. Nineteen cerebrospinal fluid samples were positive for human enterovirus B, and 8 blood samples were positive for hypoglycemic toxins present in litchi fruits. Patients who were positive for hypoglycemic toxins had shorter median times between disease onset and admission, more reports of seizures, more reports of hypoglycemia (glucose level <3 mmol/L), lower median numbers of leukocytes in cerebrospinal fluid, and higher median serum levels of alanine aminotransferase and aspartate transaminase than did patients who were positive for enteroviruses. We suggest that children with rapidly progressing acute encephalitis-like syndrome at the time of the litchi harvest have intoxication caused by hypoglycemic toxins, rather than viral encephalitis, as previously suspected. These children should be urgently treated for life-threatening hypoglycemia.

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DOI: https://doi.org/10.3201/eid2408.171004

Acute encephalitis syndrome is clinically characterized by fever, seizures, and altered mental status. This syndrome is a major public health concern in Asia; annually, >133,000 children are hospitalized with this pathology (*1*,*2*). Historically, the main etiology of acute encephalitis syndrome in Asia has been Japanese encephalitis, a vector-borne disease caused by a flavivirus (Japanese encephalitis virus), which causes >25% of cases of this syndrome in Asia. Many other viruses have been shown to cause acute encephalitis syndrome in Asia, such as enteroviruses (e.g., poliovirus, echovirus 9, enterovirus 71), and herpes simplex, measles, varicella zoster, rabies, dengue, Chandipura, and Nipah viruses.

However, for most cases of acute encephalitis syndrome, the specific etiology is unknown (*3*–*5*). Since introduction of Japanese encephalitis vaccine in the Expanded Program on Immunization in Asia (South Korea, China, Bangladesh, and Nepal) in 1997, a major shift has occurred; cases of Japanese encephalitis–attributable acute encephalitis syndrome have decreased, and cases of acute encephalitis syndrome not attributed to Japanese encephalitis have increased in countries with large vaccination coverage (*6*–*10*).

Such a shift has been observed in Vietnam, where the prevalence of Japanese encephalitis for hospitalized patients decreased from 50% in 1996 to 10% in 2009 (T.P. Nga, unpub. data). According to the Ministry of Health, 67% of the 1,800–2,300 cases of acute encephalitis syndrome reported each year occur in northern Vietnam, most

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often in Bac Giang Province (http://moh.gov.vn/Pages/Search.aspx?Key=japanese encephalitis in Vietnam). There has been a clear seasonal pattern of acute encephalitis-like syndrome in this province since 1999, with peaks in summer, particularly in young children (*11*). The symptomatology described by parents as rapid development of fever, headache, and nocturnal seizures explains the local name given to the disease (Ac Mong, meaning nightmare). Local populations had previously suggested a link with litchi cultivation because of observed synchronicity between outbreaks of acute encephalitis-like syndrome and litchi harvests.

A previous investigation of outbreaks of acute encephalitis-like syndrome in Bac Giang Province found that litchi cultivation appeared to be associated with acute encephalitis-like syndrome, but the link at the individual level remained unclear (*11*). Until 2007, results of all virologic investigations on patient samples remained inconclusive. We therefore performed next-generation sequencing (NGS) on cerebrospinal fluid (CSF) samples obtained since 2008 to identify unknown or unforeseen viruses. In addition, because hypoglycin A (HGA) and methylenecyclopropylglycine (MCPG) are suspected to be probable causes of similar outbreaks of acute encephalitis syndrome during litchi harvests in India and Bangladesh (*12–16*), we also tested serum samples for these toxins. These toxins are present in seeds and aril (flesh) of litchis (*17*,*18*) and are known to induce hypoglycemia in animal models (*19*).

Materials and Methods

Study Area and Outbreak Characteristics

Detailed characteristics for this study have been previously reported (*11*). In brief, Bac Giang Province is a rural province that has 1.6 million inhabitants and is located in northern Vietnam. The only hospital is located in Bac Giang City, the capital of the province. Outbreaks of acute encephalitis-like syndrome in Bac Giang Province are unusual and characterized by their specific location, strict seasonality, restricted age group, rapid progression to coma, and a higher case-fatality rate than that for Japanese encephalitis (*11*).

Study Design and Data Collection

We used surveillance data for case-patients with acute encephalitis-like syndrome admitted to the Bac Giang Provincial Hospital during 2008–2011. The case definition used for this syndrome was fever (temperature ≥37°5C reported by parents or at hospital admission), altered mental status or seizures, and no bacterial meningitis. We collected data from the Bac Giang Preventive Medicine Centre and the National Institute of Hygiene and Epidemiology (Hanoi, Vietnam). Patients ≤15 years of age, those who had onset of acute encephalitis-like syndrome during May 1–August 31, and those who had negative results for Japanese encephalitis virus IgM in CSF or were immunized against Japanese encephalitis virus were included in the study.

CSF and Blood Samples

For each seasonal outbreak that occurred during 2008–2011, blood samples were obtained from patients with acute encephalitis syndrome at admission to Bac Giang Provincial Hospital for standard biochemical and hematologic analysis. CSF samples from patients were also collected by physicians. Samples were cryopreserved in liquid nitrogen for transportation and stored at –80°C. Because most patients were young children, only small volumes (20 μL–300 μL) of CSF were available for most samples. Because of age of patients and local cultural practices, no brain biopsies or necropsies were performed for children who died of acute encephalitis-like syndrome.

Virologic Analyses

We conducted virus isolation in RD, Vero E6, or C6/36 monolayer cells and PCR for known viruses (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/17-1004-Techapp1.pdf). In addition, we also performed random NGS for 16 selected CSF samples that matched the case definition and were collected during 2008. We performed specific PCRs for contigs identified by NGS to confirm results, which also enabled comprehensive phylogenetic analysis by using other methods (online Technical Appendix). Primers (online Technical Appendix Table 1) were used to test CSF samples obtained from patients who had available clinical data (3 in 2008, 3 in 2009, 14 in 2010, and 21 in 2011).

Toxicologic Analysis

We tested 20 blood samples obtained during 2010–2011 (4 in 2010 and 16 in 2011, the only ones available from patients who had clinical data at the time when the hypoglycemic toxins hypothesis was proposed) for HGA and its metabolites and metabolites of MCPG by using a modified analytical method that has been reported (*20*,*21*). This method is based on ultra–high-performance liquid chromatography/tandem mass spectrometry.

Because these toxins can cause hypoglycemia by blocking the fatty acid β-oxidation pathway, we also measured concentrations of glycine and carnitine conjugates of short-to-medium chain length fatty acids in the same samples by using the same method. In addition, we quantified a spectrum of carnitine esters of 24 saturated and unsaturated fatty acids ranging from short to long chain molecules (C2–C18), including hydroxyl and dicarboxylic acids, by using tandem mass spectrometry without preceding chromatographic separation (*22*,*23*) (online Technical Appendix).

Statistical Analyses

We compared proportions of continuous variables across groups by using the Fisher exact test and distributions of continuous variables across groups by using the Mann-Whitney U test (R version 3.2.3; R Foundation for Statistical Computing, Vienna, Austria). We performed principal component analysis for age, number of days between symptoms and disease onset, glycemia at admission, number of leukocytes in CSF, and serum levels of liver enzymes to identify grouping of characteristics that might help differentiate between infectious and toxic causes of acute encephalitis-like syndrome. We conducted principal component analysis by using Qlucore Omics Explorer software (Qlucore, Lund, Sweden).

Ethics

Informed consent was obtained by physicians from parents of hospitalized children before sampling was conducted. The study protocol was reviewed and approved by institutional review boards at the National Institute of Hygiene and Epidemiology and the Institut Pasteur (Paris, France).

Results

A total of 185 children met the inclusion criteria over the study period (2008–2011). Median age was 5 years (interquartile range 2–8 years), and the sex ratio (male:female) was 1.4:1. The annual number of cases was higher in 2008 (70) and 2011 (61) than in 2009 (27) and 2010 (27) (Figure 1). Because of logistical constraints, CSF and blood samples were available for only 61 of the 185 children, of which 58 also had detailed clinical data. Therefore, these 58 patients represent the study population analyzed (Figure 1), including 10 patients from a previous study (*11*).

Virologic Analyses

NGS analysis of a pool of 16 CSF samples from the 2008 outbreak provided 116,615 nonhuman contigs from 61,291,294 nonduplicated reads with an average length of 70 nt. Among these contigs, 57 with an average length of 292 nt (range 103 nt–815 nt) matched the human enterovirus B species. Fourteen contigs were assigned to the human echovirus 30 species strain Zhejiang/17/03/CSF (GenInfo Identifier DQ246620) as best hit, with nucleotide identities ranging from 83% to 98%. The second most common reference strain matched (7 contigs assigned, with best-hit ranging from 78% to 88%) was human echovirus 33 strain Toluca-3 (GenInfo Identifier 34485451). Distinct contigs mapped at same genomic locations of these 2 reference genomes and suggested that the pool of samples presumably contained ≥4 different virus strains. PCRs using primers designed for these contigs confirmed their sequences, identified distinct viruses in the pool, and identified patients from which the sequences had been isolated.

We then conducted individual NGS on 4 selected CSF samples from the pool and acquired virus genome sequences after amplification by using specifically designed PCRs. This sequencing identified 4 distinct enterovirus genomes (120486, 120492, 120488, and 120495); the first 2 genomes were closely related (online Technical Appendix Figures 1–3). Prevalence of enterovirus infection was screened by PCR for CSF samples from patients with clinical data available and showed highly variable results (from 13/19 in 2008 to 4/21 in 2011) (Figure 2). Virus isolations were attempted for 10 CSF samples per annual outbreak; all showed negative results.

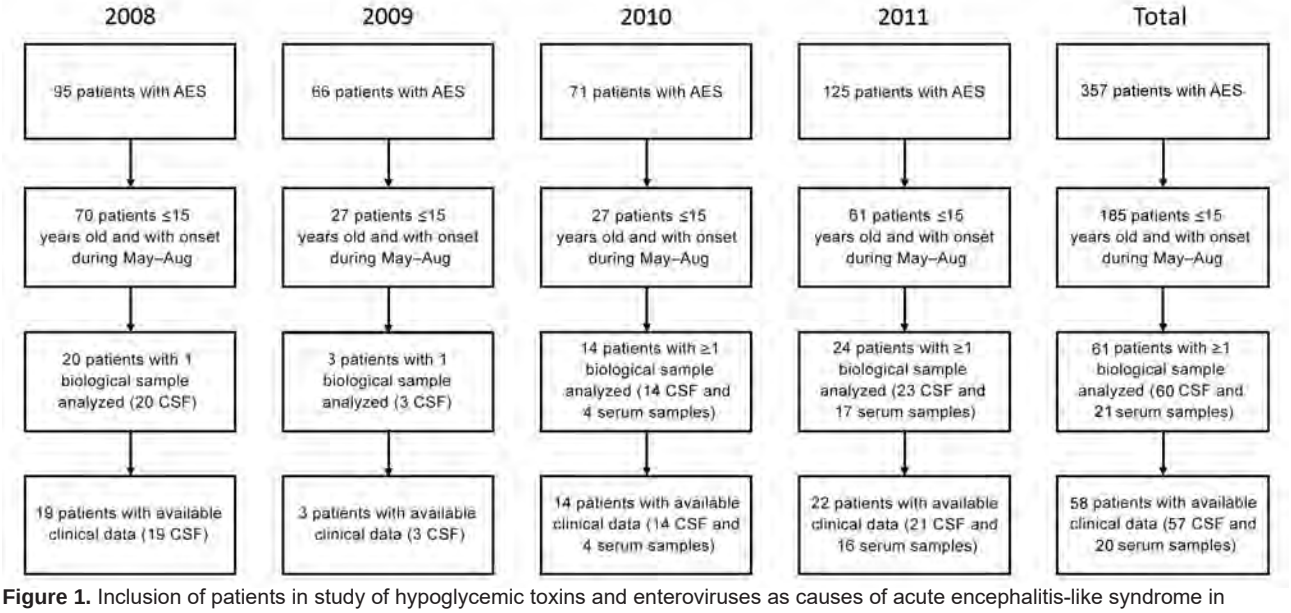

Figure 1. Inclusion of patients in study of hypoglycemic toxins and enteroviruses as causes of acute encephalitis-like syndrome in children, Bac Giang Province, northern Vietnam, 2008–2011. AES, acute encephalitis syndrome; CSF, cerebrospinal fluid.

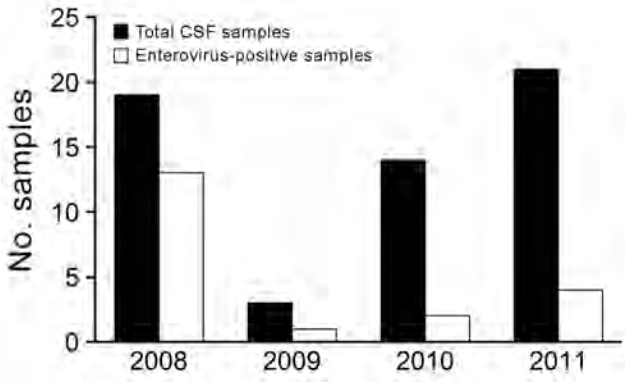

Figure 2. PCR-based prevalence of enterovirus infections per year in study of hypoglycemic toxins and enteroviruses as causes of acute encephalitis-like syndrome in samples (n = 57) from children, Bac Giang Province, northern Vietnam, 2008–2011. CSF, cerebrospinal fluid.

Toxicologic Analysis

Although enteroviruses accounted for most (68%) cases in 2008, these viruses accounted for <20% of cases in 2009–2011. Moreover, identification of multiple and distinct enterovirus strains did not correlate with the model of an epidemic diffusion that would otherwise explain seasonal outbreaks. Therefore, we were interested in exploring alternative explanations, including 2 candidate toxins, MCPG and HGA (*13–15*,*17*,*18*,*24*). Twenty blood samples (4 from 2010 and 16 from 2011), which were obtained near the time of onset of symptoms, were available for this analysis. After serum analysis (Figure 3; online Technical Appendix Table 2), we categorized children into 2 groups: 9 had high (>100 nmol/L) serum values of HGA and 11 had serum values of HGA below the lower limit of quantification (10 nmol/L), including 10 below the lower limit of detection (1 nmol/L).

All patients with high levels of HGA had quantifiable concentrations of methylenecyclopropylformyl (MCPF) carnitine, a metabolite of MCPG, and 6 of the 9 patients had detectable MCPF glycine, also derived from MCPG, although below the lower limit of quantification. Methylenecyclopropylacetyl conjugates, derived from HGA, were present in all of these samples, but concentrations did not reach quantifiable levels in all cases. The β-oxidation of fatty acids (online Technical Appendix Table 2) was shown to be inhibited in all patients with high serum levels of HGA. As a result, concentrations of glycine and carnitine conjugates of fatty acids of short to long chain length were increased. Increased concentrations were also detected for even and odd chain length acyl compounds and unsaturated compounds in the same samples, which demonstrated complete inhibition of β-oxidation of fatty acids. Of the 9 children who had high levels of HGA/MCPG, 8 had a CSF sample tested: 1 child was positive for enteroviruses, and 7 children were negative. Of the 11 children with low levels of HGA/MCPG, 4 children were positive for enteroviruses, and 7 children were negative.

Relationship between Epidemiologic, Clinical, and Biological Findings and Etiologies

Of the 9 children with high levels of HGA/MCPG, 8 were hospitalized in July 2011 and came from the same eastern district (Luc Ngan), a district known to have the highest levels of litchi production in Bac Giang Province (50% of province production) and in which the harvest occurs each year during June–July (Figure 4). Toxin-negative samples and enterovirus-positive samples were predominantly identified in the western part of the province.

On the basis of results of virologic and toxicologic analyses, we compared clinical and biologic characteristics among 4 patient groups: 1) the 19 children who were positive for enteroviruses and who had either low levels of HGA/MCPG (n = 4) or no blood sample tested for toxins (n = 15); 2) the 8 children with high blood levels of HGA/MCPG and who were negative for enteroviruses (n = 7) or not tested for enteroviruses (n = 1); 3) the 7 children who were negative for enteroviruses and toxins; and 4) the 23 children who were negative for enteroviruses and who were not tested for toxins (Table; online Technical Appendix Table 3). One child was positive for enteroviruses and toxins and was therefore not included in statistical comparisons. All children included in the study had fever either before admission (reported by parents) or at admission, except for 1 child who had high levels of toxins, but no reported fever, who was included in the study because of severity of the neurologic condition of the child (repeated seizures and coma).

Children with high blood levels of HGA/MCPG had shorter median time between disease onset and admission (0 days vs. 2 days; p = 0.008), more seizures (88% vs. 28%; p = 0.009), more hypoglycemia (glucose level <3 mmol/L) (71% vs. 0%; p = 0.001), lower median numbers of leukocytes in CSF (3 cells/mm^3 vs. 50 cells/mm^3; p = 0.001), and higher median serum levels of alanine aminotransferase (48 IU/L vs. 24 IU/L; p = 0.04) and aspartate aminotransferase (68 IU/L vs. 28 IU/L; p = 0.01) than patients infected with enteroviruses. Two (25%) of 8 children who were positive for toxins died, whereas only 1 (5.3%) of the 19 children with enterovirus encephalitis died, but this difference was not significant (p>0.05).

Principal component analysis showed that children with high levels of HGA/MCPG clustered differently in the projection space (online Technical Appendix Figure 4) than children with evidence of infection with enteroviruses (online Technical Appendix Table 4). Furthermore, children not infected with enteroviruses for whom HGA/MCPG showed negative results or was not tested had

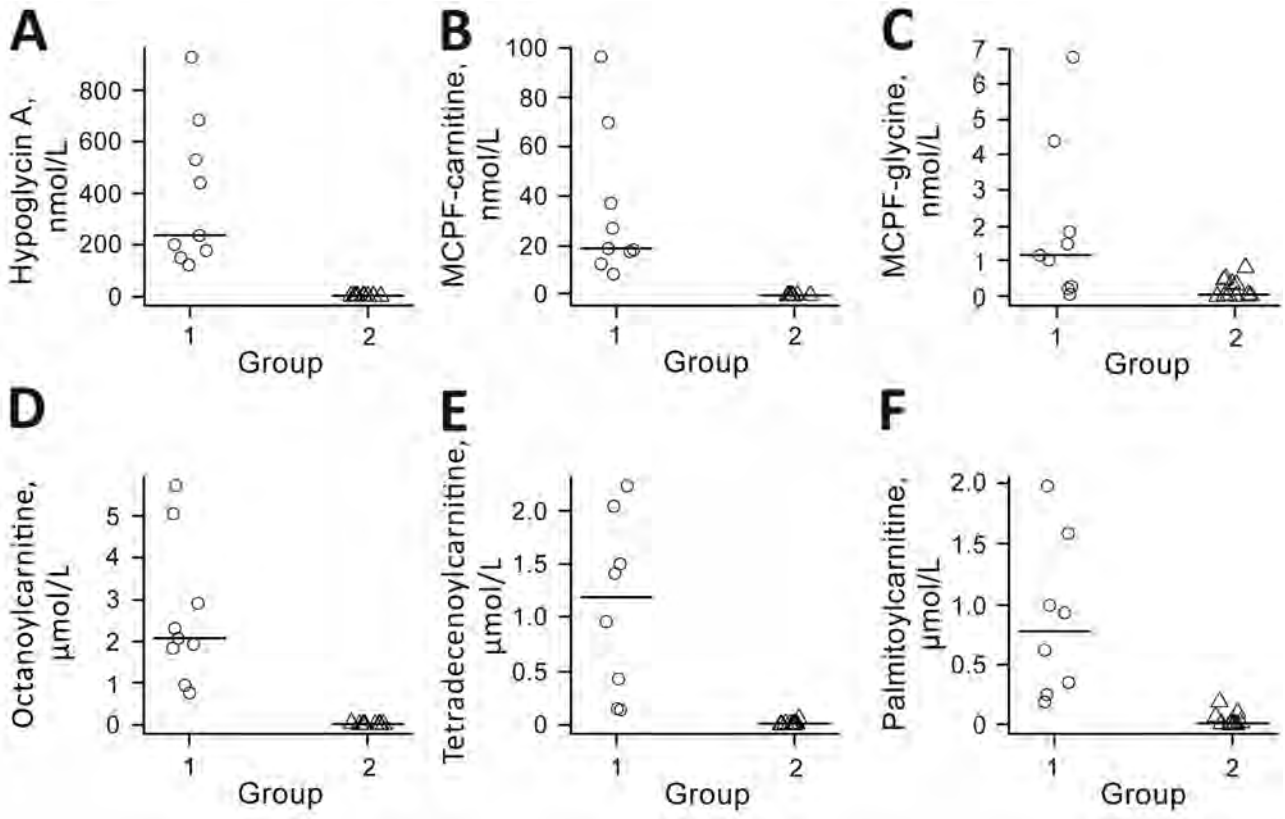

Figure 3. Serum concentrations of toxins and fatty acids in children with encephalitis-like syndrome, Bac Giang Province, northern Vietnam, 2008–2011. Children were grouped by high (group 1, n = 9 [circles]) and low (group 2, n = 11 [triangles]) serum concentrations of toxins. A) Hypoglycin A; B) MCPF-carnitine (methylenecyclopropylglycine metabolite); C) MCPF-glycine (methylenecyclopropylglycine metabolite); D) octanoylcarnitine (medium-chain fatty acid); E) tetradecenoylcarnitine (long-chain fatty acid in the form of acylcarnitine); and F) palmitoylcarnitine (long-chain fatty acid in the form of acylcarnitine). Horizontal lines indicate medians. MCPF, methylenecyclopropylformyl.

profiles more similar to children infected with enteroviruses. In particular, these children had glycemia (glucose level >3 mol/L), and leukocyte counts in CSF were slightly increased (online Technical Appendix Table 3).

Discussion

This study suggests that acute encephalitis-like syndrome previously associated spatially and temporally with litchi harvests (*11*) was caused by intoxication, rather than viral encephalitis, as initially suspected. In this context of recurrent acute encephalitis-like syndrome outbreaks since 1999, no consistent viral etiology had been identified by using standard laboratory diagnostic techniques. Because of the high number of viruses known to be associated with encephalitis (*3*,*5*,*25*), we used NGS to analyze samples of patients after the annual outbreak in 2008. This hypothesis-free technique identified human enterovirus B serotypes that were further confirmed by PCR. Other pathogens were not identified.

Despite the link shown in this study between several acute encephalitis-like syndrome cases and enteroviruses, the frequency of enterovirus infection among clinical cases was highly variable from year to year (Figure 2). Therefore, we tested serum samples of a subset of enterovirus-negative and enterovirus-positive cases for HGA and MCPG metabolites. Of 20 children tested, 9 (45%) were positive for HGA and MCPG metabolites and 8 showed inhibition of the β-oxidation catabolic process. Eight of these 9 case-patients came from the same district, which is known for having the highest litchi production in the province. These case-patients were hospitalized in July 2011, at the time of the litchi harvest in the district. These 8 case-patients appeared to have distinct characteristics in comparison with those who had enterovirus acute encephalitis syndrome, including younger age, more rapid progression, higher frequency of seizures, severe hypoglycemia, lack of increased numbers of leukocytes in CSF, and moderate increases in levels of liver enzymes.

The clinical and biochemical presentation of these case-patients clearly matches that of case-patients reported during outbreaks linked to litchi harvests in India and Bangladesh (*12*,*15*,*16*) and of case-patients with Jamaican

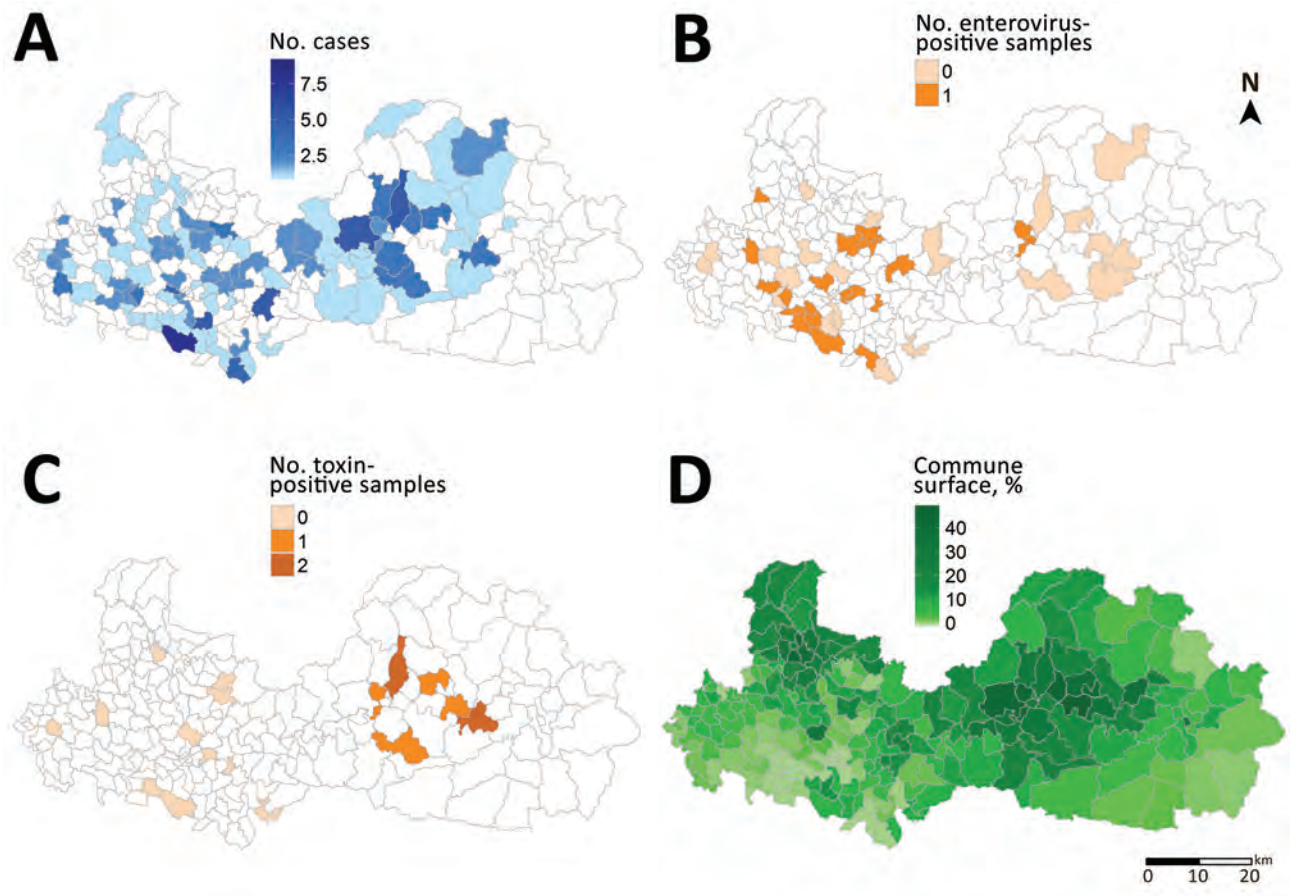

Figure 4. Geographic distribution of acute encephalitis-like syndrome in children, samples, and litchi cultivation at the commune level in Bac Giang Province, northern Vietnam, 2008–2011. A) No. cases of acute encephalitis-like syndrome meeting the inclusion criteria (n = 185); B) no. enterovirus-positive samples among all cerebrospinal fluid samples analyzed (n = 57); C) no. toxin-positive samples among all blood samples analyzed (n = 20); D) percentage of commune surfaces devoted to litchi cultivation.

vomiting sickness (*24*). Investigation of an outbreak in Muzaffarpur, India, recently concluded that intoxication with HGA and MCPG was responsible for the outbreak, a finding that is consistent with our results (*26*), which also identified HGA/MCPG in young patients with hypoglycemic encephalopathy. Our study also provides a direct comparison of clinical and biologic profiles of acute encephalitis-like syndrome related to enterovirus infection versus intoxication with HGA/MCPG. Thus, we provide useful information that can be used to guide clinical decision making, particularly the need for glycemia testing for management of patients with acute encephalitis-like syndrome.

Our study was limited by comparison of children subjected to different tests at different times (only blood samples obtained during 2010 and 2011 were available for testing of toxins, whereas testing for enterovirus was available throughout the study) and by having used fever as an inclusion criteria. In the study by Shrivastava et al. in India, in which this inclusion criterion was not used, 61% of children with litchi intoxication were afebrile (*26*). As a result of using fever as an inclusion criteria, our study might have missed several children with HGA/MCPG intoxication. Apart from 1 patient who had enterovirus infection and high serum concentrations of hypoglycemic toxins, patients with enterovirus infections did not have higher toxin levels than patients without enterovirus infections, suggesting that subtoxic concentrations of HGA/MCPG were not associated with increased risk for enterovirus infection. However, studies with larger numbers of patients are needed to rule out this hypothesis.

We also tried to elucidate the link between these 2 etiologies (enteroviruses and hypoglycemic toxins) and litchi harvesting in northern Vietnam. For enteroviruses, it is likely that temperature and humidity conditions required for enterovirus circulation match those of litchi maturation and harvest. For intoxication with HGA and MCPG, the link is more obvious because toxins have been previously identified in the litchi aril and litchi seeds (*17*,*18*). In our study, cases of intoxication clustered geographically in areas of large production of litchi. Levels of hypoglycemic

Table. Characteristics of 27 children hospitalized with acute encephalitis syndrome who were positive for enteroviruses or toxins, northern Vietnam, 2008–2011*

Characteristic	Enterovirus positive and toxin negative (n = 4) or not tested (n = 15)		Toxin positive and enterovirus negative (n = 7) or not tested (n = 1)		p value
	No. with data	No. (%) or median (IQR)	No. with data	No. (%) or median (IQR)	
Sex	19		8		0.80
F	NA	6 (32)	NA	3 (38)	NA
M	NA	13 (68)	NA	5 (62)	NA
Age, y	19		8		0.47
<2	NA	4 (21)	NA	3 (38)	NA
2–4	NA	5 (26)	NA	3 (38)	NA
5–9	NA	7 (37)	NA	1 (12)	NA
10–15	NA	3 (16)	NA	1 (12)	NA
Symptoms/signs before and at admission					
Temperature at admission, °C	18	38.0 (37.6–38.5)	7	37.5 (37.4–37.8)	0.14
Fever before admission	18	18 (100)	7	5 (71)	0.07
Headache	19	11 (58)	6	3 (50)	1.0
Seizures	18	5 (28)	8	7 (88)	0.009
Coma	14	4 (29)	7	4 (57)	0.35
Meningeal symptoms	18	12 (67)	7	4 (57)	0.67
Limb paralysis	18	1 (6)	5	0	1.0
Vomiting	18	14 (78)	7	6 (86)	1.0
Diarrhea	14	3 (21)	6	0	0.52
Days from disease onset to admission	19	2.0 (0.5–2.5)	8	0.0 (0.0–0.2)	0.008
Blood sample					
Leukocytes, × 10^9 cells/L	18	10.5 (7.5–15.8)	8	19.5 (18.4–29.9)	0.004
Platelets/μL	12	254 (197–306)	7	340 (274–487)	0.20
Hemoglobin, g/L	7	114 (108–118)	6	116 (92–122)	0.77
Glucose, mmol/L	14	4.5 (3.9–5.0)	7	2.0 (1.6–3.8)	0.67
Glucose <3 mmol/L	14	0	7	5 (71)	0.001
Cerebrospinal fluid sample					
Leukocytes/mm^3	12	50 (6–100)	6	3 (1–3)	0.001
Lymphocytes/mm^3	8	80 (73–80)	1	45 (45–45)	0.31
Protein level >0.5 g/L	15	4 (27)	6	0	0.28
Transparent appearance of CSF	15	15 (100)	7	7 (100)	1.0
Liver enzymes at or after admission, IU/L					
Alanine aminotransferase	8	24 (12–33)	8	48 (37–56)	0.04
Aspartate aminotransferase	8	28 (20–46)	8	68 (62–79)	0.01

*CSF, cerebrospinal fluid; IQR, interquartile range; NA, not applicable.

amino acids in the litchis are not known. Results from 2 studies suggest that MCPG concentration is highest in the seeds, followed by arils of semiripe litchis and then ripe litchis (*17*,*26*). Further investigations should compare levels of toxins across cultivars and soil, climate, and harvest conditions, as recommended by Spencer et al. (*27*). To further investigate a causal link between HGA/MCPG levels and acute encephalitis-like syndrome, healthy children exposed to the same litchi intake would need to be tested. Nevertheless, the evidence of inhibited β-oxidation of fatty acids in all HGA/MCPG–positive patients in this study is a convincing demonstration that intoxication was a key driver of symptoms in these patients.

Intoxication with HGA/MCPG is attributed mainly to a hypoglycemic encephalopathy, secondary to inhibition of β-oxidation and an inability to produce glucose from fatty acids. This metabolic process usually takes hours, which might explain why most children have initial symptoms during the second half of the night. Shrivastava et al. reported that children who had no evening meal were at higher risk for developing hypoglycemic encephalopathy (*26*). Young children, and even more so undernourished children, have limited glycogen stores, which increases their vulnerability to the effects of intoxication with HGA/MCPG on metabolism (*13*,*15*). Concentrations of glycine and carnitine conjugates measured in serum samples might appear rather low. These conjugates would be better measured in urine samples (*28*). However, such samples were not available in this study.

In conclusion, this study has shown that within a context of largely viral encephalitis, particularly encephalitis caused by enteroviruses, acute hypoglycemic encephalopathy developed in some children in Vietnam during the litchi harvest, possibly after absorption of a toxin present in the aril of litchi fruits. Local populations should be sensitized to the risks associated with young children eating litchis. Also, for children coming to healthcare facilities because of acute encephalitis-like syndrome during the litchi harvest season, measurement of blood glucose concentrations and immediate infusion with dextrose for those children with hypoglycemia

should be critical elements of clinical management. Use of these elements will likely increase patient survival.

Acknowledgments

We thank all collaborators, especially Ong The Vien, Dang Thanh Minh, Lam Van Tuan, and other medical staff in Bac Giang Province, for their involvement in the study; Marie-Line Joffret, Jean Balanant, and Francis Delpeyroux for advice on interpreting enterovirus data; and Antoine Gessain, Matthew Albert, Matt Buckwalter, Darragh Duffy, Michael Terhardt, Ana-Maria Burguière, Christophe Batéjat, India Leclerq, Maurice Demanoux, Allel Chabli, Laura Schaeffer, Thomas Bénet, Hugues Cordel, Alexandre Blake, Evguenia Krastinova, Louise Lu, Aude Deloumeau, Stéphane Béchet, and Rebecca Grant for their contributions to the study.

This study was supported by the Agence Nationale de la Recherche (grant ANR-08-MIE-003); the Institut Pasteur through the Inter-Pasteurian Concerted Actions; the Banque Nationale de Paris Paribas-Simplidons; the Institut de Médecine et d'Epidémiologie Appliquée; the French Government Investissement d'Avenir Programme (Labex Integrative Biology of Emerging Infectious Diseases grant ANR-10-LABX-62-IBEID); and Geneviève and Georges Deyme.

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Enhanced Surveillance for Coccidioidomycosis, 14 US States, 2016

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Although coccidioidomycosis in Arizona and California has been well-characterized, much remains unknown about its epidemiology in states where it is not highly endemic. We conducted enhanced surveillance in 14 such states in 2016 by identifying cases according to the Council of State and Territorial Epidemiologists case definition and interviewing patients about their demographic characteristics, clinical features, and exposures. Among 186 patients, median time from seeking healthcare to diagnosis was 38 days (range 1–1,654 days); 70% had another condition diagnosed before coccidioidomycosis testing occurred (of whom 83% were prescribed antibacterial medications); 43% were hospitalized; and 29% had culture-positive coccidioidomycosis. Most (83%) patients from nonendemic states had traveled to a coccidioidomycosis-endemic area. Coccidioidomycosis can cause severe disease in residents of non–highly endemic states, a finding consistent with previous studies in Arizona, and less severe cases likely go undiagnosed or unreported. Improved coccidioidomycosis awareness in non–highly endemic areas is needed.

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DOI: https://doi.org/10.3201/eid2408.171595

Coccidioidomycosis is a fungal infection caused by inhalation of soil-dwelling *Coccidioides* spp. organisms. Symptomatic infection occurs in ≈40% of cases and usually presents as a self-limiting, influenza-like illness (also called Valley fever) after a 1–3-week incubation period. A small proportion of patients have life-threatening severe pulmonary or disseminated disease (*1*,*2*). In the United States, coccidioidomycosis is known to be endemic in the southwestern states, with hyperendemic foci in Arizona's Sonoran Desert and California's southern San Joaquin Valley (*3*). The disease is also endemic in parts of Nevada, New Mexico, Utah, and Texas (*3*) but to a lesser extent. The actual endemic areas are likely broader than previously recognized; for example, *Coccidioides* was found in soil in south central Washington in 2013 and was implicated in locally acquired cases (*4*).

Coccidioidomycosis is reportable in 22 states. To meet the Council of State and Territorial Epidemiologists (CSTE) coccidioidomycosis case definition, cases must fulfill clinical and laboratory criteria (*5*). Approximately 10,000 cases are reported each year through the National Notifiable Diseases Surveillance System (NNDSS), although the number varies markedly by year. NNDSS captures basic demographic information about coccidioidomycosis cases, including the patients' state and county of residence, age, sex, race, and ethnicity. Some states routinely collect additional information, such as travel history, that is not available in NNDSS. Approximately 65% of cases are reported from Arizona and ≈30% from California (*6*), and the epidemiology and burden of coccidioidomycosis in these states has been well described. Enhanced

surveillance in Arizona during 2007–2008 showed substantial disease, including prolonged illness, and quantified the burden on the healthcare system, including an estimated $85 million total hospital charges in 2007 (*7*). In addition, patients who were aware of coccidioidomycosis before seeking healthcare were diagnosed sooner than those who did not know about the disease (*7*), suggesting that community awareness might prevent unnecessary diagnostic workup and antibacterial administration through earlier coccidioidomycosis diagnosis.

Although cases reported from states other than Arizona and California constitute a small proportion of total reported cases (4% in 2015), the number generally increased during the past decade, similar to the overall trend (*6*), indicating that coccidioidomycosis remains a public health problem on a national scale. However, features of cases in non–highly endemic areas have not been systematically described; previous studies of non–outbreak-associated cases are limited to single-state retrospective reviews of existing surveillance data or medical chart data (*8–10*). Therefore, we conducted enhanced surveillance in 14 states to better describe the epidemiology, diagnosis, and outcomes of these cases to help inform current routine surveillance practices and guide future awareness and educational efforts in these areas.

Methods

During January 1–December 31, 2016, routine surveillance conducted in 14 states (Louisiana, Michigan, Minnesota, Missouri, Montana, Nevada, New Mexico, North Dakota, Ohio, Oregon, Pennsylvania, Utah, Wisconsin, and Wyoming) identified coccidioidomycosis cases according to the CSTE case definition. State or local health department personnel contacted patients to participate in a voluntary telephone interview. A parent or guardian was interviewed for patients <18 years of age, and a relative or medical provider could complete the interview if the patient was incapacitated or deceased. Using a standardized questionnaire, health department personnel asked patients about symptoms, healthcare-seeking behaviors, diagnosis, treatment, outcomes, underlying medical conditions, and travel history. They also collected information about laboratory tests used to diagnose coccidioidomycosis from electronic surveillance databases. Some patients who met the CSTE case definition by laboratory criteria and symptoms but who clearly had a different diagnosis (such as histoplasmosis) or whose illnesses were not believed to be clinically consistent with coccidioidomycosis were not contacted for an interview. We further excluded from the analysis interviewed case-patients with compelling evidence that their illness was caused by something other than coccidioidomycosis.

We classified Nevada, New Mexico, and Utah as low-endemic because the risk for coccidioidomycosis is lower in those states than in Arizona and California. The 11 other states in this analysis were not known to be endemic for coccidioidomycosis and were considered nonendemic. We performed descriptive analysis and examined differences between cases in low- versus nonendemic states. We analyzed categorical variables by using χ^2 or Fisher exact tests and used Wilcoxon rank-sum tests to compare continuous variables. A human subjects review by CDC determined this project to be nonresearch.

Results

Interviewed and Noninterviewed Patients

We identified 339 patients who met the CSTE coccidioidomycosis case definition. Of those, 144 (43%) were not interviewed. Forty-five (31%) of those patients were not interviewed because a different illness etiology was identified or the illness was not believed to be clinically consistent with coccidioidomycosis; another 45 (31%) were unable to be contacted, 19 (13%) died, 14 (10%) refused, and no reason was provided for the remaining 21 (15%). Among the 45 noninterviewed patients with a different illness identified or an illness not clinically consistent with coccidioidomycosis, most had histoplasmosis (18 [40%]), 4 (9%) had aspergillosis, and the remainder had other or unspecified diagnoses. In addition, we excluded 9 interviewed patients thought not to have coccidioidomycosis based on laboratory test results and lack of travel to endemic areas, leaving 186 interviewed patients in the final analysis. Therefore, 16% (54/339) of all patients did not have coccidioidomycosis, all but 1 from nonendemic states. Excluding all patients who did not have coccidioidomycosis, the response rate was 65% (186/285). Interviewed and noninterviewed patients were similar in age and sex.

Demographic Features and Underlying Medical Conditions

Sixty-four (34%) patients were from low-endemic states, and 122 (66%) were from nonendemic states; 109 (59%) patients were male, 89% were white, and the median age was 65 (range 7–91) years (Table 1). Patients in nonendemic states were less likely than those in low-endemic states to be Hispanic or Latino (4% vs. 25%; odds ratio [OR] 0.10, 95% CI 0.03–0.33), were older (median 67 vs. 60 years; p = 0.01), and were more likely to have a yearly household income >$50,000 (61% vs. 41%; p = 0.043). The most common underlying medical conditions were diabetes (19%), heart disease (19%), and cancer (17%). Patients in nonendemic states were more likely to have heart disease than those in low-endemic states (24% vs. 9%; OR 3.0, 95% CI 1.2–7.7). Thirty-six (19%) patients were considered to be immunosuppressed, 61 (34%) reported no underlying conditions, and 12 (7%) reported a previous history of coccidioidomycosis.

Table 1. Demographic features and underlying medical conditions of coccidioidomycosis patients in 14 low-endemic and nonendemic US states, 2016*

Characteristic	Value
Total no. patients	186 (100)
Demographics	
Sex	
M	109 (59)
F	77 (41)
Median age, y (range), n = 185	65 (7–91)
Race, n = 170	
White	151 (89)
Black/African American	9 (5)
Asian	1 (0.6)
American Indian or Alaska Native	3 (2)
Other	6 (4)
Hispanic or Latino, n = 175	19 (11)
Health insurance coverage, n = 158	148 (94)
Some college education or higher, n = 137	91 (66)
Annual household income >$50,000, n = 104	56 (54)
Underlying medical conditions	
Smoking, n = 171	
Currently	10 (6)
In the past	74 (43)
None	87 (51)
Asthma requiring an inhaler	20 (11)
COPD or emphysema	18 (10)
Other lung disease	13 (7)
Diabetes	35 (19)
HIV/AIDS	2 (1)
Heart disease	35 (19)
Cancer	32 (17)
Transplant	2 (1)
Liver disease	9 (5)
Kidney disease	9 (5)
Other major illness	49 (26)
No underlying medical conditions reported	61 (34)
Immunosuppressed†	36 (19)
Immunosuppressive medications, n = 165	32 (19)
History of coccidioidomycosis, n = 174	12 (7)

*Values are no. (%) patients except as indicated. n values are provided for categories with <186 responses.
†Defined as HIV/AIDS, solid organ or bone marrow transplant, or immunosuppressive medication use.

Symptoms, Healthcare Use, and Diagnosis

The most common symptoms were cough (65%), fatigue (62%), and shortness of breath (52%) (Table 2). Less than half of patients reported fever (n = 85 [46%]). Patients in nonendemic states were less likely than those in low-endemic states to report chest pain (25% vs. 53%; OR 0.30, 95% CI 0.16–0.57), headache (24% vs. 41%; OR 0.46, 95% CI 0.24–0.87), joint pain (21% vs. 36%; OR 0.48, 95% CI 0.25–0.94), or muscle pain (18% vs. 31%; OR 0.48, 95% CI 0.24–0.98). Patients first sought healthcare a median of 5.5 (range 0–488; interquartile range [IQR] 1–17) days after symptom onset. Most patients first sought care at a primary care office (36%) or emergency department (36%). Seventy percent of patients reported receiving a diagnosis of another illness before being tested for coccidioidomycosis; among those, 63 (55%) said they received a pneumonia diagnosis, and 82 (83%) were prescribed antibacterial medication. Patients in nonendemic states were more likely to have had a chest radiograph performed than those in low-endemic states (94% vs. 73%; OR 5.4, 95% CI 2.1–13.9). More than half of patients (54%) visited a healthcare provider ≥3 times before being tested for coccidioidomycosis. Patients in nonendemic states were more likely than those in low-endemic states to ask for coccidioidomycosis testing (23% vs. 10%; OR 2.8, 95% CI 1.1–7.2). Most patients were tested by a primary care physician (30%) or a pulmonologist (26%). Median time between seeking healthcare and diagnosis was 38 (range 1–1,654, IQR 16–73) days. Patients in nonendemic states were more likely than those in low-endemic states to have a positive coccidioidomycosis culture (36% vs. 16%; OR 3.0, 95% CI 1.4–6.5) or immunodiffusion test (36% vs. 16%; OR 3.0, 95% CI 1.4–6.5) and less likely to have a positive enzyme immunoassay test (20% vs. 69%; OR 0.12, 95% CI 0.06–0.23) (Table 3).

Treatment and Outcomes

Seventy-seven (43%) patients were hospitalized (median duration 8 [range 3–60] days). Among 115 (68%) patients prescribed antifungal medication, most (95 [83%]) were prescribed fluconazole. Patients in nonendemic states were

Table 2. Symptoms and healthcare use among coccidioidomycosis patients in 14 low-endemic and nonendemic US states, 2016*

Characteristic	Value
Symptoms	170 (91)
Cough	121 (65)
Fatigue	116 (62)
Shortness of breath	96 (52)
Fever	85 (46)
Night sweats	71 (38)
Chest pain	65 (35)
Chills	60 (32)
Weight loss	60 (32)
Headache	55 (30)
Rash	54 (29)
Joint pain	49 (26)
Muscle pain	42 (23)
Wheezing	38 (20)
Sore throat	35 (19)
Stiff neck	30 (16)
Coughing up blood	13 (7)
Other symptoms	38 (20)
Type of facility where patient first sought care, n = 160	
Emergency room	57 (36)
Primary care	57 (36)
Urgent care	32 (20)
Specialist	9 (6)
Other	5 (3)
Patient first sought care in an endemic state, n = 166†	105 (63)
Patient first sought care in Arizona, n = 166	46 (28)
Ever went to the emergency room, n = 162	91 (56)
No. visits before being tested for coccidioidomycosis, n = 130	
1	33 (25)
2	27 (21)
>2	70 (54)
Type of doctor who first tested for coccidioidomycosis, n = 172	
Primary care	51 (30)
Urgent care	6 (4)
Emergency room	16 (9)
Infectious disease	30 (17)
Pulmonologist	45 (26)
Other	24 (14)
Site of infection, n = 127‡	
Pulmonary	105 (83)
Disseminated	22 (17)
Total no. healthcare visits for coccidioidomycosis, n = 139	
1	28 (20)
2–3	43 (31)
>3	68 (49)
Prescribed antifungal medication, n = 169	115 (68)
Fluconazole	95 (83)
Itraconazole	13 (11)
Voriconazole	4 (4)
Amphotericin B	3 (3)
Median symptom duration, d (range), n = 56	60 (7–1800)
Median symptom duration among patients recovered at interview, d (range), n = 44	38.5 (7–1800)
Median symptom duration among patients not recovered at interview, d (range), n = 12	90 (28–360)
Median time between symptom onset and interview, d (range), n = 107	115 (12–1672)

*Values are no. (%) patients except as indicated. n values are provided for categories with <186 responses.
†Arizona, California, Nevada, New Mexico, Texas, Utah, or Washington.
‡Site of infection was defined as pulmonary if lungs were the only body site involved and disseminated if another body part was involved, based on patient self-report.

more likely than those in low-endemic states to be prescribed antifungals (74% vs. 57%; OR 2.1, 95% CI 1.09–4.1). Fifty-four percent of patients were still symptomatic at the time of the interview; the most common symptom that these patients were still experiencing was fatigue (55%). Among patients who had recovered at the time of the interview, median symptom duration was 38.5 (range 7–1,800, IQR 21–90) days. Coccidioidomycosis interfered with 71% of patients' usual daily activities (median number of days affected 40 [range 2–1,080] days). Among 55 (31%) patients who had a job or were in school, 77% missed work or school (median 19 [range 1–240] days). Four (2%) patients died.

Table 3. Positive laboratory tests for coccidioidomycosis among patients in 14 low-endemic and nonendemic US states, 2016*

Characteristic	Value
Enzyme immunoassay IgM	52 (28)
Enzyme immunoassay IgM only	20 (11)
Enzyme immunoassay IgG	40 (22)
Enzyme immunoassay IgG only	13 (7)
Enzyme immunoassay IgM or IgG	69 (37)
Enzyme immunoassay IgM or IgG only	45 (24)
Immunodiffusion	53 (29)
Immunodiffusion only	18 (10)
Complement fixation	64 (35)
Median highest complement fixation titer, n = 55	8 (2–1024)
Complement fixation only	23 (12)
Complement fixation titer 1:2 only	7 (4)
Histopathology	9 (5)
Histopathology only	1 (0.5)
Culture	53 (29)
Bronchoalveolar lavage	16 (30)
Lung tissue	16 (30)
Sputum	3 (6)
Other body site	12 (23)
Unknown body site	6 (11)
Culture only	26 (14)
Molecular evidence	14 (8)
Culture, histopathology, or molecular evidence	61 (33)

*Values are no. (%) patients except as indicated. n values are provided for categories with <186 responses.

Travel to Known Endemic Areas

Overall, 124 (68%) patients (26 [41%] from low-endemic states and 98 [83%] from nonendemic states) traveled to Arizona, California, Nevada, New Mexico, Texas, Utah, Washington, Mexico, or Central or South America in the 4 months before symptom onset (or before testing positive, if asymptomatic) (Figures 1, 2). Among 88 patients for whom information was available, median travel duration was 74.5 (range 1–720) days. Five patients, all of whom traveled to Arizona, reported that a travel partner also had coccidioidomycosis. Among patients from nonendemic states, 59 (48%) reported part-time residence in Arizona. Of the 24 patients from nonendemic states who did not travel to known endemic areas in the 4 months before developing coccidioidomycosis, 16 reported ever traveling to those areas in their lifetime; 7 had incomplete travel histories because someone other than the patient was interviewed (n = 5) or because the patient did not complete the interview (n = 2). The remaining patient was a north-central Oregon resident with no underlying medical conditions whose only potentially relevant travel was to Mexico ≈12 years before symptom onset.

Knowledge of Coccidioidomycosis

Patients in nonendemic states were more likely than those in low-endemic states to know about their positive coccidioidomycosis test results before the interview (90% vs. 59%; OR 6.1, 95% CI 2.8–13.4) and were more likely to have heard of coccidioidomycosis before their diagnosis (57% vs. 40%; OR 2.0, 95% CI 1.04–3.7). Among patients who knew about coccidioidomycosis before diagnosis, 73% first heard about it from a family member, friend, or co-worker.

Discussion

These enhanced surveillance data provide much-needed insight into coccidioidomycosis-related illness among patients with cases reported from states where the disease is not highly endemic. Our findings are generally consistent with the similar surveillance conducted in Arizona during 2007–2008 in terms of effects on patients (*7*). Delays in diagnosis, unnecessary antibacterial use, and prolonged symptoms were common, and a high proportion of patients had culture-positive coccidioidomycosis, suggesting that less severe cases might go undiagnosed. We found several differences between patients in low- and nonendemic states, which appear to be related to the underlying populations and testing patterns.

Patients experienced considerable diagnostic delays. Although the median time from symptom onset to seeking healthcare (5.5 days) was shorter than in Arizona surveillance (11 days), the median time from seeking healthcare to diagnosis (38 days) was longer than in Arizona (23 days) and in a study of patients in Missouri (25 days from onset to diagnosis) (*7*,*10*). Compared with Arizona surveillance, the shorter time from onset to seeking care in this investigation could be related to an older patient population, whereas the longer time from seeking healthcare to diagnosis is likely related to lower coccidioidomycosis awareness in low-endemic and nonendemic states. Many patients also reported initial misdiagnosis before being tested for coccidioidomycosis; however, it is unclear whether those who said they were first diagnosed with pneumonia did, in fact, receive a correct initial coccidioidomycosis diagnosis but were either not informed of the specific etiology or did not remember being told, or whether they were truly misdiagnosed

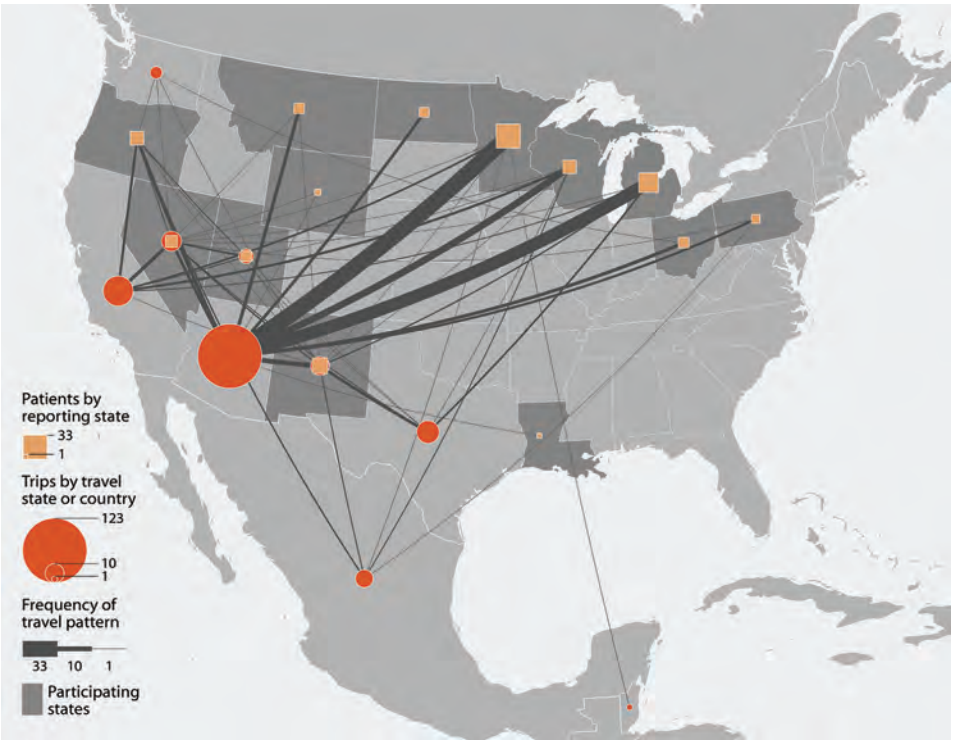

Figure 1. Reporting state and frequency of travel to coccidioidomycosis-endemic areas (Arizona, California, Nevada, New Mexico, Texas, Utah, Washington, Mexico, and Central or South America) in the 4 months before symptom onset or first positive coccidioidomycosis test among coccidioidomycosis patients reported from 14 low-endemic and nonendemic US states, 2016.

with bacterial pneumonia. Misdiagnosis seems likely because of the high proportion of patients who reported being diagnosed with another illness and receiving antibacterial medication, similar to other studies (*7*,*11*). We did not observe statistically significant differences in misdiagnosis or delays in diagnosis between low-endemic and nonendemic states, indicating a need for increased healthcare provider awareness about coccidioidomycosis in all areas.

The differences between patients from low-endemic and nonendemic states appear to reflect underlying population demographics and provider testing practices rather than differences in disease severity. Enzyme immunoassay is commonly used in highly endemic areas as an initial test for coccidioidomycosis because it is high-throughput, requires less expertise, and is more sensitive (though less specific) than other serologic methods (*2*). Providers and laboratories in low-endemic areas might be more familiar with this test than those in nonendemic areas, whereas patients in nonendemic areas were more likely to have positive coccidioidomycosis cultures. Providers in nonendemic areas might not be testing specifically for coccidioidomycosis, but rather diagnosing it incidentally, given that *Coccidioides* organisms can grow on various culture media. Nearly all cultures came from invasive procedures and <10% from sputum, suggesting that diagnosis by culture was uncommon among patients with uncomplicated primary pulmonary disease not warranting invasive procedures. Some patients from nonendemic states first sought care in endemic areas and might have had coccidioidomycosis diagnosed there; however, we did not collect data on diagnosis location, making geographic differences in testing patterns difficult to fully understand. In contrast to possible differences in providers' knowledge of coccidioidomycosis tests, patients themselves were more likely to have known about coccidioidomycosis before being diagnosed with it and were more likely to ask for coccidioidomycosis testing if they were reported from nonendemic states. The modest awareness among these patients is probably related to the fact that a high proportion resided part-time in Arizona, where public outreach about coccidioidomycosis is frequent and awareness is likely to be greater than in other areas. Patients in nonendemic states were also more likely to know about their positive results before the interview. Possible reasons that patients did not know of their positive test results include that the patient misunderstood or did not remember their diagnosis or that the provider did not inform the patient because they believed the results were not clinically relevant. The second explanation would also support the finding that patients in low-endemic states were less likely to be prescribed antifungal medications.

The most common symptoms (cough, fatigue, and shortness of breath) and prolonged symptom duration (median 38.5 days among patients who had recovered at the time of the interview) were similar to those in Arizona patients (42 days) (*7*). Comparable to our findings, other studies show that coccidioidomycosis symptoms, particularly fatigue, can take months to resolve and profoundly impair physical activities, resulting in missed workdays and

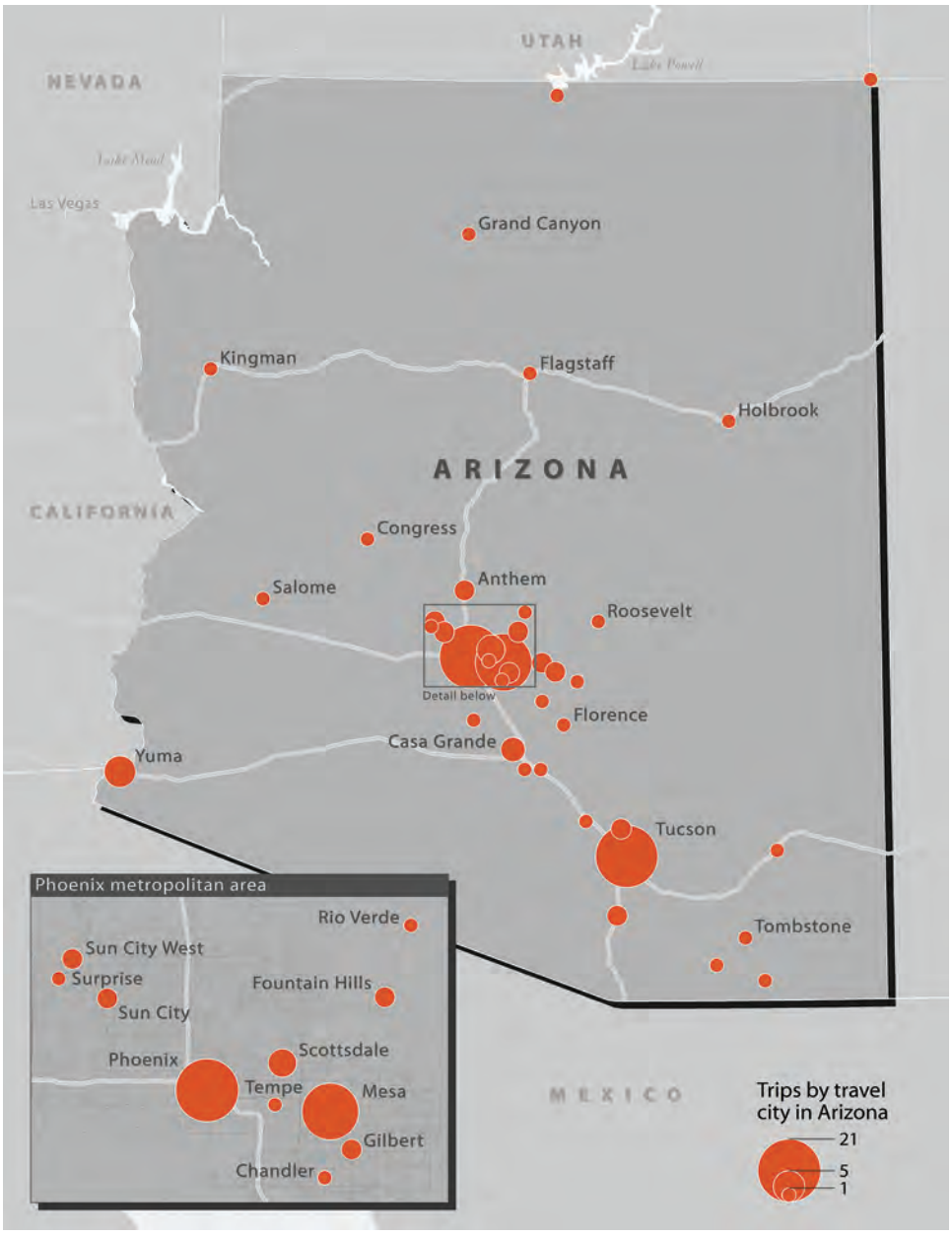

Figure 2. Frequency of trips to Arizona in the 4 months before symptom onset or first positive coccidioidomycosis test among coccidioidomycosis patients reported from 14 low-endemic and nonendemic US states, 2016.

inability to perform usual daily activities (*7*,*12*,*13*). The reasons that patients in low-endemic states were more likely to report chest pain, headache, joint pain, and muscle pain than patients in nonendemic states are unclear but could further reflect geographic differences in testing practices if physicians in low-endemic areas are more likely to suspect and test for coccidioidomycosis based on those symptoms. In previous studies of coccidioidomycosis test positivity among community-acquired pneumonia (CAP) patients in highly endemic areas, myalgia (*11*) and rash (*14*) were the only clinical features that differentiated coccidioidal CAP from noncoccidioidal CAP. Another study found that chest pain was a significant predictor of being tested for coccidioidomycosis among CAP patients (*15*). In addition, approximately half of patients in our surveillance reported fever, similar to findings in Arizona surveillance (*7*).

Potential recall bias is our investigation's main limitation. However, patient interviews can yield insightful data about effects on patients' daily activities and other information that might not be routinely available from medical records, such as detailed travel histories.

Travel to or part-time residence in Arizona was frequent among patients reported from nonendemic states. Approximately 40 million persons visited Arizona in 2015 (*16*), but the number of seasonal residents is more challenging to measure; the most recent figures estimated

Arizona's winter-only resident population to be ≈300,000 during 2000–2001 (*17*). These seasonal residents, also known as snowbirds, are typically retired, older adults who have higher socioeconomic statuses than others in their age group and who usually cite a more enjoyable climate as the reason for their part-time residence outside their home state (*18*). Overall, the risk for acquiring coccidioidomycosis during travel to Arizona is likely small. One expert estimated that only 1 in 17,000 visitors would experience an infection serious enough to seek medical care (*19*). However, the total number of cases estimated to occur in Arizona visitors is estimated to be ≈1,300 per year (*19*), suggesting a public health problem far larger than surveillance detects. The high proportion of cases from nonendemic states whose coccidioidomycosis was diagnosed by culture (36%, compared with <10% in Arizona surveillance) also indicates that less severe cases go undiagnosed or unreported. Obtaining a patient's history of travel to or residence in coccidioidomycosis-endemic areas is essential for early diagnosis (*2*).

Some patients (59% from low-endemic states and 17% from nonendemic states) did not report travel to endemic areas in the 4 months before symptom onset. A study of cases in Missouri residents also found that approximately one quarter of patients did not report travel to endemic areas during the 3 weeks before symptom onset (*10*). Incomplete travel histories or travel that occurred >4 months before symptom onset likely explain the lack of recent travel to endemic areas among patients from nonendemic states in our surveillance. In low-endemic states, most cases in patients without recent travel to other endemic areas could presumably be locally acquired. A deeper understanding of the highest-risk geographic areas in those states is needed.

We classified Oregon as nonendemic; although *Coccidioides* spp. DNA was identified from several soil samples in central Oregon in 2016, the fungus has not been cultured from environmental samples (*20*). Our surveillance identified culture-confirmed coccidioidomycosis in 1 Oregon patient who did not recently travel to known endemic areas, and the acute nature of his illness did not suggest reactivation of infection acquired during his earlier travel to Mexico. The patient reported extensive exposure to alfalfa hay (of unknown source), and rare coccidioidomycosis cases have been transmitted by similar fomites (*21*), suggesting that acquisition from the local environment and remote travel are not the only possible sources of infection. Unfortunately, a suitable clinical isolate was not available for whole-genome sequencing and comparison to isolates from nearby states. Such testing, in combination with environmental isolates, has enabled identification of cases acquired from the natural environment in south-central Washington (*4*,*22*) and could allow for discovery of similar transmission in Oregon, if present.

Our results could be used to inform minor revisions to the CSTE case definition. In nonendemic areas or areas with unknown endemicity, interpretation of positive coccidioidomycosis serologic test results can be challenging if the patient is asymptomatic, has no relevant travel or an unknown travel history, or has laboratory evidence of a different disease. In this surveillance, ≥16% (54/339) of patients whose illness met the CSTE definition likely did not have coccidioidomycosis; all but 1 were from nonendemic states, and many had histoplasmosis, which is known to cause cross-reactions with coccidioidomycosis serologic tests (*23*). Some states are already excluding such cases from their case-counts even though the CSTE definition does not specify exclusion criteria. Similarly, the CSTE definition does not state whether cases counted in a previous year should be counted again if subsequent positive laboratory tests are reported. Most states, including Arizona, only count cases once because infection is thought to confer lifelong immunity. Seven percent of patients in our analysis self-reported a history of coccidioidomycosis, but we were not able to determine if their cases had been previously reported in other states. Last, 9% of patients we interviewed did not report symptoms, although the actual proportion could be higher because some patients were not contacted for an interview because their illnesses were thought to be clinically incompatible with coccidioidomycosis. In Arizona's enhanced surveillance, 5% of patients had no symptoms or symptoms inconsistent with coccidioidomycosis according to the CSTE definition, suggesting that the CSTE definition's laboratory component alone is sufficiently specific for public health surveillance (*7*). Further characterization of clinical scenarios involving asymptomatic patients with positive coccidioidomycosis tests could inform clinical practice and disease surveillance. Overall, the contributions of false-positive laboratory tests, previously reported cases, and asymptomatic cases to national-level case-counts are undoubtedly small, but they serve as examples of ways that coccidioidomycosis surveillance could be improved.

Although *Coccidioides* is most common in Arizona and California, coccidioidomycosis is a disease of national importance. Our investigation revealed many cases associated with travel to or part-time residence in highly endemic areas, as well as cases presumably acquired in Nevada, New Mexico, and Utah. Patients experienced substantial delays in diagnosis and prolonged symptoms, leading to lost productivity. The high proportion of culture-positive cases suggests that less severe cases go undiagnosed, resulting in underestimates of the actual number of cases, which is typical for public health surveillance. Greater awareness nationwide among clinicians and the public about coccidioidomycosis is needed to minimize delays in diagnosis and appropriate treatment.

Acknowledgments

We thank Rajal K. Mody and Sekai Chideya-Chihota for contributions to the project design and William M. Hartnett for creating the figures. We also thank Zach Baucom, Jeff Brasel, Jim Collins, Carol Conroy, Michelle Feist, Louise Saw, Emily Smith, Maximilian Wegener, and state and local public health investigators who conducted patient interviews.

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Human Norovirus Replication in Human Intestinal Enteroids as Model to Evaluate Virus Inactivation

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Human noroviruses are a leading cause of epidemic and endemic acute gastroenteritis worldwide and a leading cause of foodborne illness in the United States. Recently, human intestinal enteroids (HIEs) derived from human small intestinal tissue have been shown to support human norovirus replication. We implemented the HIE system in our laboratory and tested the effect of chlorine and alcohols on human norovirus infectivity. Successful replication was observed for 6 norovirus GII genotypes and was dependent on viral load and genotype of the inoculum. GII.4 viruses had higher replication levels than other genotypes. Regardless of concentration or exposure time, alcohols slightly reduced, but did not completely inactivate, human norovirus. In contrast, complete inactivation of the 3 GII.4 viruses occurred at concentrations as low as 50 ppm of chlorine. Taken together, our data confirm the successful replication of human noroviruses in HIEs and their utility as tools to study norovirus inactivation strategies.

Human noroviruses are the leading cause of epidemic and endemic acute gastroenteritis worldwide (*1*). A major barrier to studying the pathogenesis, virus–host interactions, and effect of control measures to prevent and treat norovirus gastroenteritis has been the lack of a robust and reproducible cell culture system. Since the discovery of norovirus in 1972, many research groups have attempted to grow human norovirus. Initial studies included a comprehensive number of primary and continuous cell lines that had been used successfully to grow other viruses, but none supported human norovirus replication (*2*). Other attempts included the use of a differentiated human embryonic small-intestinal cell line (INT 407) (*3*), but the results could not be confirmed by others (*4*–*6*). The discovery of murine norovirus (MNV) in 2003 and the fact that this virus successfully replicated in a murine macrophage cell line in vitro and in primary immune cells in vivo suggested that immune cells may also support replication of human norovirus (*7*). However, immune cells isolated from healthy adults did not support replication (*8*). A more recent study reported that BJAB cells, a continuous human B cell line, supported replication of a GII.4 norovirus strain in the presence of bacteria (*9*). Although initially promising, these results have not been consistently confirmed by other groups (*10*).

The first steps toward a new cell culture system for human norovirus included the detection of human norovirus antigen in duodenal and jejunal enterocytes in tissue sections from human norovirus–infected transplant patients (*11*) and the development of human intestinal enteroids (HIEs) derived from nontransformed small intestine and colonic tissues (*12*). The HIEs, or "mini-guts," are generated from stem cells present in the intestinal crypts isolated from human intestinal tissue and cultured indefinitely as ex vivo, 3-dimensional (3D) cultures in growth-factor–enriched media (*13*–*15*). HIE cultures recapitulate the complexity and cell diversity of the gastrointestinal tract in relatively the same proportions as in the intestine itself (*14*,*15*) and successfully support the replication of human rotavirus (*13*) and human norovirus (*16*,*17*). These studies confirmed the role of enterocytes as the major site for human norovirus replication and host restriction based on genetic factors, as well as the role of bile as a strain-specific requirement or enhancer for virus infectivity.

During the past 40 years, the efficacy of inactivation and disinfection procedures for human norovirus could be evaluated only by human challenge studies (*18*,*19*) or by using cultivable surrogate viruses such as MNV, feline calicivirus, or Tulane virus (*20*–*23*). However, without direct confirmation that any of these surrogates correlate

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DOI: https://doi.org/10.3201/eid2408.180126

with inactivation of infectious human norovirus, no consensus has been reached on the best surrogate for human norovirus (*24*,*25*). In this study, we demonstrate the successful implementation of HIE cultures, show successful replication of several human norovirus genotypes, and demonstrate the applicability of HIEs to evaluate the efficacy of chlorine and alcohols on reducing virus infectivity.

Materials and Methods

Detailed methods and description of HIE cultures, gene expression analysis, viral infections, norovirus detection, and statistical analyses are provided in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/8/18-0126-Techapp1.pdf). This investigation was determined by the Centers for Disease Control and Prevention (CDC) to be public health nonresearch and therefore not subject to institutional review board review.

Fecal Samples

We included 80 human norovirus-positive fecal samples (12 genogroup [(G)] I, 65 GII, and 3 GIV) collected during 2000–2017 in the study (Table 1). Samples were stored at 4°C or –70°C from collection time until the time of testing. All samples were tested during April 2016–December 2017.

Human Intestinal Enteroid Culture

Baylor College of Medicine (Houston, TX, USA) provided secretor-positive jejunal HIE cultures (J2 and J3 lines) and Wnt3a-producing cells (CRL-2647 cells). Calvin Kuo (Palo Alto, CA, USA) kindly provided R-spondin-producing cells. Gijs van den Brink (University of Amsterdam, Amsterdam, the Netherlands) kindly provided Noggin-producing cells. Complete media with growth factors ($CMGF^+$) and without growth factors ($CMGF^-$), differentiation media, and 3 conditioned media (Wnt3a, R-spondin, and Noggin) were prepared as reported previously (*13*,*16*,*17*).

We grew jejunal HIE cultures (J2 or J3 lines) as undifferentiated 3D cultures in the presence of $CMGF^+$ supplemented with 10 μmol/L Y-27632, as described previously (*17*). After 7 days, highly dense 3D cultures were either split 1:2, frozen in LN_2, or dissociated into a single cell suspension and plated as undifferentiated monolayers. After culture for 24 h at 37°C in 5% CO_2, we replaced $CMGF^+$ supplemented with 10 μmol/L Y-27632 with differentiation medium to induce monolayer differentiation.

Infection Experiments and Viral Replication

We performed all infections in triplicate on 100% confluent 4-day-old differentiated HIE (J3 line) monolayers, except when specified that the J2 line was used. In some experiments, we pretreated monolayers with 1% sow bile included in the differentiation medium 48 h before infection. In other experiments, we differentiated HIE monolayers without pretreatment, and infected them in the presence of 500 μmol/L of glycochenodeoxycholic acid (GCDCA; Sigma, St. Louis, MO, USA) or with 500 μM GCDCA plus 50 μM ceramide.

To determine viral infectivity, we inoculated duplicate 96-well plates with 100 μL of fecal filtrate (online Technical Appendix) at 1:10, 1:100, and 1:1000 dilution. After 1 h incubation at 37°C in 5% CO_2, we washed the monolayers twice with $CMGF^-$ and added 100 μL of differentiation medium containing 1% sow bile, 500 μmol/L GCDCA, or 500 μM GCDCA plus 50 μmol/L ceramide to each well. For each set of infections, we immediately froze 1 plate at –70°C and incubated a duplicate plate at 37°C in 5% CO_2 for 72 h and froze it at –70°C. We used quantitative reverse transcription real-time PCR to determine the amount of norovirus RNA from input virus and from HIE monolayers at 1 hour postinfection (hpi) and at 72 hpi. Standard curve based on quantified RNA transcripts was included.

Inactivation Treatments

Alcohol Treatment

We diluted 10% fecal filtrates 1:10 in 70% ethanol or isopropanol solutions and incubated them for 1 min or 5 min. We then neutralized the alcohols in the samples by adding 9 volumes of CMGF supplemented with 10% fetal bovine serum (FBS). We included a nontreatment control and an alcohol neutralization control in each experiment.

Chlorine Suspension Assays

We prepared fresh chlorine stock solutions at 1,000 ppm and 10,000 ppm by diluting commercial bleach (6% sodium hypochlorite) in cell culture–grade water. We diluted 20 μL of 10% fecal filtrates in an appropriate volume of chlorine stock solutions to achieve a series of total chlorine concentrations of 5–5,000 ppm. After incubating the solutions for 1 min at room temperature, we added sodium thiosulfate (final concentration 50 mg/L) to neutralize free chlorine. We included a nontreatment control and a chlorine neutralization control in each experiment.

Results

HIE Model and Small Intestine Complexity

We recovered jejunal HIE cultures from 2 donors (J2 and J3) frozen at passage 7 (P7) from LN_2 and grew them as 3D cultures in Matrigel (BD Biosciences, San Jose, CA, USA). Within 24 hours, cells formed small cystic or multilobular HIEs and continued to grow in the presence of $CMGF^+$ medium (Figure 1). With each passage, the number of 3D HIEs doubled, reaching a maximum Matrigel capacity of 100/plug. We were able to culture HIEs for ≥4 months (16–17 consecutive passages) (Figure 2, panel

A). We confirmed that the number of highly proliferative stem cells increased over time, as shown by enhanced transcriptional levels of LGR5+ (Figure 2, panel B), whereas differentiated monolayers' LGR5+ expression levels were greatly reduced and lineage-specific markers (SI, ALPI, TFF3, MUC2, FFA4R) were increased (Figure 2, panel C).

Table 1. Human norovirus–positive fecal samples tested on 80 jejunal HIEs in study of human norovirus replication in HIEs

Genotype*		No.	Mean norovirus	Patient age group		Collection	Storage	Outbreak or	No. inactivated/
Capsid	RdRp	samples	RNA copies/μL	Years	No.	date†	condition‡	sporadic	no. samples %
GI.1	GI.P1	4	$0.5–19.3 \times 10^3$	>18	4	2000	−70°C	Sporadic	0/4 (0)
GI.3	GI.P3	2	$0.3–59.4 \times 10^4$	0–12	1	2013 Jul	−70°C	Sporadic	0/4 (0)
				>18	1	2005	−70°C	Outbreak	
	GI.Pd	2	$1.2–8.7 \times 10^4$	0–12	1	2015 Nov	−70°C	Sporadic	
					1	2017 Feb	−70°C	Sporadic	
GI.4	GI.P4	1	2.2×10^2	>18	1	2000	−70°C	Outbreak	0/1 (0)
GI.7	GI.P7	3	$2.6–16.4 \times 10^3$	0–12	1	2010 Nov	−70°C	Sporadic	0/3 (0)
					1	2014 Mar	−70°C	Sporadic	
					1	2016 Dec	−70°C	Sporadic	
GII.1	GII.Pg	1	1.4×10^5	0–12	1	2017 May	−70°C	Sporadic	1/1 (100)
GII.2	GII.P16	3	$0.2–52.6 \times 10^3$	0–12	2	2017 Feb	−70°C	Sporadic	1/2 (50)
					1	2017 Mar	−70°C	Sporadic	0/1 (0)
GII.3	GII.P21	2	$1.0–6.4 \times 10^5$	0–12	1	2012 Mar	−70°C	Sporadic	0/1 (0)
					1	2015 May	−70°C	Sporadic	
	GII.P12	2	$1.6–4.4 \times 10^6$	0–12	2	2012 Aug	−70°C	Sporadic	1/2 (50)
	GII.P16	4	$0.2–141.1 \times 10^4$	0–12	1	2012 Mar	−70°C	Sporadic	0/4 (0)
					1	2012 Jun	−70°C	Sporadic	
					1	2015 Oct	−70°C	Sporadic	
					1	2016 Dec	−70°C	Sporadic	
GII.4 Den Haag	GII.P4 Den Haag	3	$1.3–161.6 \times 10^4$	>18	1	2010 May	4°C	Outbreak	0/1 (0)
				0–12	1	2013 Jun	−70°C	Sporadic	0/1 (0)
					1	2015 May	−70°C	Sporadic	1/1 (100)
GII.4 New Orleans	GII.P4 New Orleans	1	4.1×10^5	0–12	1	2013 Apr	−70°C	Sporadic	0/1 (0)
GII.4 Sydney	GII.Pe	22	$3.5 \times 10^3–2.1 \times 10^7$	0–12	3	2012	−70°C	Sporadic	1/3 (30)
					1	2015 Jan	−70°C	Sporadic	1/1 (100)
					7	2015 Feb	−70°C	Sporadic	3/7 (40)
					1	2015 Mar	−70°C	Sporadic	1/1 (100)
					1	2015 Apr	−70°C	Sporadic	1/1 (100)
					1	2015 Oct	−70°C	Sporadic	0/1 (0)
					1	2016 Apr	−70°C	Sporadic	0/1 (0)
				>18	1	2011	−70°C	Outbreak	0/1 (0)
					1	2013	−70°C	Outbreak	0/1 (0)
					1	2012 May	4°C	Outbreak	0/1 (0)
					2	2016 Apr	4°C	Outbreak	1/2 (50)
					2	2016 May	4°C	Outbreak	0/2 (0)
	GII.P16	13	$0.4 \times 10^3–6.1 \times 10^6$	0–12	2	2016 Dec	−70°C	Sporadic	1/2 (50)
					6	2017 Feb	−70°C	Sporadic	0/6 (0)
					5	2017 Mar	−70°C	Sporadic	0/5 (0)
	GII.P4 New Orleans	4	$0.6–14.9 \times 10^4$	0–12	1	2015 Jan	−70°C	Sporadic	0/4 (0)
					1	2017 Jan	−70°C	Sporadic	
					1	2017 Feb	−70°C	Sporadic	
					1	2017 Mar	−70°C	Sporadic	
GII.5	GII.P22	1	1.1×10^4	0–12	1	2010 Nov	−70°C	Sporadic	0/1 (0)
GII.6B	GII.P7	1	7.6×10^4	0–12	1	2015 Jan	−70°C	Sporadic	0/1 (0)
GII.6	GII.P7	4	$0.1–8.4 \times 10^6$	0–12	1	2012 Dec	−70°C	Sporadic	0/4 (0)
					2	2015 Jan	−70°C	Sporadic	
					1	2017 Jan	−70°C	Sporadic	
GII.7	GII.P7	2	$0.3–9.1 \times 10^6$	>18	1	2010 Oct	−70°C	Sporadic	0/2 (0)
				0–12	1	2012 Aug	−70°C	Sporadic	
GII.14	GII.P7	1	6.1×10^4	0–12	1	2016 Dec	−70°C	Sporadic	1/1 (100)
GII.17	GII.Pe	1	3.4×10^5	0–12	1	2010 Oct	−70°C	Sporadic	1/1 (100)
GIV	GIV.P1	3	$0.3–13.6 \times 10^3$	>18	3	2016 May	4°C	Outbreak	0/3 (0)
Total		80			80				16/80 (20)

*Dual genotyping based on sequencing partial RdRp and capsid regions (*26,27*). HIE, human intestinal enteroids; RdRp, RNA-dependent RNA polymerase.
†When month was not available, only year of collection is reported.
‡Samples were stored at the indicated temperature.

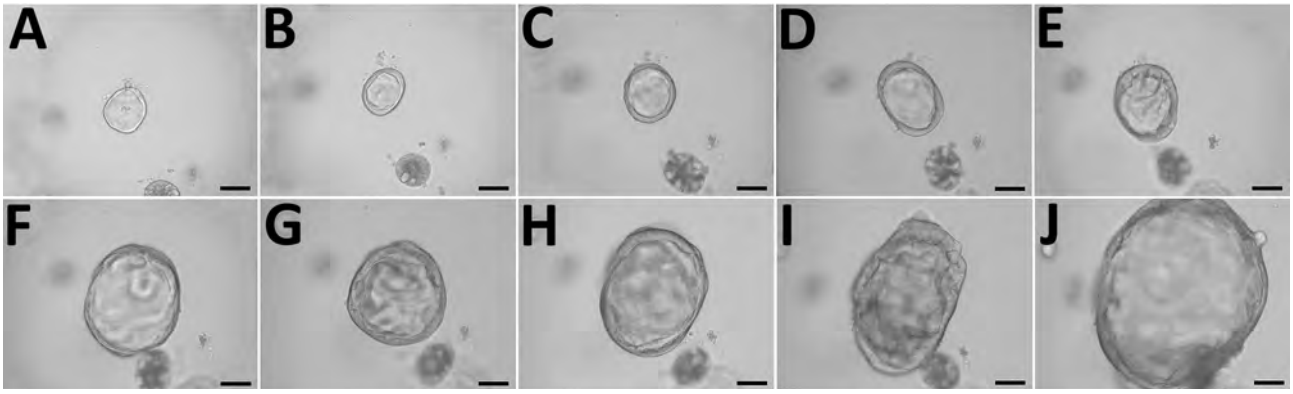

Figure 1. Growth of 3-dimensional human intestinal enteroid (HIE) embedded in Matrigel in the presence of complete media with growth factors containing Wnt3a, R-spondin, and Noggin as part of a study of human norovirus replication in HIEs. Microscopy images show the growth of a representative undifferentiated HIE: A) day 1; B) day 2; C) day 3; D) day 4; E) day 5; F) day 6; G) day 7; H) day 8; I) day 9; J) day 10. Scale bars indicate 100 μm.

Human Norovirus Infection of Jejunal HIEs

A previous study demonstrated the replication of GII.4 norovirus in HIEs (*16*). To evaluate whether those results could be reproduced, we infected jejunal HIEs (line J3) with GII.4 fecal filtrates (GII.P4 Den Haag-GII.4 Den Haag, GII.P4 New Orleans-GII.4 New Orleans, GII.Pe-GII.4 Sydney, and GII.P16-GII.4 Sydney). At 72 hpi, we detected 100- to 1,100-fold increases in viral RNA copies per well for all GII.4 fecal filtrates compared with viral RNA levels detected at 1 hpi (Figure 3, panel A; Table 2).

To further evaluate the reproducibility of the system, we included GII.4 fecal filtrates (GII.4 Den Haag, GII.4 New Orleans, and GII.4 Sydney) in each infection experiment conducted during August 2016–2017. We observed consistent replication of the 3 strains without significant differences in viral titers (Figure 3, panel B). The mean $\log_{10}$ increase was 2.7 (95% CI 2.68–2.82; n = 35) for GII.4 Sydney, 2.4 (95% CI 2.29–2.45; n = 33) for GII.4 Den Haag, and 2.3 (95% CI 2.17–2.46; n = 18) for GII.4 New Orleans. We observed significantly higher-fold increases on viral RNA titers for GII.4 Sydney compared with GII.4 Den Haag and GII.4 New Orleans ($p<0.0001$).

To evaluate whether HIEs support replication of non-GII.4 strains, we inoculated monolayers with different GI-, GII-, and GIV-positive fecal filtrates (Table 1). We observed viral replication for GII.Pg-GII.1 (2.6 $\log_{10}$), GII.P16-GII.2 (2.8 $\log_{10}$), GII.P12-GII.3 (2.3 $\log_{10}$), GII.P7-GII.14 (1.7 $\log_{10}$), and GII.Pe-GII.17 (1.9 $\log_{10}$) strains (Table 2; Figure 3, panel C). We did not observe replication of GI, GIV, and other GII genotypes. We also confirmed that both J2 and J3 HIE lines support human norovirus replication without significant differences in -fold change between the 2 cell lines (Figure 3, panel D). Most (15/16; 94%) samples that successfully replicated had been stored at –70°C (Table 2) and were collected from children <2 years of age (13/16; 81%) (Figure 3, panel E). CDC830 was stored at 4°C for 2 months before cultivation; this sample was collected from an adult 83 years of age.

We next evaluated replication of human norovirus in HIEs by assessing the kinetics of infection for 4 GII genotypes (GII.1, GII.2, GII.3, and GII.4 Sydney). Consistent with a successful infection, norovirus RNA levels increased at 12 hpi, reaching a plateau at 24 hpi; no significant further increase at 72 hpi was observed for any of the genotypes (Figure 4). Despite a similar viral input level ($3.3–9.3 \times 10^5$ copies/well), GII.4 Sydney infected HIEs with higher efficiency than did the other 3 genotypes (Figure 4), as shown by higher levels of viral RNA in cells and supernatant.

To further confirm the production and release of norovirus from cells, we quantified viral titers in supernatants collected from cell cultures and replaced the differentiation media every 24 hours after infection (Figure 5). We detected viral RNA in supernatants collected at 24 hpi and 48 hpi for all infections. Higher and more consistent levels of norovirus RNA were detected in HIE infected with GII.4 Sydney up to 96 hpi, whereas the initial RNA levels detected for GII.1, GII.2; and GII.3 declined and became undetectable after 48 hpi for GII.1 and GII.2. These data clearly demonstrate that, although HIEs are permissive to infection with different norovirus genotypes, GII.3 and GII.4 replicated with higher efficiency.

We also compared the amount of input viral RNA with the success of replication. Samples that replicated successfully had a significantly higher input titer compared with strains that did not replicate ($p<0.0001$) (Figure 6, panel A). Stratified by genotype, the effect of the initial input amount of virus was observed for infections performed with GII.1, GII.2, GII.4 Den Haag, GII.4 New Orleans, and GII.4 Sydney viruses (Figure 6, panels B, C). To further confirm the role of the amount of virus

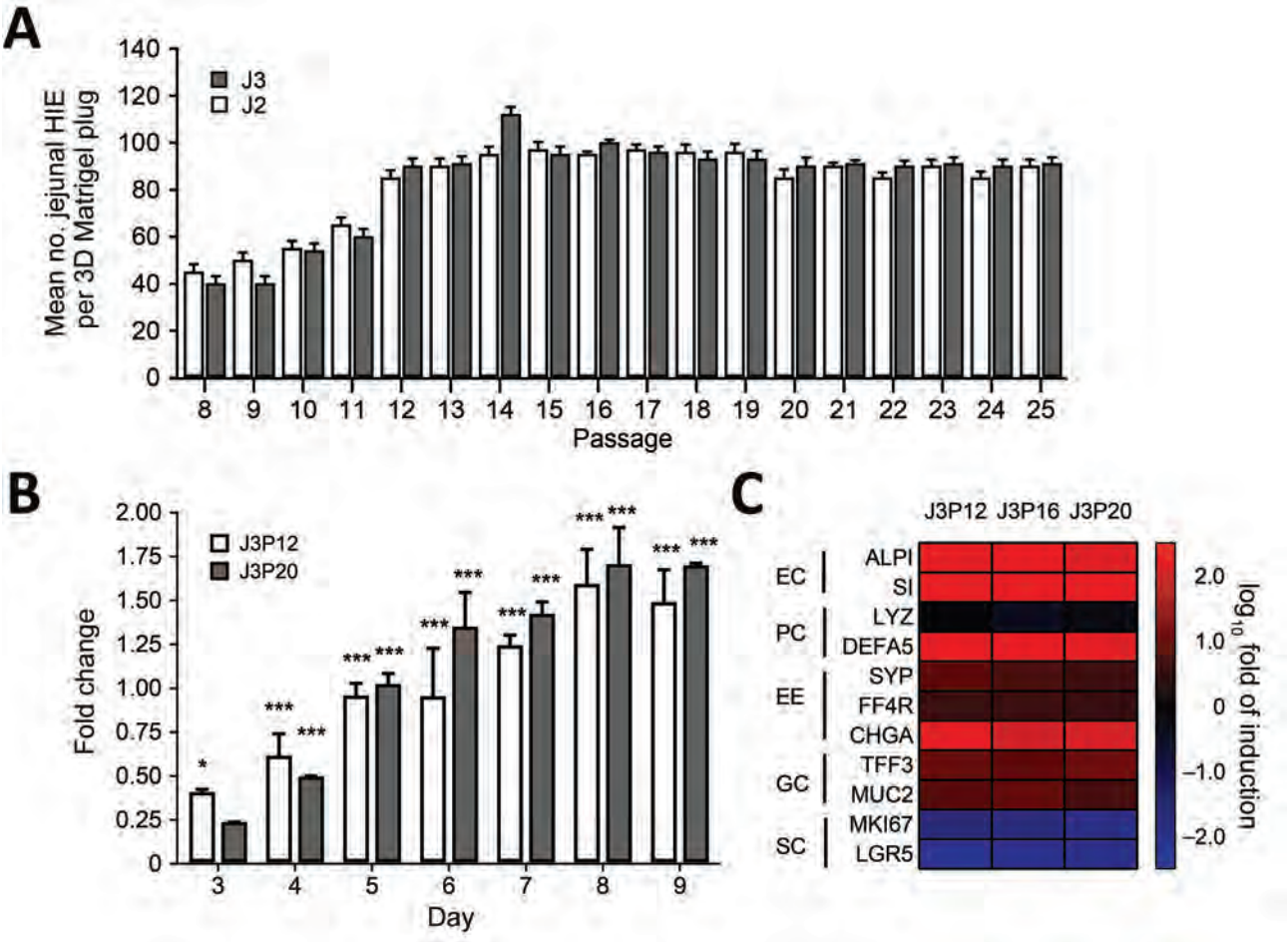

Figure 2. Characterization of differentiated and undifferentiated HIE in a study of human norovirus replication in HIEs. A) Quantification of undifferentiated HIE generated on each passage. Undifferentiated HIEs derived from 2 donors (J2P7 and J3P7) frozen at passage 7 (P7) were recovered from LN_2 and embedded in Matrigel (BD Biosciences, San Jose, CA, USA) (4 plugs per HIE). Cell count was performed at day 7. On that day, undifferentiated HIEs were split 1:2 or 1:3, depending on density, and seeded again in Matrigel. All available wells ($n \geq 4$) per passage were counted. Error bars indicate SD. B) Analysis of stem cell proliferation marker gene LGR5 expression by quantitative reverse transcription PCR in undifferentiated HIEs. HIEs were embedded into Matrigel, seeded in individual wells, and cultured in the presence of complete media with growth factors. RNA was isolated from 2 wells at 1 hour postseed (day 0) and each day during days 3–9. LGR5 expression was normalized to GAPDH and expressed as fold change relative to day 0 (n = 2 wells/bar). Two different passages were assayed (P12 and P20). Error bars indicate SDs; asterisks indicate significant difference from day 0: *p<0.05; ***p<0.001. C) Heat map based on log ($2^{-\Delta\Delta Ct}$) comparing gene expression levels for markers of differentiated small intestinal epithelial cells between undifferentiated and 4-day differentiated HIE monolayers. Experiments were performed with 3 independent cell passages (P12, P16, and P20). Transcripts were normalized to GAPDH levels. Shown are markers for enterocytes (EC), Paneth cells (PC), enteroendocrine cells (EE), goblet cells (GC), and stem cells (SC). Gene symbols: ALPI, intestinal-type alkaline phosphatase; CHGA, chromogranin A; DEFA5, defensin α 5; FFA4R, free fatty acid receptor 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LGR5, leucin-rich repeat-containing G-protein-couple receptor 5; LYZ, lysosyme; MKI67, marker of proliferation Ki-67; MUC2, mucin 2; SI, sucrose isomaltase; SYP, synaptophysin; TFF3, trefoil factor 3. 3D, 3-dimensional; HIE, human intestinal enteroid.

inoculum on the success of replication, we infected HIE monolayers with 10-fold serial dilutions of GII.3 and GII.4 fecal filtrates. The dose required to produce infection in 50% of the inoculated wells (ID_{50}) was 2.1×10^3 genome copies/well for GII.4 Den Haag, 4.4×10^2 genome copies/well for GII.4 Sydney, and 4.0×10^3 genome copies/well for GII.3, based on the Reed-Muench method (Figure 7) (*28*).

Inactivation of Human Norovirus by Alcohols

We next evaluated the efficacy of alcohols to inactivate infectious human norovirus by using 3 successfully replicating GII.4 viruses (GII.4 Den Haag, GII.4 New Orleans, and GII.4 Sydney). Although replication levels of fecal filtrates exposed to 70% ethanol for 1 and 5 minutes were significantly lower compared with nontreated fecal filtrates (p<0.05), none of the GII.4 viruses was completely inactivated by ethanol

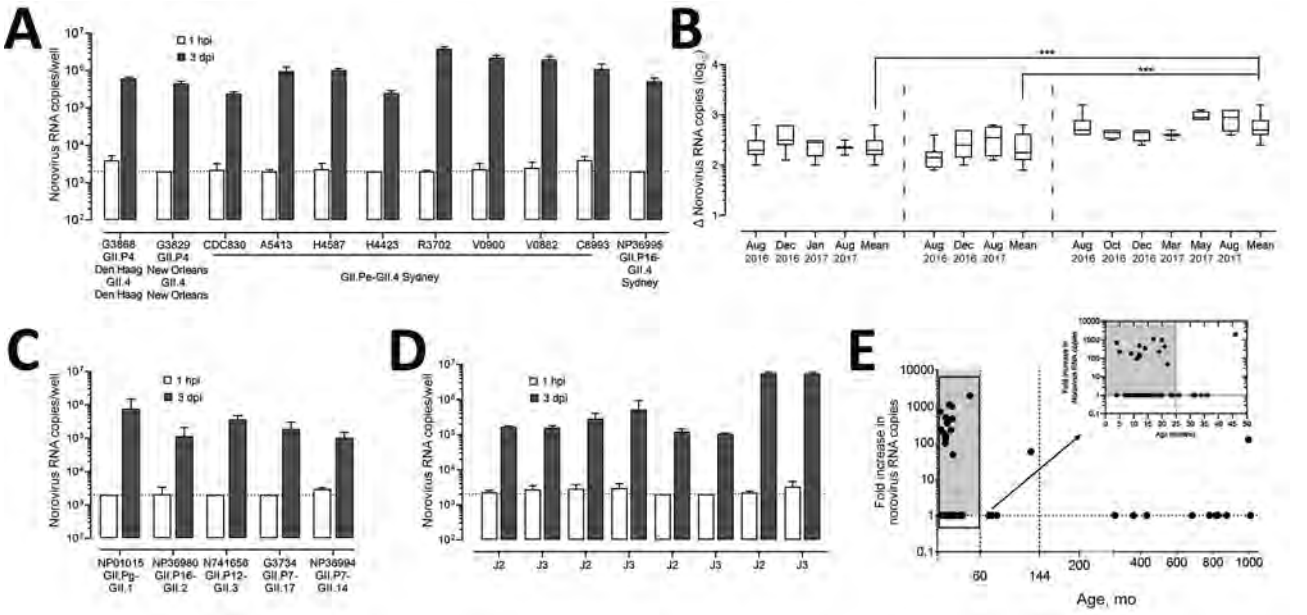

Figure 3. Evaluation of human norovirus replication in human intestinal enteroids (HIEs). A) Jejunal HIE monolayers (J3 line) inoculated with GII.4 P4 Den Haag-GII.4 Den Haag (6.2×10^4 RNA copies/well), GII.4 P4 New Orleans-GII.4 New Orleans (5.3×10^5 RNA copies/well), GII.4 P16-GII.4 Sydney (1.5×10^6 RNA copies/well) or GII.4 Pe-GII.4 Sydney (1.8×10^5 to 3.0×10^6 RNA copies/well). Each experiment was performed 3 times with 3 technical replicates each. B) Replicated infections with fecal filtrates. GII.4 P4 Den Haag-GII.4 Den Haag (6.2×10^4 RNA copies/well), GII.4 P4 New Orleans-GII.4 New Orleans (5.3×10^5 RNA copies/well), GII.4 Pe-GII.4 Sydney (1.8×10^5 RNA copies/well) were included as positive controls throughout 1 year to assess the reproducibility of the HIE culture system. Data are shown as log increase in norovirus RNA per well at 3 dpi compared with 1 hpi. Boxes represent 25th percentile, median, and 75th percentile, and whiskers show minimum and maximum values. Each box represents all experiments performed during the indicated month (GII.P4 New Orleans-GII.4 New Orleans, n = 4–8, total: 18; GII.P4 Den Haag-GII.4 Den Haag, n = 6–15, total: 33; GII.Pe-GII.4 Sydney, n = 6–20, total: 35). ***p<0.0001 comparing log increase in norovirus RNA between 2 GII.4 variants. C) Jejunal HIE monolayers inoculated with NP01015 (GII.Pg-GII.1) at 4.8×10^6 RNA copies per well, NP36980 (GII.P16-GII.2) at 1.51×10^6 RNA copies/well, N741656 (GII.P12-GII.3) at 1.5×10^6 RNA copies/well, G3734 (GII.P7-GII.17) at 2.0×10^3 RNA copies/well or NP36994 (GII.P7–GII.14) at 5.9×10^6 RNA copies/well. Data represent mean ± SD of 3 experiments with 3 technical replicates for each experiment. D) Jejunal HIE monolayers (lines J2 and J3) inoculated with G3734 (GII.P7-GII.17) at 2.0×10^3 RNA copies/well, N741656 (GII.P12-GII.3) at 1.5×10^6 RNA copies/well, NP36980 (GII.P16-GII.2) at 1.51×10^6 RNA copies/well, or R3702 (GII.P16-GII.4 Sydney) at 7.7×10^6 RNA copies/well. Data represent mean ± SD of 2 experiments with 3 technical replicates for each experiment. E) Relationship between age of patient from whom fecal sample was collected and success of replication in HIE. We collected 80 fecal samples during 2000–2017 from children <12 years of age (n = 62) and from adults (n = 18). No replication is indicated as 1. For panels A, C, and D, dotted lines represent quantitative RT-PCR limit of detection. dpi, days postinfection; hpi, hours postinfection.

(Figure 8, panel A). In addition, we treated 2 GII.4 Sydney fecal filtrates (R3702 and CDC830) with 70% ethanol or 70% isopropanol for 5 minutes to rule out the possibility that the observed inactivation patterns were sample specific (Figure 8, panel B). We observed no complete inactivation for any of the tested samples, although the replication levels after treatment with 70% ethanol were up to 0.7 $\log_{10}$ lower, and we found no reduction after exposure to isopropanol (Figure 8, panel B). Following treatment of the fecal filtrates with 70% isopropanol for 5 minutes, norovirus input RNA was still detectable, whereas input titers after 70% alcohol treatment were reduced (1.3–2.9 $\log_{10}$).

Inactivation of Human Norovirus by Chlorine

To evaluate the ability of chlorine to effectively inactivate infectious human norovirus, we treated fecal filtrates of 3 GII.4 viruses with increasing concentrations of chlorine (5–5,000 ppm) for 1 minute. Compared with nontreated controls, all chlorine concentrations ≥50 ppm completely inactivated GII.4 Den Haag, GII.4 Sydney (Figure 8, panels C, D), and GII.4 New Orleans (data not shown). Norovirus input RNA was detectable in all samples that were treated with ≤600 ppm of chlorine.

Discussion

Since the discovery of Norwalk virus, many attempts have been made to culture human noroviruses; most efforts were unsuccessful, or the results were not reproducible in other laboratories (*2–6,9,10*). The successful long-term expansion of intestinal epithelial organoids has been a major breakthrough in the field of in vitro culture of intestinal epithelium (*12,14,15*). Recent studies show that HIEs support

Table 2. Norovirus fecal filtrates successfully cultivated in study of human norovirus replication in human intestinal enteroids

Sample ID	Genotype, RdRp-Capsid*	Mean norovirus RNA copies/μL	Participant age, mo	Collection date†	Storage condition‡	Fold virus RNA increase, log_{10}§
NP01015	GII.Pg-GII.1	1.4×10^5	4	2017 Mar	–70°C	696 (2.8)
NP36980	GII.P16-GII.2	1.9×10^3	132	2017 Feb	–70°C	56 (1.8)
N741656	GII.P12-GII.3	1.4×10^5	9	2012 Aug	–70°C	181 (2.3)
G3868	GII.P4 Den Haag-GII.4 Den Haag	2.0×10^5	12	2015 May	–70°C	155 (2.2)
G3829	GII.P4 New Orleans-GII.4 New Orleans	4.1×10^5	5	2013 Apr	–70°C	227 (2.4)
A5413	GII.Pe-GII.4 Sydney	1.6×10^6	12	2012	–70°C	487 (2.7)
R3702	GII.Pe-GII.4 Sydney	7.7×10^6	46	2015 Jan	–70°C	1236 (3.0)
V0882	GII.Pe-GII.4 Sydney	4.0×10^6	20	2015 Feb	–70°C	998 (3.0)
V0900	GII.Pe-GII.4 Sydney	5.1×10^5	17	2015 Feb	–70°C	1102 (3.0)
H4423	GII.Pe-GII.4 Sydney	5.9×10^5	12	2015 Feb	–70°C	125 (2.1)
H4587	GII.Pe-GII.4 Sydney	4.4×10^6	21	2015 Apr	–70°C	448 (2.7)
C8993	GII.Pe-GII.4 Sydney	1.7×10^7	19	2015 Mar	–70°C	223 (2.3)
CDC830¶	GII.Pe-GII.4 Sydney	2.9×10^6	996	2016 Apr	4°C	121 (2.1)
NP36995	GII.P16-GII.4 Sydney	2.1×10^6	14	2016 Dec	–70°C	340 (2.5)
NP36994	GII.P7-GII.14	6.1×10^4	22	2016 Dec	–70°C	47 (1.6)
G3734	GII.Pe-GII.17	3.4×10^5	11	2010 Oct	–70°C	96 (1.9)

*Dual genotyping based on sequencing partial RdRp and capsid regions (*26*,*27*). RdRp, RNA-dependent RNA polymerase.
†When month was not available, only year of collection is reported.
‡Samples were kept from collection time until testing at the indicated temperature.
§Virus RNA increase after 3 days postinfection expressed as fold increase and log_{10} fold increase. For each fecal filtrate, data represent mean viral RNA increase from 3 experiments with 3 technical replicates each.
¶CDC830 was stored at 4°C for 2 mo before cultivation.

replication of human norovirus and other enteric viruses (*13*,*16*,*29*) and enable analysis of the innate immune response against these viruses (*30*). In this study, we showed successful replication of 6 GII norovirus genotypes (GII.1, GII.2, GII.3, GII.4, GII.14, and GII.17), including 3 GII.4 variants. Repeated infections conducted over a 1-year period showed consistent increase in viral titers of these 3 GII.4 variant strains, demonstrating that the HIE model is robust. Our data also demonstrate that, after initial confirmation of infectivity, storage of fecal samples at –70°C will preserve virus infectivity for at least 1 year.

We showed successful replication for 6 of the 14 genotypes tested in this study, although the success rate varied. Strain-specific differences have been reported for other viruses grown in HIEs (*13*,*29*). For example, compared with echovirus 11 and coxsackievirus B, enterovirus 71 replicates to significantly lower levels in HIEs (*29*). Enteroids also support robust replication of human rotavirus

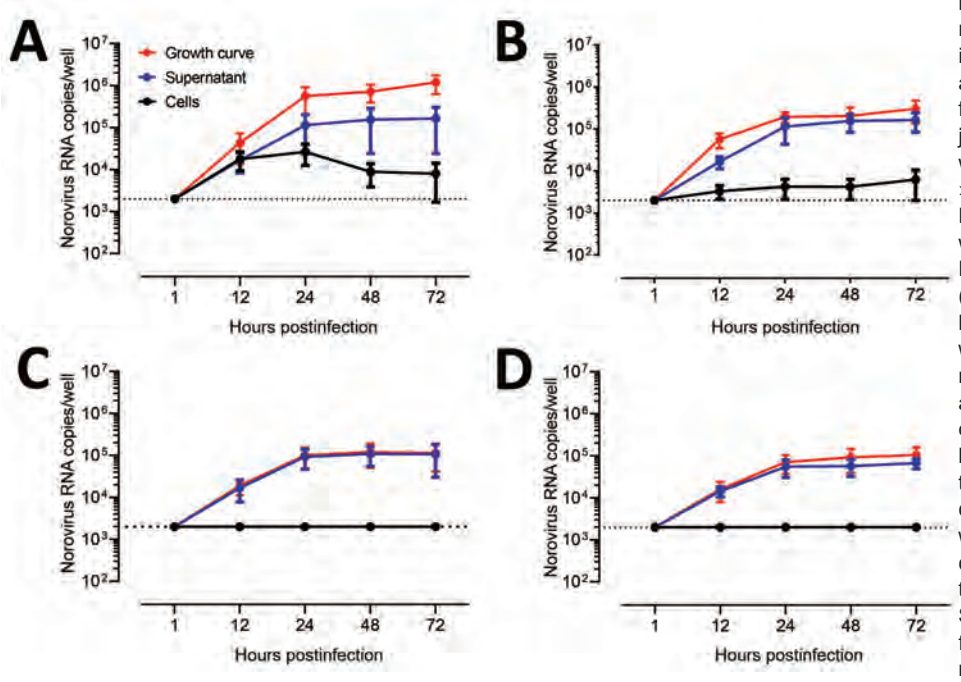

Figure 4. Evaluation of human norovirus replication in human intestinal enteroids (HIEs) by assessment of kinetics of infection for 4 GII genotypes. We inoculated jejunal HIE monolayers (J3 line) with A) GII.4 Pe-GII.4 Sydney (3.3×10^5 RNA copies/well), B) GII.P12-GII.3 (5.3×10^5 RNA copies/well), C) GII.P16-GII.2 (3.2×10^5 RNA copies/well), or D) GII.Pg-GII.1 (9.3×10^5 RNA copies/well). After 1 h at 37°C in 5% CO_2, monolayers were washed, and medium was replaced with differentiation media and incubated for 3 d. For the growth curve, we extracted RNA from frozen lysates (cells and supernatant) at the indicated time points. For the cells vs. supernatant experiment, we removed supernatants by centrifugation before harvesting the cells. Data represent mean ± SD of 2 experiments with 3 wells for each time point. Dotted lines represent quantitative RT-PCR limit of detection.

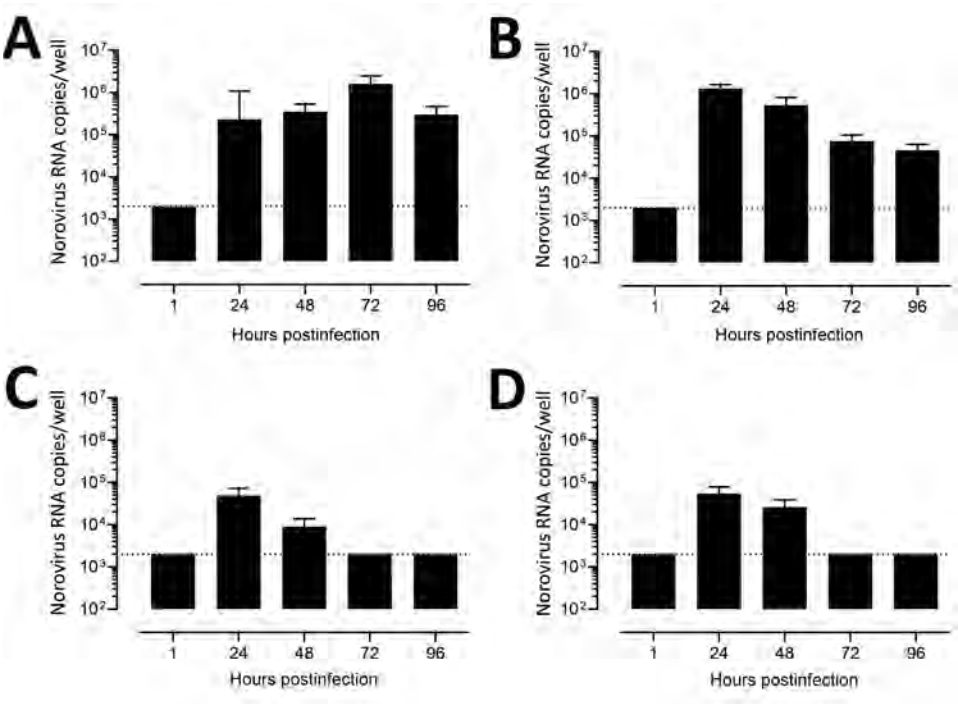

Figure 5. Confirmatory testing for human norovirus replication in human intestinal enteroids (HIEs). We inoculated jejunal HIE monolayers (J3 line) with A) GII.4 Pe-GII.4 Sydney (3.3×10^5 RNA copies/well), B) GII.P12-GII.3 (5.3×10^5 RNA copies/well), C) GII.P16-GII.2 (3.2×10^5 RNA copies/well), or D) GII.Pg-GII.1 (9.3×10^5 RNA copies/well). After 1 h at 37°C in 5% CO_2, monolayers were washed, and media was replaced with differentiation media and incubated at 37°C and 5% CO_2. At 24, 48, and 72 hours postinfection, we removed the medium and added fresh differentiation media. At 96 hours postinfection, we removed the medium. We extracted RNA and quantified it by quantitative reverse transcription PCR from the supernatant at each time point. Data represent mean ± SD of 2 experiments with 3 wells for each time point. Dotted lines represent RT-qPCR limit of detection.

strains Ito (G3P[8]) and Wa (G1P[8]) but not the attenuated G1P[8] human rotavirus vaccine strain (*13*). Ettayebi et al. also demonstrated that GII.4 Sydney strains infect enteroids with higher efficiency than do GI.1, GII.3, and GII.17 viruses (*16*); in our study, GII.4 norovirus strains replicated at higher efficiency than did GII.1, GII.2, and GII.3 viruses. In addition, the ID_{50} values for GII.4 and GII.3 viruses were slightly lower (2–5×) than reported previously (*16*). Taken together, these results indicate that high viral RNA titers are not a guarantee for successful replication, perhaps suggesting that fecal specimens that do show norovirus replication may contain large numbers of noninfectious particles.

Until now, evaluation of control measures for human norovirus, including disinfection measures, has relied primarily on the use of cultivable surrogate viruses (*24*). Although these viruses are similar in size and genome organization, none completely mimics the inactivation patterns of human norovirus based on reduction of viral RNA titers. We demonstrated that the HIE model can be used to evaluate the effectiveness of alcohols and chlorine against human norovirus. Although 5 minutes of exposure to 70% ethanol and isopropanol slightly reduced viral RNA levels, overall, the alcohols did not inactivate GII.4 viruses. These results are in agreement with a previous study that, based on lack of reduction of viral RNA titers, suggested that GII human noroviruses are not affected by alcohol (*24*). In a comprehensive study comparing different cultivable surrogate viruses for human norovirus, Cromeans et al. (*24*) showed that Tulane virus, but not feline calcivirus or MNV, was resistant to alcohols. Using HIEs, we now demonstrate that human norovirus closely resembles Tulane virus when measuring inactivation by alcohols.

For chlorine, our data showed that complete inactivation of 3 different GII.4 strains could be achieved with concentrations as low as 50 ppm. These results are consistent with a recent report indicating that treatment with chlorine concentrations <50 ppm were not sufficient to inactivate human norovirus in secondary effluents from water treatment plants (*31*). In conclusion, our inactivation data demonstrate that chlorine, but not alcohol, completely inactivates human norovirus and that evaluation of inactivation strategies based only on detection of viral RNA does not always reflect the effectiveness of the treatment.

Our study has several limitations. First, the success rate of samples with a moderate viral RNA titer was relatively low, and thus far we have had no success with GI and GIV samples. However, because we were also not able to replicate several high viral load GII samples, other, currently unknown, factors also contribute to successful replication. Second, although we demonstrated that infectious particles are produced and we were able to measure complete inactivation by chlorine treatment, we analyzed only viral RNA levels, not protein levels. Further work is needed on the amount of chlorine required to inactivate human norovirus because we used fecal samples, which inherently have

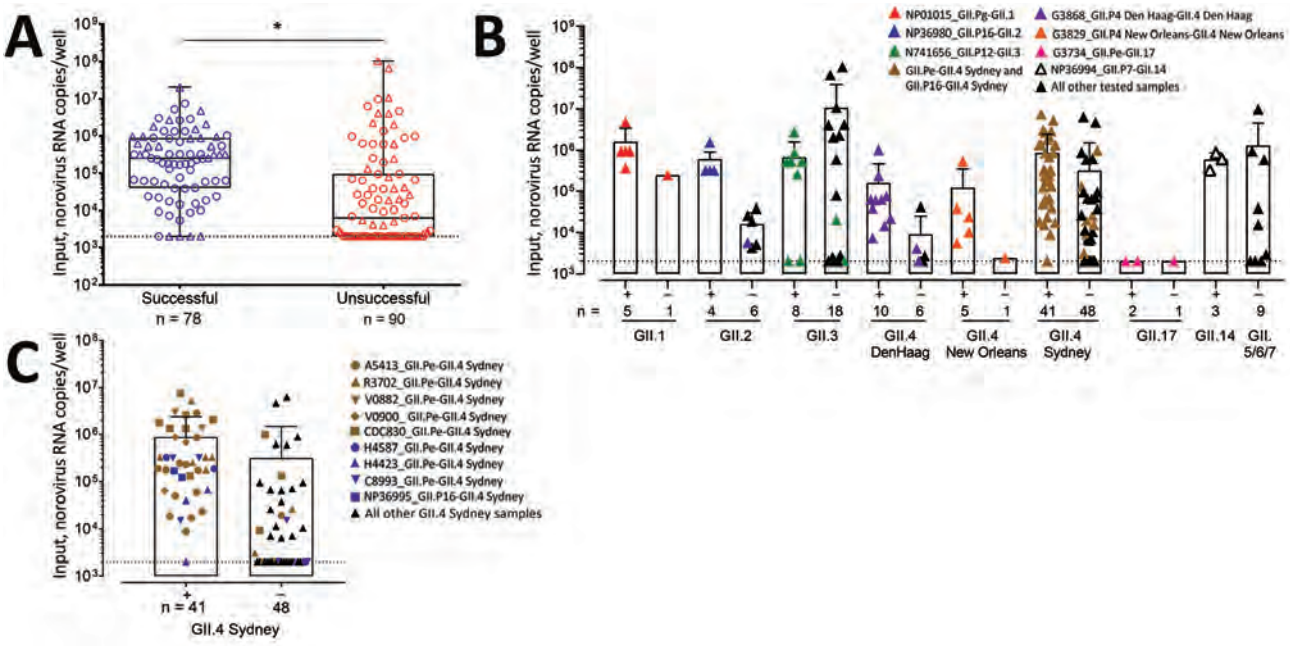

Figure 6. Comparison of amount of input viral RNA with success of human norovirus replication in human intestinal enteroids (HIEs). A) We infected HIE monolayers with undiluted or prediluted (1:10; 1:100; 1:1000) 10% fecal filtrates. Each dot represents the input norovirus RNA per well of a single experiment (n = 168) that resulted in successful (n = 78) or unsuccessful (n = 90) virus replication. Boxes represent 25th percentile, median, and 75th percentile, and whiskers show the minimum and maximum values. Circles indicate GII.4 genotypes and triangles non-GII.4 genotypes. ***p<0.001 by Mann-Whitney test. B, C) Role of initial norovirus RNA input in successful (+) and unsuccessful (–) human norovirus infections. We infected HIE monolayers with undiluted or prediluted (1:10; 1:100; 1:1000) 10% fecal filtrates and incubated them at 37°C in 5% CO_2 for 3 d. We extracted RNA and quantified it by quantitative reverse transcription PCR from frozen lysates (cells and supernatant) at 1 hour postinfection and 3 days postinfection. Data points represent individual experiments. Bars represent mean ± SD. Dotted lines represent RT-qPCR limit of detection. Samples that successfully replicate at high, but not low, concentration colored are colored and listed in Table 2.

a high chlorine burden, and we measured total chlorine, whereas the level of free chlorine is what actually determines inactivation. Finally, the HIE model is costly and labor intensive; additional improvements are required to make it more affordable and widely available.

In conclusion, we confirmed that the HIE system to culture human norovirus (*16*) can be successfully implemented in another laboratory. The culture system supported identical levels of replication of a panel of human norovirus strains consistently for >1 year. The success of replication depends on genotype and initial virus titer but also on other unknown factors. Additional HIE cell lines need to be tested or cultures need to be enriched for enterocytes (*32*) because replication of some noroviruses is restricted by cell line characteristics (*16*). In addition, whether infectivity is limited by the presence of virus-specific fecal antibodies and the possibility that cellular host factors may prevent or limit replication of certain

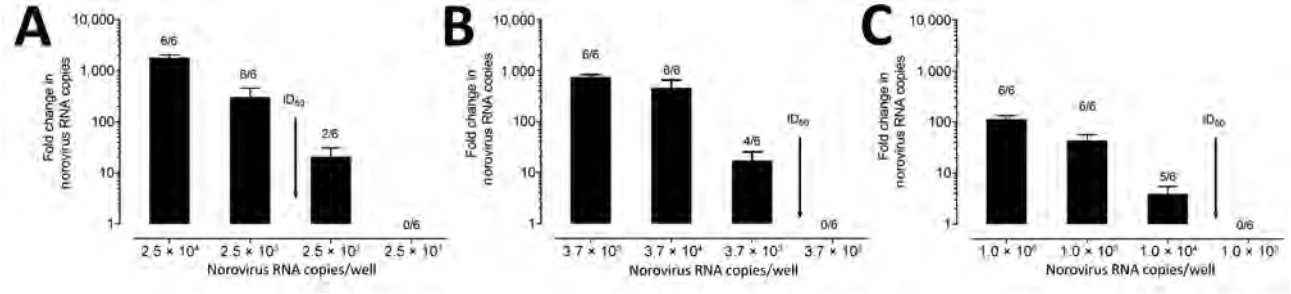

Figure 7. Determination of ID_{50} required for human norovirus replication in human intestinal enteroids (HIEs). We inoculated HIE monolayers in triplicate with 10-fold serial diluted fecal filtrates A) A5413_GII.4 Sydney, B) G3868_GII.4 Den Haag, or C) N741656 _ GII.3 RNA copies and incubated them for 1 h at 37°C. We washed the monolayers 3 times and cultured them in differentiation media for 3 d. We extracted RNA and quantified it by quantitative reverse transcription PCR from frozen lysates (cells and supernatant) at 1 hour postinfection and 3 days postinfection. We calculated ID_{50} using the Reed-Muench method (*28*). ID_{50}, 50% infectious dose.

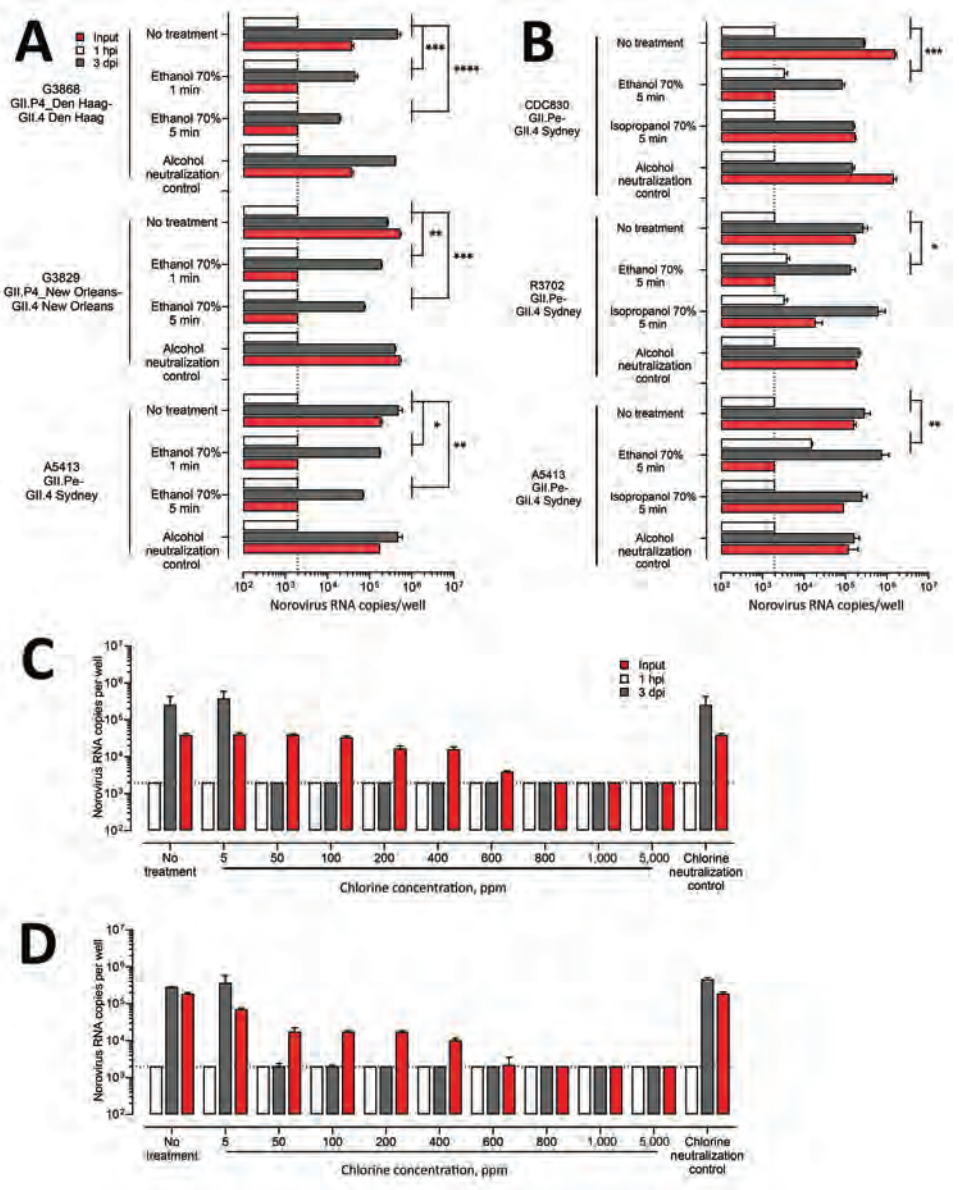

Figure 8. Inactivation of human norovirus with 70% alcohol or chlorine solution in suspension. A) Ten percent fecal filtrates (G3868 [GII.4 Den Haag], 2.04 × 10^6 RNA copies; G3829 [GII.4 New Orleans], 4.14 × 10^6 RNA copies; A5413 [GII.4 Sydney], 1.58 × 10^7 RNA copies) were either treated or not treated with 70% ethanol for 1 min or 5 min at room temperature. We added complete media without growth factors supplemented with 10% fetal bovine serum to neutralize remaining ethanol. B) Ten percent fecal filtrates from 3 GII.4 Sydney strains (CDC830, 2.89 × 10^7 RNA copies; R3702, 7.73 × 10^7 RNA copies; A5413, 1.58 × 10^7 RNA copies) were either treated or not treated with 70% ethanol or 70% isopropanol for 5 min at room temperature and neutralized with complete media without growth factors supplemented with 10% fetal bovine serum. C, D) Ten percent fecal filtrates (G3868, 2.04 × 10^6 RNA copies [C]; A5413, 1.58 × 10^7 RNA copies [D]) were either treated or not treated with freshly prepared chlorine solutions of increasing concentrations (5, 50, 100, 200, 400, 600, 800, 1,000, and 5,000 ppm) for 1 min at room temperature. Sodium thiosulfate (final concentration 50 mg/L) was added to neutralize the remaining free chlorine. For all experiments, HIEs were then infected with 100 μL of treated or not treated fecal filtrate. After 1 h at 37°C in 5% CO_2, we washed the monolayers, added differentiation media, and incubated for 3 d. Data represent mean ± SD of 2 experiments with 3 wells for each treatment and time point. For each fecal filtrate, we performed 1-way analysis of variance followed by Dunnett's test. Dotted lines represent RT-qPCR limit of detection. p values are compared with the nontreated fecal filtrate: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. dpi, days postinfection; hpi, hours postinfection.

genotypes all indicate that more research is needed to further optimize this cultivation system.

This study was supported by the intramural food safety program at the Centers for Disease Control and Prevention and in part by Agriculture and Food Research Initiative Competitive Grant 2011-68003-30395 from the US Department of Agriculture, National Institute of Food and Agriculture and PHS grants P01AI 057788 and P30 DK 56338. Names of specific vendors, manufacturers, or products are included for public health and informational purposes; inclusion does not imply endorsement of the vendors, manufacturers, or products by the Centers for Disease Control and Prevention or the US Department of Health and Human Services.

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Clonal Expansion of Macrolide-Resistant Sequence Type 3 *Mycoplasma pneumoniae*, South Korea

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To investigate the genetic background for the emergence of macrolide resistance, we characterized the genetic features of *Mycoplasma pneumoniae* using multilocus sequence typing. Of the 146 *M. pneumoniae* strains collected during the 5 consecutive outbreaks of *M. pneumoniae* pneumonia during 2000–2016 in South Korea, macrolide resistance increased from 0% in the first outbreak to 84.4% in the fifth. Among the 8 sequence types (STs) identified, ST3 (74.7%) was the most prevalent, followed by ST14 (15.1%). Macrolide-susceptible strains comprised 8 different STs, and all macrolide-resistant strains were ST3 (98.3%) except 1 with ST14. The proportion of macrolide-resistant strains in ST3 remained 2.2% (1/46) until the 2006–2007 outbreak and then markedly increased to 82.6% (19/23) during the 2010–2012 outbreak and 95.0% (38/40) during the 2014–2016 outbreak. The findings demonstrated that clonal expansion of ST3 *M. pneumoniae* was associated with the increase in macrolide resistance in South Korea.

Mycoplasma pneumoniae is one of the major causes of community-acquired pneumonia in children and adolescents (*1*). *M. pneumoniae* pneumonia develops with a gradual onset of constitutional symptoms over several days to a week (*2*). Although most patients may have self-limited symptoms resembling those of an upper respiratory infection, *M. pneumoniae* is recognized for producing a broad array of extrapulmonary manifestations that include hemolysis, rash, and joint involvement (*1*,*3*). Previous studies have established that the P1 adhesin (P1), a 170-kd surface protein located at the tiplike structure of virulent *M. pneumoniae*, mediates its cytadherence to the surface of respiratory epithelial cells, which is a critical step in the infection process (*4*).

Epidemiologic studies have shown that outbreaks of *M. pneumoniae* pneumonia occur every 3–7 years, varying from region to region on the basis of underlying low-grade endemic activity (*5*,*6*). Because the P1 is a major determinant of virulence, most studies have targeted the genetic variations of the *p1* gene to explain specific genotypes that link to the outbreaks (*7*–*9*). However, because *M. pneumoniae* has a small genome size, its genomic diversity is known to be limited among strains, and any associations of specific genotypes with disease outbreaks are rarely found (*7*,*10*,*11*). Since the first report of a macrolide-resistant *M. pneumoniae* isolated from a child in Japan in 2001 (*12*), several countries in Asia, including South Korea, Japan, and China, have reported increased prevalence of macrolide resistance (*13*–*16*). Point mutations in domain V of 23S rRNA are responsible for macrolide resistance. High antimicrobial consumption may provide selective pressure for the development of macrolide resistance (*13*). However, rapid dissemination of multiple clones that exhibit macrolide resistance also can lead to the increase in macrolide resistance rates in the community (*17*).

The multilocus sequence typing (MLST) scheme was first applied in *Neisseria meningitidis* and is a tool widely used for strain differentiation in many types of bacteria (*18*). Recently, Brown et al. developed an MLST scheme for *M. pneumoniae* using 8 housekeeping genes with a relatively high discriminatory ability (*19*). MLST has the potential to be used as a tool to characterize strains isolated during epidemic outbreaks of *M. pneumoniae* pneumonia and to

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DOI: https://doi.org/10.3201/eid2408.180081

[1]These authors contributed equally to this article.

investigate relatedness of specific genetic background to the emergence of macrolide resistance.

To clarify the genetic diversity of *M. pneumoniae* strains between outbreaks, we conducted an MLST analysis of *M. pneumoniae* detected from children in whom community-acquired pneumonia was diagnosed during 5 consecutive epidemics of *M. pneumoniae* pneumonia during 2000–2016 in South Korea. We also sought to find the genetic background that may explain the emergence of macrolide resistance among *M. pneumoniae* strains.

Materials and Methods

M. pneumoniae Strains

This study comprised *M. pneumoniae* strains detected from children with pneumonia at 5 hospitals during 5 consecutive outbreaks of *M. pneumoniae* pneumonia in South Korea: 2000, 2003–2004, 2006–2007, 2010–2012, and 2014–2016. Epidemic periods were previously defined by an interval spanning an increase of ≥5 cases/2 months over a 4-month period to a decrease of <5 cases/2 months over a 4-month period in the primary site of this study (*6*,*14*). *M. pneumoniae* pneumonia was diagnosed using the following criteria: 1) the presence of rales on auscultation or infiltration of the lung demonstrated on chest radiograph and 2) a positive PCR result for *M. pneumoniae* or isolation of *M. pneumoniae* on culture. Five hospitals participated in this study: Seoul National University Children's Hospital (Seoul), Seoul National University Bundang Hospital (Seongnam), Eulji Hospital (Seoul), Chungnam University Hospital (Daejeon), and Chonnam University Hospital (Gwangju). Two hospitals in Seoul and 1 hospital in Seongnam cover the Seoul metropolitan area, where almost half of the South Korea population resides. Daejeon is representative of central South Korea, and Gwangju represents the south. *M. pneumoniae* strains detected from community-acquired cases were included for further analysis. We excluded healthcare-associated infections and intrafamilial infections.

DNA Extraction from *M. pneumoniae* and Macrolide Resistance

We extracted DNA directly from the cultivated *M. pneumoniae* or from nasopharyngeal aspirates using an extraction kit (DNeasy Kit; QIAGEN, Hilden, Germany), according to the manufacturer's instructions. We amplified the *p1* gene by PCR for the detection of *M. pneumoniae*. Starting in 2010, *M. pneumoniae* was cultivated using pleuropneumonia-like organism broth and agar for nasopharyngeal aspirates or pleural fluid obtained from the patient as previously described (*20*). The mutations responsible for macrolide resistance were confirmed by sequencing analysis of the amplified PCR products for domain V of the 23S rRNA gene. Primers MP23SV-F 5′-TAACTATAACGGTCCTAAGG-3′ and MP23SV-R 5′-ACACTTAGATGCTTTCAGCG-3′ were used, and PCR products were sequenced to identify mutations (*14*).

MLST Analysis and P1 Typing

We performed MLST on the *M. pneumoniae* DNA samples as previously described. Each allele was assigned to the 8 housekeeping genes (*ppa*, *pgm*, *gyrB*, *gmk*, *glyA*, *atpA*, *arc*, and *adk*), and a corresponding sequence type (ST) was given for each sample (*19*). We submitted new alleles and allelic profiles to the PubMLST database for MLST assignment (http://pubmlst.org/mpneumoniae/). We used eBURST version 3 software (http://eburst.mlst.net/) to estimate the relationships among the strains and to assign strains to a clonal complex (CC) (*21*). We also conducted P1 typing for the samples from 2000–2016 by sequencing 2 of the repetitive elements located in the *p1* gene of *M. pneumoniae* genome: *RepMP2/3* and *RepMP4*. We assigned P1 subtypes and each subtype variant by comparison with previously published data (*22*).

Statistical Analysis

We conducted statistical analysis using IBM SPSS Statistics for Windows version 23.0 (IBM Corp., Armonk, NY, USA). A linear-by-linear association model was used for Pearson's χ^2 test for trend analyses. We considered a p value of <0.05 as significant.

Ethics Statement

The institutional review board of Seoul National University Hospital approved the study protocol (IRB no. H-1012–007–341). Informed consent was exempted because nasopharyngeal aspirates were obtained as a standard of patient care to identify the etiologic agents of acute lower respiratory tract infections.

Results

M. pneumoniae Strains and Macrolide Resistance

Our study comprised 146 *M. pneumoniae* DNA samples. Samples included for each outbreak were selected as follows: 21 samples from 2000, 14 samples from 2003–2004, 29 samples from 2006–2007, 37 samples from 2010–2012, and 45 samples from 2014–2016. Until the 2006–2007 outbreak, DNA samples were directly collected from respiratory samples (64 samples), and DNA samples from the 2010–2012 outbreak were extracted from cultured *M. pneumoniae* (82 samples).

For the samples before 2010, we included all the available specimens because of a limited number of archived samples relative to the 2010–2012 and 2014–2016 outbreaks. During the 2 outbreaks for which we have a larger

number of samples, we selected samples to represent geographic region, month of isolation, and ages of patients. The proportions of selected samples were 28.5% (2010–2012) and 32.4% (2014–2016) of the archived samples. Of the study population, 56.1% were male. The mean age of children was 6.5 years; 7.4% were <2 years of age, 32.1% were 2–5 years of age, 60.5% were ≥5 years of age. The remaining 187 samples that were not selected for this study did not differ significantly from selected samples with respect to mean patient age and geographic region.

From the 146 *M. pneumoniae* strains investigated, 59 (40.4%) strains expressed macrolide resistance associated with either an A2063G (58 [98.3%]) or A2064G (1 [2.7%]) mutation in the 23S rRNA gene. Differences in macrolide resistance were recognized in each of the outbreaks (Figure 1). Strains from the 2000 and the 2003–2004 outbreaks were all susceptible to macrolide. The proportion of macrolide-resistant strains for each outbreak was 3.4% for 2006–2007, 54.1% for 2010–2012, and 84.4% for 2014–2016. The trend analysis for macrolide resistance across the 5 periods showed a significant increase (0% to 84.4%; p<0.0001).

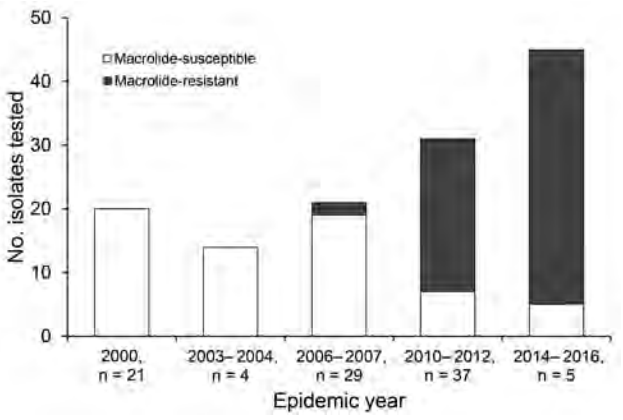

Figure 1. Macrolide resistance of *Mycoplasma pneumoniae*, South Korea, 2000–2016. Each number on the bar graph indicates the macrolide resistance of each epidemic year. The proportion of macrolide-resistant strains by each outbreak were as follows: 0% (2000 and 2003–2004), 3.4% (2006–2007), 54.1% (2010–2012), and 84.4% (2014–2016).

MLST and P1 Typing of *M. pneumoniae*

MLST analysis identified 8 STs during the study period: ST1, ST2, ST3, ST7, ST14, ST15, ST17, and ST31 (Table 1). The epidemic distribution of STs is shown in Figure 2. During the study period, ST3 (109 [74.7%]) was the most commonly identified ST, followed by ST14 (22 [15.1%]). ST3 was also the predominant ST found during all 5 outbreaks. A total of 3–5 STs circulated during each outbreak, and several minor STs (ST7, ST15, and ST31) that circulated in the earlier outbreaks were not found in the recent outbreaks. The distribution of ST did not differ by geographic region.

We conducted P1 typing for 85 strains from 2000–2016. Overall, we identified 5 subtypes and subtype variants of P1 (1, 2, 2a, 2b, and 2c). P1 subtype 1 was the main subtype at 70.6% (60 strains), followed by subtype 2 with 29.4% (25 strains). P1 subtype 1 accounted for 85.7% of the 2000 outbreak, 42.9% in 2003–2004, 75.0% in 2006–2007, 71.9% in 2010–2012, and 50.0% in 2014–2016. P1 subtypes 2 and 2a were observed up until the 2003–2004 outbreak, and subtype variant 2c was observed from the 2006–2007 outbreak. P1 subtype variant 2c (13 [52.0%]) was most common within P1 subtype 2, followed by subtype variants 2a (7 [28.0%]), 2 (4 [16.0%]), and 2b (1 [4.0%]). Each ST was associated with a single P1 subtype or subtype variant, except for ST14 strains, which possessed both P1 subtype variants 2a and 2c.

Sequence Type and Macrolide Resistance

Macrolide-susceptible strains consisted of 8 different STs identified. Among 8 STs, ST3 (58/109 [53.2%]) and ST14 (1/22 [4.5%]) were the only ones that expressed macrolide resistance (Table 2). One ST14 strain that expressed macrolide resistance was from the 2010–2012 outbreak and harbored the A2063G mutation.

We found a correlation between the increasing proportion of macrolide resistance and the proportion of macrolide-resistant strains within ST3. All of the strains in ST3 were macrolide susceptible until the 2003–2004 outbreak, and only 1 of the 22 strains in ST3 showed macrolide resistance in the 2006–2007 outbreak. The proportion of macrolide-resistant strains within ST3 dramatically increased to 82.6% (19/23) during the 2010–2012 outbreak and to

Table 1. *Mycoplasma pneumoniae* STs and allelic profile of each ST with corresponding P1 type, South Korea, 2000–2016*

ST	No. (%) isolates	Allelic profile								P1 type
		ppa	*pgm*	*gyrB*	*gmk*	*glyA*	*atpA*	*arcC*	*adk*	
ST1	2 (1.4)	1	2	1	1	1	3	2	1	1
ST2	1 (0.7)	2	3	2	2	2	4	1	1	2b
ST3	109 (74.7)	1	2	1	1	1	3	1	1	1
ST7	2 (1.4)	2	3	2	2	2	4	1	2	2
ST14	22 (15.1)	2	3	2	2	4	4	1	5	2a, 2c
ST15	4 (2.7)	2	3	2	2	4	4	1	1	2a
ST17	4 (2.7)	1	5	1	1	1	3	1	1	1
ST31†	2 (1.4)	2	8	2	2	2	4	1	1	2

*ST, sequence type.
†Newly identified ST in this study.

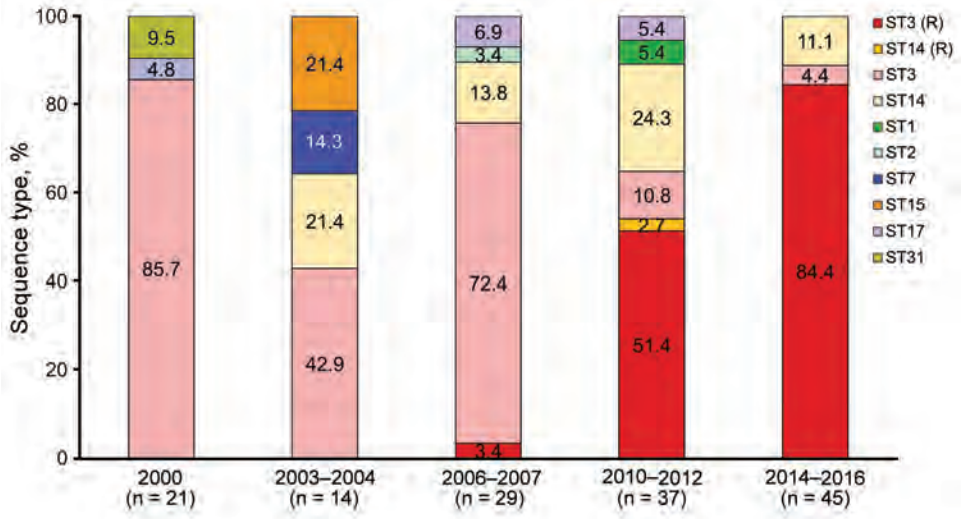

Figure 2. *Mycoplasma pneumoniae* ST distribution by each outbreak and macrolide resistance within specific STs, South Korea, 2000–2016. Each number of the box indicates proportion of each ST. (R) designates macrolide resistance. ST, sequence type.

95.0% (38/40) during the 2014–2016 outbreak (Table 3). These data strongly suggest that a clonal expansion of ST3 *M. pneumoniae* is responsible for the increasing proportion of macrolide resistance.

eBURST Analysis

Two CCs were identified on eBURST analysis (Figure 3). CC1 contained 115 (78.8%) strains with 3 STs, and CC2 contained 31 (21.2%) strains with 5 STs. ST3 and ST2 were predicted to be the founder ST for each CC. The newly identified ST in this study was ST31, which was part of CC2 and a single-locus variant of ST2.

Discussion

In this study, molecular microbiological analysis of MLST found that the increase of macrolide resistance in South Korea during a 17-year period (2000–2016) was related to changes in genetic backgrounds of *M. pneumoniae* strains. Traditionally, diagnosis of *M. pneumoniae* pneumonia relied on the increase of mycoplasma antibody or the presence of IgM. However, with the emergence of macrolide resistance, it became important to know the presence of macrolide resistance of *M. pneumoniae*. Thus, direct detection of *M. pneumoniae* either by culture or by PCR is crucial for testing macrolide resistance. Although no treatment strategy for macrolide-resistant *M. pneumoniae* pneumonia has yet been established, alternative antimicrobial drugs (tetracyclines and fluoroquinolones) can be considered when patients remain febrile at least 48–72 hours after macrolide treatment (*23*,*24*).

Table 2. Distribution of *Mycoplasma pneumoniae* STs by macrolide susceptibility, South Korea, 2000–2016*

ST (no. isolates)	No. (%) isolates	
	Macrolide susceptible	Macrolide resistant
ST1 (2)	2 (2.3)	0
ST2 (1)	1 (1.1)	0
ST3 (109)	51 (58.6)	58 (98.3)
ST7 (2)	2 (2.3)	0
ST14 (22)	21 (24.1)	1 (1.7)
ST15 (4)	4 (4.6)	0
ST17 (4)	4 (4.6)	0
ST31 (2)	2 (2.3)	0
Total (146)	87 (100)	59 (100)

*ST, sequence type.

The whole genome of *M. pneumoniae* is ≈820 kb and has up to 700 coding operons. On the basis of the data of the comparative analysis of 58 strains, the genome appears to be highly conservative among the strains (*1*). Because of relatively low sequence variations and many repetitive elements within the genome of *M. pneumoniae*, epidemiologic investigations on genetic diversity have focused on the sequence variations of *p1* gene. Several studies explored an association between P1 subtypes and epidemic outbreaks. The postulation was based on the idea that development of temporary immunity to 1 type by an outbreak might enable reemergence of the other (*7*). Despite experimental grounds and the scientific reasoning, studies that followed did not support the hypothesis. A study from Germany that examined the P1 molecular typing of 467 *M. pneumoniae* did not support predominance of 1 of the 2 major P1 subtypes or switching of the subtypes during the endemic situation before and during the outbreak period (*10*). Furthermore, Diaz et al. examined 199 *M. pneumoniae* samples from 17 investigations of cases, small clusters, and outbreaks that were supported by the Centers for Disease Control and Prevention (Atlanta, GA, USA) to determine the association of P1 subtypes with macrolide resistance (*11*). In that study, the distribution of P1 subtypes did not differ between macrolide-resistant and macrolide-susceptible *M. pneumoniae* strains, suggesting that an individual P1 subtype is not associated with macrolide-resistant genotype.

Table 3. Distribution of macrolide susceptibility within *Mycoplasma pneumoniae* sequence type 3, South Korea, 2000–2016

Macrolide susceptibility	Epidemic years, no. (%) strains				
	2000	2003–2004	2006–2007	2010–2012	2014–2016
Susceptible	18 (100)	6 (100)	21 (95.5)	4 (17.4)	2 (5.0)
Resistant			1 (4.5)	19 (82.6)	38 (95.0)
Total	18	6	22	23	40

Molecular typing methods other than P1 typing have attempted to discriminate strains in each outbreak and to find correlations between strain diversity and macrolide resistance of *M. pneumoniae*. Recent studies use sophisticated technologies such as quantitative PCR for the diagnosis and multilocus variable-number tandem-repeat analysis (MLVA) for characterization and classification. Waller et al. reported 7 different MLVA profiles associated with certain P1 subtypes from 12 *M. pneumoniae* strains during an outbreak in the United States (*25*). MLVA and MLST also were adopted for studying *M. pneumoniae* in recent years (*19*,*26*). MLVA uses naturally occurring variations in the number of tandem repeated DNA sequences found in many different loci of the genome. MLST characterizes the isolates of microbial species using DNA sequences from internal fragments of multiple housekeeping genes. Of the 2 molecular typing methods, the discriminatory power of MLST scheme with the 8 loci was 0.784 for the collection of 57 isolates, whereas MLVA scheme was 0.633 (*19*,*27*). This finding was due partly to removal of the *Mpn1* locus in MLVA scheme because of inconsistency in nomenclature and identification of repeat regions (*28*).

Sun et al. reported that the rates of resistance mutations increased in parallel with an increase in MLVA type 4572 during 2003–2007 and 2008–2013 and decreased in parallel with a decrease in type 4572 during 2014 and 2015, based on 480 *M. pneumoniae* isolates from children in Beijing, China, during 2003–2015 (*29*). A study of MLVA typing of *M. pneumoniae* strains isolated during 2004–2014 in Yamagata, Japan, reported that the prevalence of macrolide resistance–associated mutations in type 4572 was 59.7% (108/181), which was significantly higher than in other MLVA strains (*30*). The prevalence of the A2063G mutation in type 4572 strains was 0.9% (1/107) during 2004–2010 but became 83.8% (62/74) during 2011–2014. A recent study from Hong Kong reported an increased prevalence in macrolide resistance as well and identified type 4572 strain as the contributor (*31*). In that study, the authors reported that the macrolide resistance rate for type 4572 significantly increased from 25.0% in 2011 to 100% in 2014. In contrast to those studies, a study of 152 *M. pneumoniae* strains conducted by Liu et al. suggested that macrolide-resistant strains were multiclonal origin (*17*). The results of that study clustered 137 macrolide-resistant strains into 15 MLVA types, indicating the high rate of macrolide resistance could result from dissemination of the multiple resistant clones. This conflicting result might have resulted from the 5 loci MLVA, which applied the earlier MLVA method, including the unstable *Mpn1* locus (*26*).

We cannot, at this point, answer with confidence why an ST3 strain became the most prevalent strain among macrolide-resistant *M. pneumoniae*. We can, however, speculate. Mutation or some other mechanism could have caused the previously macrolide-susceptible ST3 strains to become macrolide resistant, and the new strain could have developed an ability to disseminate through high-density populations. Antimicrobial selective pressure could have aided this development. An alternative possibility is that the macrolide-resistant strains were introduced to and spread rapidly through the community. Our data demonstrate that ST3 and ST14 are not genetically related; they share 1 of 8 allelic loci and differ in P1 subtypes. Analysis with eBURST shows they exist in different clonal complexes. Further research with whole-genome sequencing can reveal the distinguishing characteristics of macrolide-resistant and -susceptible strains within ST3 strains (*32*,*33*). In addition, whole-genome sequencing may reveal how the macrolide-resistant ST3 became predominant.

Antimicrobial pressure would have played a role to some extent because South Korea is a high antimicrobial drug use country. Trend analyses of the national data on antimicrobial drug consumption (expressed in defined daily doses [DDD]/1,000 inhabitants/day [DID]) demonstrated an increase in macrolide use in the community during 2005–2014 (*34*). Macrolide use remained steady until 2007; however, DID increased consistently from 2007 (2.59 DID) through 2014 (4.14 DID). In particular, in children ≤6 years

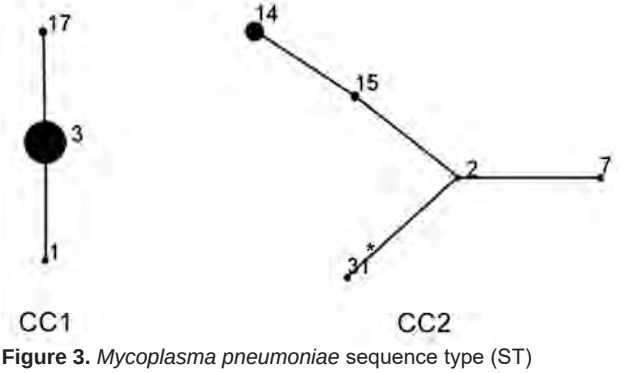

Figure 3. *Mycoplasma pneumoniae* sequence type (ST) relationship of 146 strains by eBURST analysis (http://eburst.mlst.net/), South Korea, 2000–2016. Two main CCs were defined without any singleton. ST3 and ST2 were the predicted founder of each CC. Numbers on the diagram correspond to STs. The size of each circle correlates with the number of isolates of each ST. CC, clonal complex.

of age, the increase measured from 7.73 DID (2007) to 9.51 DID (2014), with a peak of 11.99 DID in 2011. Therefore, the increase in macrolide consumption might explain in part the 17-year (2000–2016) change of macrolide resistance in *M. pneumoniae*. Nevertheless, the expansion of a single clone, which was demonstrated by our study, makes us assume another possible explanation. We think the macrolide-resistant clone, which we characterize as an ST3 clone by MLST, might have been introduced before the 2010–2012 outbreak and spread extensively among the community where the population density is high, supported by antimicrobial selective pressure.

Although various studies have investigated the genetic association of *M. pneumoniae* with epidemic outbreaks or macrolide resistance, research using MLST is currently insufficient. Our study demonstrates the predominance of ST3 throughout the entire study period. ST14 was the second most common ST found in all of the epidemics, except for the 2000 epidemic year, implying that 2 clonal complexes and their STs have existed simultaneously. As for ST and macrolide resistance, our study demonstrates ST3 as a major ST that harbors macrolide resistance and found a single macrolide-resistant ST14 strain. Until the 2006–2007 outbreak, most of the ST3 strains were macrolide susceptible, but ST3 from outbreaks since 2010–2012 showed macrolide resistance >90% on an increasing tendency. This finding confirms the concept that the increased prevalence of macrolide resistance is related to a single clone expansion. The results of our study can be compared with previous studies using MLST and MLVA. Brown et al. demonstrated an association among MLVA type 4572, P1 subtype 1, and CC1 (ST1, ST3, ST5, ST9, ST11, and ST12) (*19*). Our study also shows a relationship between P1 subtype 1 and CC1 (ST1 and ST3), which is consistent with their report. This relationship could have occurred by acquisition of 23S rRNA mutation within certain STs from predisposing genetic factors or by an introduction of a macrolide-resistant strain. On the basis of the studies we have described and our own results, clonal expansion of certain molecular types, 4572 in MLVA and ST3 in MLST, is likely to be the reason for the marked increase in macrolide resistance.

Our study has several limitations. First, even though *M. pneumoniae* strains were collected from 5 hospitals, the data might not represent reality nationwide. However, this was the best possible multicenter-based study we could devise, given that no nationwide surveillance system exists. Second, despite our best efforts, the numbers of strains in the earlier outbreaks were smaller than those collected in the later outbreaks, when culture was performed prospectively. However, this study is of value because the distribution of STs in a certain region for a relatively long period of time is well described. The finding that an expansion of a single ST contributed to the increase in macrolide resistance is a potentially powerful insight for further research. Additional studies that investigate the epidemiology and mechanism of acquiring macrolide resistance will give further insight into better treatment strategies. In particular, further studies should be addressed to in silico methods for the analysis of genetic background that can explain macrolide resistance within ST3 strains. Recent advances in microbiology have made whole-genome sequencing a valuable investigation tool that can lead to the identification of a specific genotype associated with macrolide resistance or virulence of *M. pneumoniae*.

In summary, we found that, during outbreaks of *M. pneumoniae* pneumonia that showed substantial increase in macrolide resistance in South Korea, all but 1 macrolide-resistant strain was ST3. These findings demonstrate that clonal expansion of an ST3 *M. pneumoniae* was associated with the increase in macrolide resistance in South Korea.

Acknowledgments

We thank Sun Jung Kim, Seong Yeon Lee, and Chanbin Lee for their technical assistance and Sangho Sohn for sharing his opinion on the epidemiology described in this research.

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea, which is funded by the Ministry of Education, Science and Technology (NRF-2015R1D1A1A09059589).

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Ancylostoma ceylanicum Hookworm in Myanmar Refugees, Thailand, 2012–2015

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Release date: July 11, 2018; Expiration date: July 11, 2019

Learning Objectives

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

- Evaluate the epidemiology of hookworm infection among Myanmar refugees living in 3 camps along the Thailand-Myanmar border, based on a cohort study
- Analyze the response to treatment of hookworm infection assessed through molecular analyses among Myanmar refugees living in 3 camps along the Thailand-Myanmar border, based on a cohort study
- Identify risk factors for hookworm infection among Myanmar refugees living in 3 camps along the Thailand-Myanmar border, based on a cohort stud.

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DOI: https://doi.org/10.3201/eid2408.180280

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During 2012–2015, US-bound refugees living in Myanmar–Thailand border camps (n = 1,839) were surveyed for hookworm infection and treatment response by using quantitative PCR. Samples were collected at 3 time points: after each of 2 treatments with albendazole and after resettlement in the United States. Baseline prevalence of *Necator americanus* hookworm was 25.4%, *Ancylostoma duodenale* 0%, and *Ancylostoma ceylanicum* (a zoonosis) 5.4%. Compared with *N. americanus* prevalence, *A. ceylanicum* hookworm prevalence peaked in younger age groups, and blood eosinophil concentrations during *A. ceylanicum* infection were higher than those for *N. americanus* infection. Female sex was associated with a lower risk for either hookworm infection. Cure rates after 1 dose of albendazole were greater for *A. ceylanicum* (93.3%) than *N. americanus* (65.9%) hookworm (p<0.001). Lower *N. americanus* hookworm cure rates were unrelated to β-tubulin single-nucleotide polymorphisms at codons 200 or 167. *A. ceylanicum* hookworm infection might be more common in humans than previously recognized.

Hookworm infection affects >470 million persons worldwide (*1*). Childhood infection has been associated with growth stunting, severe anemia, and iron deficiency (*2*,*3*). Ancylostoma duodenale and Necator americanus hookworms are believed to be the most prevalent species that infect humans. Infections with these species are acquired by transdermal penetration of the hookworm larvae or by the fecal-oral route (A. duodenale hookworm only), and infection is limited to humans (*4*). Dogs and cats infected with Ancylostoma ceylanicum hookworm have been found in close association with human populations (*5*–*9*). With use of molecular techniques, an increased number of human A. ceylanicum hookworm infections have been documented in parts of Asia and the Solomon Islands (*10*–*13*).

Hookworms and other soil-transmitted helminths are neglected tropical pathogens targeted for worldwide control by the World Health Organization by 2030 (*14*). This goal is being pursued through the mass administration of benzimidazole compounds (i.e., albendazole, mebendazole). However, the ubiquitous use of a single class of drug in both human and veterinary medicine has raised concern for the emergence of drug resistance (*15*–*20*). We report hookworm infection and cure rates in a large cohort of US-bound refugees from Myanmar residing in camps in Thailand along the Myanmar–Thailand border and assess the presence of β-tubulin mutations that could confer drug resistance among persons with persistent infection.

Materials and Methods

Recruitment of Participants and Sample Collection

This investigation was part of a larger program involving refugees living in camps along the Myanmar–Thailand border that was conducted by the Centers for Disease Control and Prevention during 2012–2015 (*21*). In our analysis, we included 1,839 (92%) of the 2,004 refugees ≥6 months of age from this cohort who provided fecal samples. (Figure 1; online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/18-0280-Techapp1.pdf). Fecal samples were collected at time point 1 (T1, during the required medical examination for US resettlement), T2 (before departing the refugee camp), and T3 (after US resettlement). All refugees were offered albendazole after fecal specimens were collected to treat presumptive infection with helminths.

Molecular Detection of Hookworm Species

We performed stool extraction (online Technical Appendix) as previously described (*22*). For the initial 233 samples, we performed quantitative PCR (qPCR) with primer-probe sets Ad1 (for amplifying *A. duodenale*) and Na (for amplifying *N. americanus*) (Table 1) (*23*,*24*). Then, we switched to a more sensitive primer-probe set targeting a repetitive element in *A. duodenale* (Ad2) (*24*). When qPCR with the Ad2 primer-probe set failed to amplify the samples positive by qPCR with Ad1, we subjected 7 discordant samples to PCR restriction fragment length polymorphism, as previously described (*26*). We also sequenced these PCR products using standard (Sanger) sequencing technology.

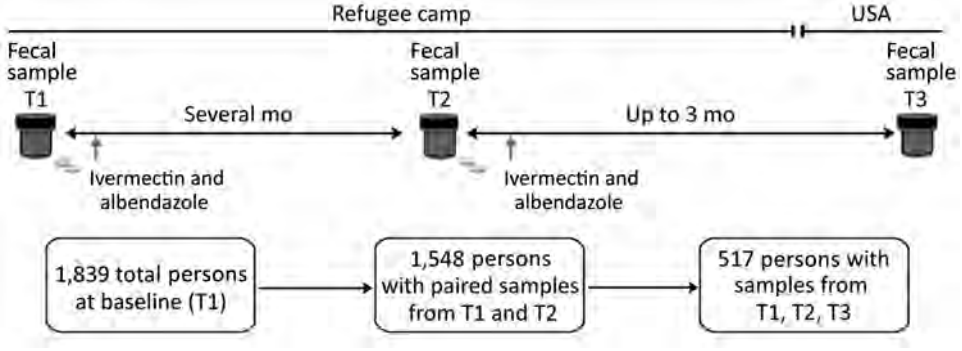

Figure 1. Study design showing collection of fecal samples from and treatment of US-bound Myanmar refugees for hookworm infection, Thailand, 2012–2015. Myanmar refugees (n = 2,004) from 3 camps in Thailand (Mae La [camp 1], Mae Ra Ma Luang [camp 2], Mae La Oon [camp 3]) along the Myanmar–Thailand border were recruited to donate fecal samples and receive treatment (ivermectin and albendazole) for parasitic infections. T1 was the time of the resettlement medical exam, T2 occurred before camp departure, and T3 was a time after resettlement in the United States. Albendazole and ivermectin were given immediately after fecal collection at T1 and T2. T1, time point 1; T2, time point 2; T3, time point 3.

Table 1. Primer-probe sets used to determine genotypes of hookworms present in US-bound Myanmar refugees in camps along Myanmar–Thailand border, Thailand, 2012–2015*

Genome target/primer-probe set name, sequence type	Sequence	Final concentration, nmol/L	Reference
ITS2/Ad1			(*23*)
Forward primer	5′-GAATGACAGCAAACTCGTTGTTG-3′	900	
Reverse primer	5′-ATACTAGCCACTGCCGAAACGT-3′	900	
Probe	5′-ATCGTTTACCGACTTTAG-3′	250	
Repetitive element/Ad2			(*24*)
Forward primer	5′-GTATTTCACTCATATGATCGAGTGTTC-3′	900	
Reverse primer	5′-GTTTGAATTTGAGGTATTTCGACCA-3′	900	
Probe	5′-TGACAGTGTGTCATACTGTGGAAA-3′	250	
Repetitive element/Ac			(*25*)
Forward primer	5′-CAAATATTACTGTGCGCATTTAGC-3′	900	
Reverse primer	5′-GCGAATATTTAGTGGGTTTACTGG-3′	900	
Probe	5′-CGGTGAAAGCTTTGCGTTATTGCGA-3′	250	
Repetitive element/Na			(*24*)
Forward primer	5′-CCAGAATCGCCACAAATTGTAT-3′	900	
Reverse primer	5′-GGGTTTGAGGCTTATCATAAAGAA-3′	900	
Probe	5′-CCCGATTTGAGCTGAATTGTCAAA-3′	250	
SNP200			This
Forward primer	5′-AATGCTACACTCTCTGTTCACCAGTT-3′	900	paper
Reverse primer	5′-CGGAAGCAGATATCATACAAAGCTT-3′	900	
Wild type FAM probe/mutant VIC probe†	5′-AATACAGATGAGACCT(T/A)CT-3′	166; 231	
SNP167			This
Forward primer	5′-TCGGGAAGAATACCCTGATAGAAT-3′	900	paper
Reverse primer	5′-CTTTTGCTCTTATTTCCATCAATAGGA-3′	900	
Wild type FAM probe/mutant VIC probe†	5′-TGTCCTCGT(T/A)TTCC-3′	125; 350	

*Ac, *Ancylostoma ceylanicum* set; Ad1, *A. duodenale* set 1; Ad2, *A. duodenale* set 2; ITS2, internal transcribed spacer 2; Na, *Necator americanus* set; SNP, single-nucleotide polymorphism.
†FAM and VIC probes (Integrated DNA Technologies, Skokie, IL, USA).

Definitions

Participants whose fecal samples were positive for hookworm DNA by qPCR and became negative at the immediate next time point were considered cured. Those whose fecal samples were negative for hookworm DNA by qPCR but then positive the immediate next time point were considered to have a newly acquired infection. We defined persistent infection as having detectable hookworm DNA at 2 successive time points.

Single-Nucleotide Polymorphism Detection in *N. americanus*–Positive Samples

For the participants who were positive for *N. americanus* hookworm at all 3 time points, we tested fecal samples from T1 and T3 for β-tubulin single-nucleotide polymorphisms (SNPs) at codon 200. We also tested refugee T3 fecal samples for SNP167 by using an allele-specific real-time PCR approach with common primers and SNP-specific probes (Table 1) and defined heterozygosity and homozygosity of SNPs on the basis of change in the cycle threshold, similar to previously described methods (*27–29*) (online Technical Appendix).

Statistical Analyses

Unless stated otherwise, we used the geometric mean to measure central tendency. We determined the odds ratios (ORs) of risk factors for infection with *A. ceylanicum* and *N. americanus* hookworms by using a generalized linear model that used overdispersion with binomial distribution and logit link. We performed a maximum likelihood analysis using JMP 12.0.1 (https://www.jmp.com/en_us/home.html). We used these models to test the following parameters: sex,

Table 2. Baseline characteristics of 1,839 US-bound Myanmar refugees in camps along Myanmar–Thailand border, by camp, Thailand, 2012–2015

Characteristic	Mae La, camp 1	Mae La Oon, camp 2	Mae Ra Ma Luang, camp 3
Total participants, no.	549	667	623
Sex, no. (%)			
F	261 (47.5)	327 (49.0)	304 (48.8)
M	288 (52.5)	340 (51.0)	319 (51.2)
Age, y, mean (range)	20.8 (0.6–79.9)	19.3 (0.86–88.9)	18.9 (0.55–83.1)
Any hookworm infection, no. (%)	180 (32.8)	173 (25.9)	182 (29.2)
Hemoglobin, g/L, geometric mean (range)	130 (71–179)	127 (71–180)	126 (71–183)
Eosinophil concentration, $\times 10^8$ cells/L, geometric mean (range)*	3.56 (0.01–55.3)	4.27 (0.01–107)	5.21 (0.01–178)

*Eosinophil concentration reference range: 0–4.5 $\times 10^8$ cells/L.

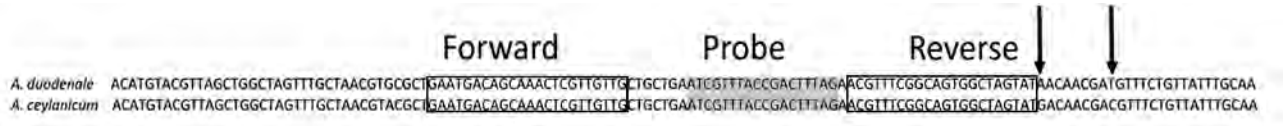

Figure 2. Partial internal transcribed spacer 2 (ITS2) sequences of *Ancylostoma duodenale* and *A. ceylanicum* hookworms. Boxes indicate the location of forward and reverse primer binding; gray shading indicates the location of probe binding. These regions of the *A. duodenale* and *A. ceylanicum* ITS2 are identical. The locations where the ITS2 sequences differ (arrows) fall outside of the primer and probe binding regions.

camp, age (infants and toddlers <2 years of age, children 2–18 years of age, and adults >18 years of age), and co-infections (online Technical Appendix). The cutoff of <2 years of age for infants and toddlers was chosen because, after this age, the rates of mouthing, a prominent cause of fecal-oral contamination, decrease (*30*,*31*). We performed cure rate comparisons using a 2-tailed Fisher exact test. We compared the geometric mean eosinophil and hemoglobin concentrations of those monoinfected with *A. ceylanicum* or *N. americanus* hookworm (excluding co-infections involving both hookworms and *Strongyloides stercoralis*, *Ascaris lumbricoides*, and *Trichuris trichiura* roundworms) by the Mann-Whitney test using Prism GraphPad 6.0e (https://www.graphpad.com/scientific-software/prism/).

Results

A total of 4,330 fecal samples from 1,839 refugees underwent DNA extraction and multiparallel qPCR. After excluding 4 participants positive for hookworm at T1 who did not receive albendazole, the number of participants with T1-T2 paired samples totaled 1,548; we had samples from all 3 time points for 517 participants. The geometric mean time between T1 and T2 sample collections was 188 (range 48–1,013) days and between T2 and T3 was 46.2 (range 14–413) days. Baseline hookworm infection (any type) in this population was high, ranging from 25.9%–32.8% depending on the camp of residence (Table 2).

Detection of *A. ceylanicum*

When a highly sensitive primer-probe set specific to the *A. duodenale* genome (Ad2) was used on samples positive for *Ancylostoma* DNA by qPCR with primer-probe set Ad1, none was positive. Because of concern that Ad1 might enable the cross-amplification of other *Ancylostoma* spp., we performed seminested PCR and *Mva*I and *Psp*1406I digestion with 7 samples positive by Ad1 but negative by Ad2. Results from restriction fragment length polymorphism PCR indicated the presence of *A. ceylanicum* hookworm (online Technical Appendix Figure); these results were further confirmed by sequencing (98% identity to *A. ceylanicum* ribosomal sequence [GenBank accession no. LC036567]). The internal transcribed spacer 2 regions of the *A. duodenale* and *A. ceylanicum* genomes, which the Ad1 primer-probe set aligned with, are identical (Figure 2). Cross-species identification with this primer-probe set has been previously predicted (*23*), although not previously demonstrated in the literature.

We then determined the prevalence of *A. ceylanicum* hookworm among the refugee population using a primer-probe set specific to a repetitive DNA element in the *A. ceylanicum* genome (Ac; Table 1). When using Ac, the total number of *A. ceylanicum*–positive samples increased from 106 (using the Ad1 set) to 124 (using the Ac set). We tested or retested these samples (n = 124) by qPCR using the Ad2 primer-probe set, and 0% were positive. All samples positive by qPCR with Ad1 were positive by qPCR with Ac.

Response to Treatment

Baseline prevalence of *N. americanus* (26.3%) hookworm was higher than that of *A. ceylanicum* (5.3%) hookworm among all participants (n = 1,839); likewise, prevalence of *N. americanus* (25.4%) species was higher than that of *A. ceylanicum* (5.4%) species among all participants who gave paired T1-T2 fecal samples (n = 1,548) (Table 3). Refugees in their sixth (50–59 years) and seventh (60–69 years) decades of life had the highest *N. americanus* hookworm prevalence (>40% positive), and refugees in their third decade of life had the highest *A. ceylanicum* hookworm prevalence (9%) (Figure 3).

A. ceylanicum infection had a higher cure rate than did *N. americanus* infection (Table 3); 92.8% of *A. ceylanicum* hookworm–infected refugees were cured by T2, despite the

Table 3. Hookworm prevalence and cure rate in US-bound Myanmar refugees in camps along Myanmar–Thailand border with paired samples, by time point, Thailand, 2012–2015*

	T1, n = 1,548	T2, n = 1,548		T3, n = 517	
Species	Baseline prevalence, no. (%)	Cure, no. (%)	Persistent infection, no. (%)	Cure, no. (%)	Persistent infection, no. (%)
Ancylostoma ceylanicum	83/1,548 (5.4)	77/83 (92.8)	6/83 (7.2)	7/7 (100)	0
Necator americanus	393/1,548 (25.4)	271/393 (69)	122/393 (31)	21/50 (42)	29/50 (58)

*The geometric mean time from T1 to T2 was 188.2 (range 48–1,013) d and from T2 to T3 was 46.2 (range 14–413) d. T1, time point 1; T2, time point 2; T3, time point 3.

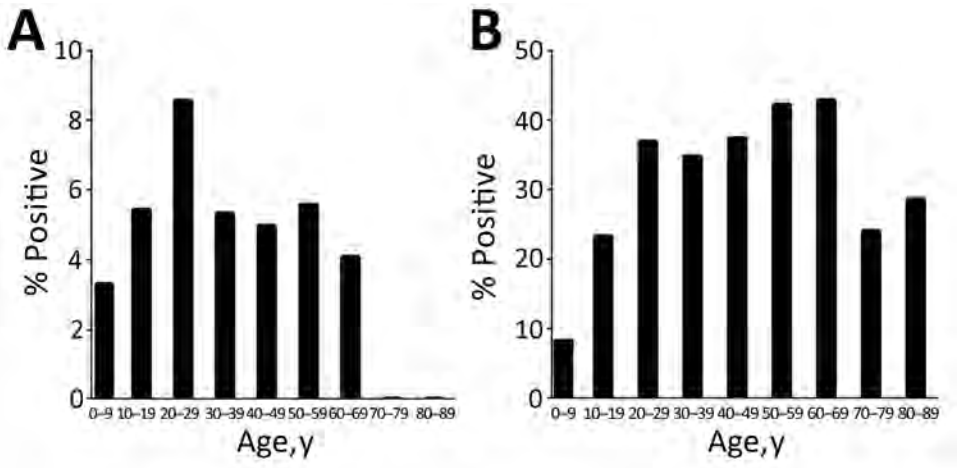

Figure 3. Baseline prevalence of hookworm infections in 1,839 US-bound Myanmar refugees at 3 camps along the Myanmar–Thailand border, by age group, Thailand, 2012–2015. A) *Ancylostoma ceylanicum* hookworm. B) *Necator americanus* hookworm.

relatively long time that elapsed between T1 and T2. Of the samples paired for T2 and T3, all 7 *A. ceylanicum* hookworm–infected refugees at T2 were cured by T3. At T2, the *N. americanus* infection cure rate was 69%, and after the second administration of albendazole at T3, 42% (21/50) were cured, resulting in 29 participants with persistent *N. americanus* infection at resettlement in the United States. Combining cure rates across all time points, the overall *A. ceylanicum* hookworm cure rate was 93.3% (84/90), higher than that for *N. americanus* hookworm (65.9%, 292/443; p<0.001).

In total, 151 refugees had persistent *N. americanus* infections, and 6 refugees had persistent *A. ceylanicum* infections. At T2, the hookworm genomic DNA relative quantity in fecal samples of those with persistent *N. americanus* infections (n = 122) decreased significantly (p<0.001) (Figure 4). Of the 6 persons with persistent *A. ceylanicum* infections at T2, 4 (67%) had a decrease and 2 (33%) an increase in hookworm genomic DNA in their fecal samples. Those infected with *N. americanus* hookworm at T2 and T3 (n = 29) had no significant decrease in parasite genomic

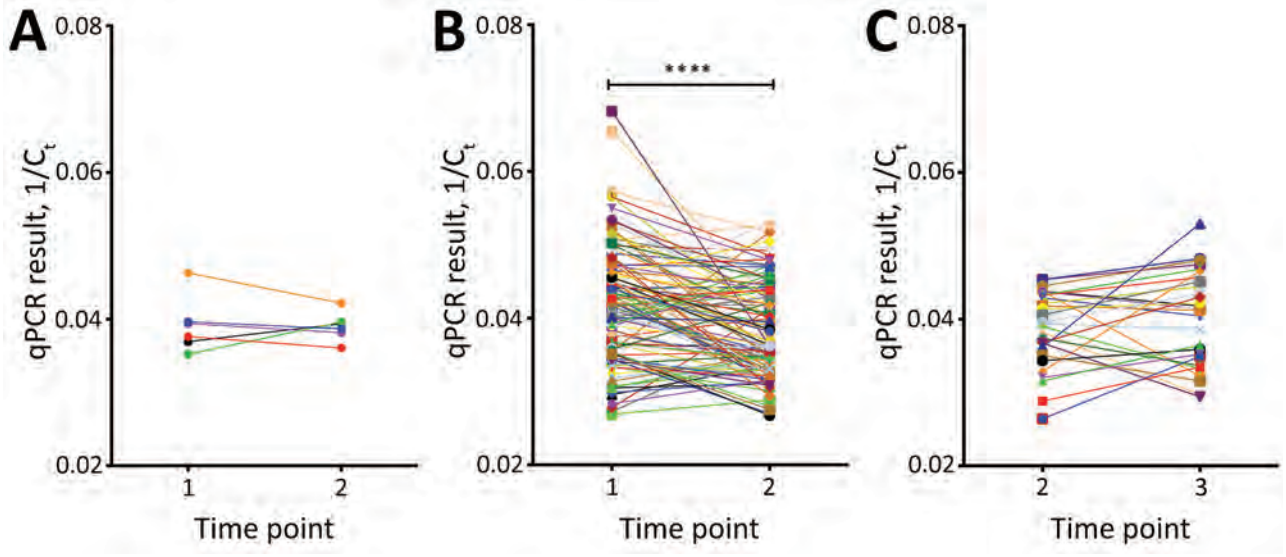

Figure 4. Change in relative quantities of *Ancylostoma ceylanicum* and *Necator americanus* hookworm genomic DNA in fecal samples from US-bound Myanmar refugees at 3 camps along the Myanmar–Thailand border after treatments with albendazole and ivermectin, Thailand, 2012–2015. Fecal samples were collected at 3 time points: time point 1 (T1, baseline), time point 2 (T2, after first treatment), and time point 3 (T3, after second treatment). Quantities were expressed as $1/C_t$ and differences were assessed by paired *t*-test. A) *A. ceylanicum* genomic DNA relative quantities in those who were persistently infected after first treatment (n = 6). The difference between T1 and T2 was not significant. B) *N. americanus* genomic DNA relative quantities in those who were persistently infected after first treatment (n = 122). The relative genomic DNA quantity was significantly reduced in those who remained infected with *N. americanus* hookworm after first treatment. C) *N. americanus* genomic DNA relative quantities in those who were persistently infected after second treatment (n = 29). No significant change in *N. americanus* relative quantity was found in those persistently infected after the second treatment. The geometric mean time from T1 to T2 was 188.2 (range 48–1,013) d and from T2 to T3 was 46.2 (range 14–413) d. ****p<0.0001. C_t, cycle threshold; qPCR, quantitative PCR.

DNA relative quantity at T3, and 26 refugees were positive for *N. americanus* hookworm at all 3 time points.

We detected a small number of newly acquired hookworm infections. Of the 1,548 participants with T1-T2 paired samples, we detected 15 (≈1%) new *A. ceylanicum* infections and 36 (2.3%) new *N. americanus* infections (Table 4). Of the 517 participants with T2-T3 paired samples, we detected 3 (0.58%) new *A. ceylanicum* infections and 13 (2.5%) new *N. americanus* infections.

Risk Factors Associated with *N. americanus* and *A. ceylanicum* Infection

A generalized linear model was used to assess whether coincident helminth or protozoa infection, age, sex, or camp affected the risk for infection with *N. americanus* or *A. ceylanicum* hookworm at T1 (Table 5). The *y*-intercept for the model of *N. americanus* hookworm was 2.4 (95% CI 2.14–2.69) and for *A. ceylanicum* hookworm 3.24 (95% CI 2.77–3.74). The strongest predictors (ORs >1.5 and p values <0.0001) of *N. americanus* infection were adult age (OR 3.83), *T. trichiura* infection (OR 1.90), and *A. lumbricoides* infection (OR 1.71). Female participants had a reduced odds of *N. americanus* infection (OR 0.68; p<0.0001). *N. americanus* infection was the only 1 of the 5 infections assessed that was associated with *A. ceylanicum* co-infection (OR 2.08; p = 0.0018); female sex (OR 0.57; p<0.0001) and residence in camp 1 (Mae La, OR 0.69; p = 0.03) were associated with reduced odds of *A. ceylanicum* infection.

We used a similar model that took age, sex, and camp into account to compare participants who cleared their infection with *N. americanus* hookworm after 1 treatment (n = 290) with those who did not clear infection after 2 treatments (n = 26). Female sex, but not age or camp, was associated with clearance after a single treatment (OR 2.14, 95% CI 1.23–4.44; p = 0.0045).

N. americanus Benzimidazole Resistance and β-Tubulin SNP Changes

Because 26 participants were positive for *N. americanus* hookworm at all 3 time points despite 2 courses of albendazole treatment, benzimidazole drug resistance was a concern. In total, 19 of 26 persistently infected participants had T1 fecal samples available for DNA reextraction and SNP200 testing; 3 samples showed no amplification, and 16 were wild type. We then performed DNA reextraction and SNP200 and SNP167 testing with the T3 fecal samples available (n = 24). Only 11 of 24 samples had sufficient quantities of *N. americanus* DNA (>150 pg/μL) to be amplified. All samples were positive for wild -type SNP200 and SNP167. None were homozygous or heterozygous for mutant alleles. Thus, no alterations in the β-tubulin gene could be detected at codons 167 or 200 to account for drug resistance.

An additional 213 samples (from multiple time points) had sufficient *N. americanus* DNA to test SNP200 variation further; 173 of 213 samples were evaluable by allelic discrimination qPCR. All were homozygous wild type for SNP200.

Blood Cell Concentration Differences Between *N. americanus* and *A. ceylanicum* Infections

We further evaluated refugees with either *N. americanus* (n = 143) or *A. ceylanicum* (n = 24) hookworm monoinfections; participants co-infected with both hookworms or other soil-transmitted helminths (i.e., *S. stercoralis*, *A. lumbricoides*, and *T. trichiura* roundworms) were excluded. Peripheral blood eosinophil concentrations were significantly higher (p<0.001) in those with *A. ceylanicum* monoinfections (geometric mean 8.49×10^8 cells/L, 95% CI $5.98–12.04 \times 10^8$ cells/L) than those with *N. americanus* monoinfections (geometric mean 3.44×10^8 cells/L, 95% CI $2.92–4.05 \times 10^8$ cells/L) (Figure 5, panel A). The hemoglobin levels did not differ between those with only *A. ceylanicum* and those with only *N. americanus* infections (Figure 5, panel B).

Discussion

A. ceylanicum hookworm is increasingly being recognized as a pathogen in humans, particularly in Southeast Asia (*32*). *A. ceylanicum* is the only hookworm species known to achieve patency in both humans and other animals (e.g., dogs and cats) (*33*). In this group of US-bound refugees from Myanmar, *N. americanus* infection was the most prevalent hookworm infection at all 3 time points tested (25.4% at baseline); *A. ceylanicum* was the only other hookworm species found, with a baseline prevalence of 5.4%. Because *A. ceylanicum* infection has been described in both Thailand (*7*,*10*) and Myanmar (*11*,*34*), whether these persons were infected in their home country or in the camps in Thailand is unknown. Camp allocation seemed to have an effect on infection status for both hookworms; camps 2 and 3 imparted a higher risk for *A. ceylanicum* hookworm acquisition, and camp 1 had a higher rate of *N. americanus* infection. Evidence of newly acquired infections for both hookworms at T2 and T3 (Table 4) indicates that active transmission of both species was ongoing at these 3 camps. However, whether the majority of hookworm infections at baseline were acquired prior to entry or while residing in the camps is unknown. Differential infection rates at the 3 camps might reflect environmental and hygiene conditions in the camps, the historical exposure of the persons at these camps, or other factors. Of note, a risk factor for infection with either *A. ceylanicum* or *N. americanus* hookworm was infection with the other hookworm (Table 5). This finding contrasts with previous surveys showing that *A. ceylanicum* and *N. americanus* co-infections are rare (*26*,*32*). Although

Table 4. New hookworm infections acquired by US-bound Myanmar refugees in camps along Myanmar–Thailand border, by time point, Thailand, 2012–2015*

Species	T2, n = 1,548, no. (%)	T3, n = 517, no. (%)
Ancylostoma ceylanicum	15 (0.97)	3 (0.58)
Necator americanus	36 (2.3)	13 (2.5)

*T2, time point 2; T3, time point 3.

A. ceylanicum infection by the fecal-oral route is thought to be possible (*33*), walking barefoot has been shown to be a major risk factor for *A. ceylanicum* and *N. americanus* hookworm infections (*35*). Thus, the high propensity for co-infection in the population we evaluated suggests a similar mode of transmission, namely transdermal penetration, for both hookworms.

Despite possible co-transmission, those infected with *A. ceylanicum* hookworm had slightly different risk factors than those infected with *N. americanus* hookworm. *A. ceylanicum* hookworm prevalence peaked in the third decade of life, compared with *N. americanus* prevalence, which peaked in the sixth and seventh decades. However, *N. americanus* prevalence remained high for many decades of life (20–69 years of age).

T. trichiura roundworm, *Entamoeba histolytica* ameba, and *A. lumbricoides* roundworm infection as risk factors for *N. americanus* hookworm co-infection reflects the high prevalence of parasitic infections in this population (*21*), in a setting with inadequate sanitary infrastructure despite improvement efforts (*36*). Why infections with these pathogens but not *S. stercoralis* roundworm (acquired similarly to hookworm) or *Giardia duodenalis* protozoa (acquired similarly to *E. histolytica* ameba) put refugees at risk for *N. americanus* infection deserves further study.

Female participants were less likely to acquire both *N. americanus* and *A. ceylanicum* hookworms, a finding that might reflect differential exposures, differences in immunity (*37*,*38*), differences in albendazole metabolism (*39*), or a combination of these factors. Although eosinophilia has been previously reported in experimental human infections with *A. ceylanicum* hookworm (*33*) and in *A. ceylanicum* case reports (*34*,*40*), a more striking eosinophilia was seen among those with *A. ceylanicum* infections than those with *N. americanus* infections. This observation supports the idea that, unlike the hookworms that only infect humans, the zoonotic *A. ceylanicum* parasite might be less able to downregulate the host's IgE-mediated response to infection (*41*). No difference in hemoglobin levels was found between those with either hookworm species. Anemia is typically seen in *A. duodenale* infection and is less commonly associated with *N. americanus* infection (*3*); for *A. ceylanicum* infection, data on anemia are scant.

The treatment for *N. americanus* infection was only modestly effective (cure rate 42%–69%). It has been suggested that deworming might have enabled the zoonotic *A. ceylanicum* hookworm to fill a niche left by a decrease in anthropophilic hookworms (*32*). That *A. ceylanicum* infection was largely cured after single courses of treatment with albendazole (cure rate 92.8%–100%) is reassuring, although follow-up time periods in this evaluation were more varied than in most controlled studies specifically assessing response to treatment. Still, reinfection and newly acquired infection rates (0.58%–0.97% for *A. ceylanicum* and 2.3%–2.5% for *N. americanus*) were modest after deworming, compared with previous projections suggesting reinfection rates as high as 30% (*14*) after 3 months. With the average time between T1 and T2 exceeding 6 months (and the wide time range of 1–33 months), this population experienced a lower reinfection rate than has been suggested for endemic areas.

Persons who were infected with *N. americanus* hookworm at all 3 time points represent a group that might have never cleared the infection or might have cleared infection but were subsequently reinfected. However, the time between sample collections was not statistically different between those who were persistently positive and those who cleared infection after a single treatment (E.M. O'Connell,

Table 5. Characteristics of US-bound Myanmar refugees in camps along Myanmar–Thailand border associated with increased risk for infection with *Ancylostoma ceylanicum* or *Necator americanus* hookworm, Thailand, 2012–2015*

Category	*N. americanus*		*A. ceylanicum*	
	Odds ratio	p value	Odds ratio	p value
Children compared with infants and toddlers	8.17	0.0025	NS	NS
Adults compared with children	3.83	<0.0001	NS	NS
Ancylostoma ceylanicum infection	2.08	0.0017	NA	NA
Trichuris trichiura infection	1.90	<0.0001	NS	NS
Entamoeba histolytica infection	1.79	0.0173	NS	NS
Ascaris lumbricoides infection	1.71	<0.0001	NS	NS
Residence at camp 1, Mae La	1.27	0.0039	0.69	0.0303
Female sex	0.68	<0.0001	0.57	<0.0001
Necator americanus infection	NA	NA	2.08	0.0018

*We built the following factors into the model: other infections (*Strongyloides* stercoralis, *A. lumbricoides*, *E. histolytica*, *Cryptosporidium* spp., *T. trichiura*, *Giardia* duodenalis); camp (1, 2, 3); age (infants and toddlers <2 years of age, children 2–18 years of age, adults >18 years of age); and sex. Associations with *Cryptosporidium* spp. and *G. duodenalis* were not significant. We used a generalized linear model that used overdispersion with binomial distribution and logit link, and we performed maximum-likelihood analysis using JMP 12.0.1 (https://www.jmp.com/en_us/home.html). NA, not applicable; NS, not significant.

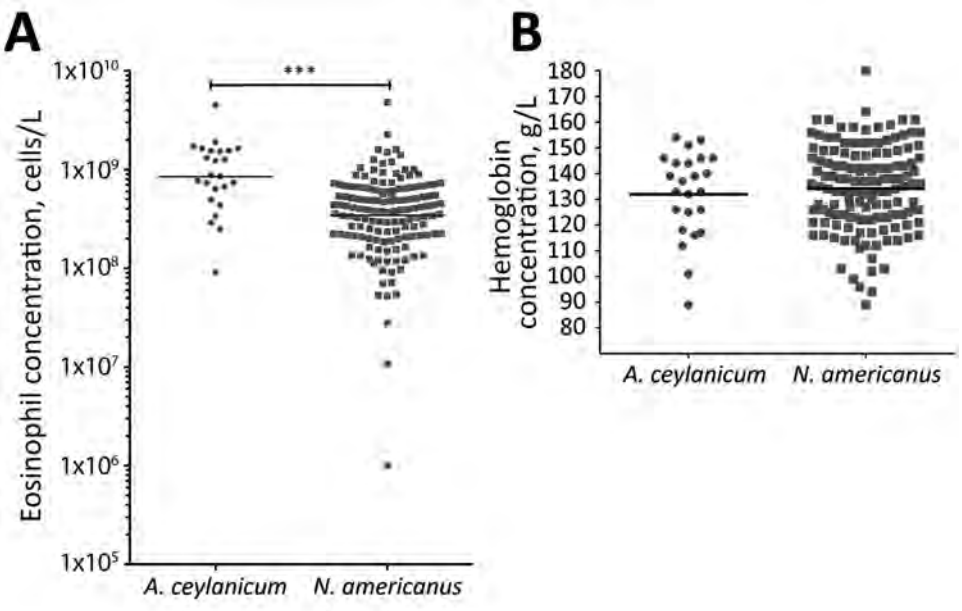

Figure 5. Eosinophil and hemoglobin concentrations in US-bound Myanmar refugees at 3 camps along the Myanmar–Thailand border who were monoinfected with *Ancylostoma ceylanicum* (n = 24) or *Necator americanus* (n = 143) hookworm at baseline, Thailand, 2012–2015. Those co-infected with both hookworms or *Strongyloides stercoralis*, *Ascaris lumbricoides*, or *Trichuris trichiura* roundworms were excluded from analysis. Horizontal line indicates geometric mean. Significance was calculated by Mann-Whitney test. A) The geometric mean eosinophil cell concentration was significantly higher in those with *A. ceylanicum* monoinfection (8.49×10^8 cells/L, 95% CI 5.98–12.0×10^8 cells/L) than those with *N. americanus* monoinfection (3.44×10^8 cells/L, 95% CI 2.92–4.05×10^8 cells/L) (***p<0.001). B) The geometric mean hemoglobin level of the 2 groups was not significantly different.

unpub. data). Reports of benzimidazole resistance are increasing in the literature on veterinary medicine; resistance in canine *Ancylostoma caninum* hookworm (*15*) and phylogenetically similar bovine intestinal nematodes (*17*) was associated with SNPs in the β-tubulin gene, particularly in codon 200 but also in codon 167 and, rarely, in codon 198. Likewise, other studies have shown low cure rates after benzimidazole administration in the setting of *N. americanus* infection, and research suggests that resistance is emerging (*42*). One group found that albendazole administration exerts selective pressure on *T. trichiura* codon 200 in Kenya and Haiti (*20*). The same group found that, in pooled *N. americanus* eggs from Haiti, the allele containing the resistant codon 200 had a mean allelic frequency of 36% (*43*). A frequency of 0% was found for the homozygous resistant genotype in hookworm eggs (species not identified) from Haiti and Panama (*20*), and although the authors reported a 2.3% frequency of the homozygous resistant genotype in hookworm eggs from Kenya, the frequency of the resistant genotype after treatment did not increase (*20*), raising questions about the significance of this allele. Our examination of codons 167 and 200 in samples persistently positive for *N. americanus* hookworm across all 3 time points, and of codon 200 in those with high levels of *N. americanus* DNA in fecal samples at any time point, revealed only homozygous wild type β-tubulin genes.

Several possibilities might explain why β-tubulin mutations were not found to account for persistent *N. americanus* infections. First, the lesser-known codon 198 or another codon within the β-tubulin gene might be responsible for resistance in the hookworms infecting these refugees. Second, whereas amplification rates for our qPCR assay were as high as or higher than those in most other reports, up to 54% of samples positive for *N. americanus* DNA at T3 did not amplify adequately to determine genotype. Therefore, mutations in SNPs at either codon 167 or 200 could have been missed. Last, β-tubulin might not be the only gene involved in drug resistance and responsible for low cure rates in *N. americanus*. In this population, 88.5% (23 of 26) of those persistently positive for *N. americanus* at all 3 time points were male. The peak concentration in serum and area under the serum concentration time curve for albendazole sulfoxide and albendazole sulfone (the main active metabolites of albendazole) have been found to be higher in female than in male volunteers (*39*). Also, male refugees were possibly more likely than female refugees to quickly reacquire infection due to differences in environmental exposures.

One lesson from this evaluation was that unbiased surveys are necessary before pursuing highly specific molecular techniques when profiling organisms that infect a population, particularly when considering emerging infectious diseases. Although the use of the highly repetitive genomic sequence for *A. duodenale* hookworm is superior in sensitivity and specificity than the internal transcribed spacer 2 region (*44*), targeting the more conserved genomic region ultimately enabled detection of an unexpected organism, *A. ceylanicum* hookworm, which otherwise would have been missed in this population.

This project had several limitations. Control and nontreatment groups were absent. Participation was completely voluntary. The number of fecal sample collections for the third time point dropped off substantially because of logistical issues relating to collecting and shipping samples from the various US states after resettlement. Unlike other studies focused on *A. ceylanicum* hookworm, this evaluation did not include surveying the local cat and dog populations to establish potential reservoirs, which might be a worthwhile future research direction.

In summary, this cohort of US-bound refugees living in 3 camps in Thailand on the Myanmar–Thailand border was found to have a high prevalence of *N. americanus* hookworm with suboptimal cure rates after albendazole administration that do not seem to be attributable to mutations in the β-tubulin gene at codons 200 or 167. In addition, *A. ceylanicum* hookworm was the only other hookworm species identified in this population. *A. ceylanicum* infection had a much higher cure rate after a single course of albendazole, and those with *A. ceylanicum* monoinfection had a similar hemoglobin level as those with *N. americanus* monoinfection. Future mapping efforts of soil-transmitted helminths should take into account the emergence of *A. ceylanicum* hookworm infection in humans to further understand its distribution across the world. The recognition of the increased importance of zoonotic *A. ceylanicum* hookworm over that of *A. duodenale* hookworm in some populations raises epidemiologic questions about transmission dynamics and the differential effect on local health of these 2 species.

Acknowledgments

We thank Diana Martin and Chaohua Li for help in processing samples. We thank Marla Petway for assistance in specimen management.

This project was funded in part through the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA, and the Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

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Susceptibility of Human Prion Protein to Conversion by Chronic Wasting Disease Prions

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Chronic wasting disease (CWD) is a contagious and fatal neurodegenerative disease and a serious animal health issue for deer and elk in North America. The identification of the first cases of CWD among free-ranging reindeer and moose in Europe brings back into focus the unresolved issue of whether CWD can be zoonotic like bovine spongiform encephalopathy. We used a cell-free seeded protein misfolding assay to determine whether CWD prions from elk, white-tailed deer, and reindeer in North America can convert the human prion protein to the disease-associated form. We found that prions can convert, but the efficiency of conversion is affected by polymorphic variation in the cervid and human prion protein genes. In view of the similarity of reindeer, elk, and white-tailed deer in North America to reindeer, red deer, and roe deer, respectively, in Europe, a more comprehensive and thorough assessment of the zoonotic potential of CWD might be warranted.

Chronic wasting disease (CWD) is a fatal contagious prion disease of cervids that is found in the United States, Canada, South Korea, and most recently in Europe (*1*,*2*). The species affected differ in these geographic areas; mule deer (*Odocoileus hemionus*), white-tailed deer (*O. virginianus*), North American elk (*Cervus canadensis*), and moose (*Alces alces*) are most commonly affected in the United States and Canada, and red deer (*C. elaphus*) and sika deer (*C. nippon*) in South Korea also have been affected (*1*,*2*). The first identification of CWD in Europe occurred in 2016 in wild moose (*A. alces*, also known as Eurasian elk) and in a free-ranging reindeer (*Rangifer tarandus*, closely related to the free-ranging caribou of North America), a species not previously known to be affected by CWD in wild or farmed animals (*2*,*3*). CWD is a pressing animal health issue, but whether it might become a human public health issue should also be considered.

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DOI: https://doi.org/10.3201/eid2408.161888

Scrapie and bovine spongiform encephalopathy (BSE) are well-characterized animal prion diseases affecting animals of consumption; scrapie affects sheep and BSE cattle. Human prion diseases occur as sporadic, genetic, and acquired forms; the variant form of Creutzfeldt-Jakob disease is zoonotic BSE, acquired through the oral route and having a long incubation period (*4*). In contrast, sheep scrapie is generally considered to be of no or only very low risk to human health, although this possibility has been questioned recently (*5*,*6*). The molecular basis of prion replication is a change in conformation of the normal cellular prion protein (PrP^{C}) into the abnormal and misfolded conformer (PrP^{Sc}) that is partially protease-resistant (PrP^{res}). However, the molecular criteria for predicting zoonotic potential for prions are unclear. Consequently, approaches to understanding the zoonotic risk for prion transmission to humans have been empirical, involving in vivo and in vitro models (*7*). Experimental transmission of mule deer CWD to nonhuman primates showed that squirrel monkeys are susceptible, whereas cynomolgus macaques appear to be resistant (*8*,*9*). Similarly, attempted transmission of deer or elk CWD to transgenic mouse lines expressing human PrP^{C} have largely failed, indicative of a species barrier (*10*–*13*), although this might be overcome in some instances (*14*).

In contrast to these in vivo approaches, we have used protein misfolding cyclic amplification (PMCA) to investigate the molecular compatibility of bovine, ovine, and cervid prions with full-length, glycosylated and glycosylphosphatidylinositol-anchored human prion protein (PrP) (*15*,*16*). We found that scrapie samples failed to convert human PrP^{C} to PrP^{res}, whereas cattle BSE converted the human protein efficiently (*15*–*17*). These observations suggest that PMCA can reproduce aspects of cross-species transmission potential and inform assessment of zoonotic risk. The single CWD-affected specimen (from a North American elk) previously available to us was found to be capable of converting human PrP^{C} to PrP^{res} (*15*). Here, we expand on the previous report; analyzing more elk specimens of 2 different genotypes (132 MM, homozygous for methionine at *Prnp* position 132, and 132 ML, methionine–leucine heterozygous at the same position), analyzing white-tailed deer CWD specimens for

the first time, and analyzing reindeer that have been experimentally infected with white-tailed deer CWD. The elk ML polymorphism at position 132 of *Prnp* is of particular interest because it corresponds to the methionine–valine (MV) *PRNP* codon 129 polymorphism in humans, which is itself a major genetic susceptibility factor associated with human prion disease (*18*). Although these examples of CWD all derive from North America, the inclusion of white-tailed deer (a relative of European roe deer, *Capreolus capreolus*), North American elk (a near relative of red deer), and, most important, reindeer might help in formulating risk assessments in Europe.

Materials and Methods

Animal Tissue

We obtained from the National and OIE Reference Laboratory for Scrapie and CWD (Ottawa, ON, Canada) elk, white-tailed deer, and reindeer frozen brain tissue from CWD-affected animals that had been confirmed positive by using a statutory diagnostic testing regimen (Table). Small pieces of tissue were obtained, and we analyzed all for the presence of PrP^{res} by using Western blot, as previously described (*15*). We used transgenic mouse brains produced by gene replacement and expressing physiologic levels of the human PrP of the 3 *PRNP* codon 129 polymorphic genotypes as the PMCA substrate (*20*).

Protein Misfolding Cyclic Amplification

Substrate and Seed Preparation

We homogenized transgenic mouse brains in conversion buffer by using a glass on glass manual grinder and a conversion buffer made of 1× phosphate-buffered saline (PBS), 150 mmol/L of NaCl, 1% Triton X-100, and a complete protease inhibitor cocktail (cOmplete; Roche, Mannheim, Germany) to obtain a final 10% weight-to-volume solution. We cleared the homogenized tissue by using centrifugation as described previously (*15*), aliquoted the supernatant (i.e., PMCA substrate) into 1.5 mL tubes, and stored at –80°C until used. We homogenized the CWD brain material (i.e., PMCA seed) by using 1.5-mL Eppendorf tubes and disposable polypropylene pestles that used the same buffer.

PMCA Procedure

We performed amplification in a programmable Q-700 sonicator attached to a microplate aluminum horn (*15*). We mixed brain homogenate CWD PMCA seeds with aliquots of PMCA substrate in a final volume of 120 µL in PCR tubes at a 1:3 ratio. We included low molecular weight heparin at 100 µg/mL (*21*) in all PMCA reactions and added EDTA to a final concentration of 6 mmol/L. To perform a comparison between samples before and after the amplification procedure, we took 19 µL of each reaction mixture before the serial cycles of sonication and incubation. Each cycle consisted of 20 s sonication (at an amplitude of 38, wattage 278–300) followed by 29 min and 40 s incubation; we repeated this procedure 96 times (48 h).

Proteolytic Treatment and Western Blotting

We evaluated the presence of PrP^{res} by Western blot after proteinase K treatment. We incubated 19 µL of each sample with proteinase K in a final concentration of 50 µg/mL for 1 h at 37°C in a standard thermoblock. Before loading, we mixed samples with an appropriate volume of 4× NuPAGE buffer (Invitrogen, Carlsbad, CA, USA) and boiled them at 100°C for 10 min. We loaded the samples on NuPAGE Novex (Fisher Scientific; Loughborough,

Table. Description of cervid CWD specimens used to evaluate the susceptibility of human prion protein to conversion by in vitro conversion analysis*

Species	Common name: North America	Common name: Europe	Specimen ID	Disease in captive animals or experimental transmission	*Prnp* genotype, known relevant polymorphisms
Cervus canadensis	North American or Rocky Mountain elk or wapiti	Closely related to red deer (*Cervus elaphus*)	Elk 0†	Captive	132 MM
			Elk 1	Captive	132 MM
			Elk 2	Captive	132 MM
			Elk 3	Captive	132 MM
			Elk 4	Captive	132 MM
			Elk 5	Captive	132 ML
			Elk 6	Captive	132 ML
Odocoileus virginianus	White-tailed deer	Related to roe deer (*Capreolus capreolus*)	WTD 1	Experimental	96 GG
			WTD 2	Experimental	96 GG
Rangifer tarandus subspecies	Caribou (free-ranging) or reindeer (captive)	Reindeer (free-ranging and captive)	Reindeer 1	Experimental (transmission of WTD [CWD 96GG])‡	§
			Reindeer 2	Experimental [transmission of WTD (CWD 96GG)]¶	§

*CWD, chronic wasting disease; WTD, white-tailed deer.
†Previously reported in Barria et al. (*15*).
‡Case 12 previously reported in Mitchell et al. (*19*).
§Reportedly identical to the case of CWD in a wild reindeer in Norway (Benestad et al. [*2*]).
¶Case 47 previously reported in Mitchell et al. (*19*).

United Kingdom) 10% Bis-Tris gels (1.0 mm, 10 wells) and subjected them to electrophoresis at 200 volts for 55 min. We transferred proteins to a polyvinylidene difluoride membrane by using 800 mA for 60 min (*15*) and blocked membranes with 2% milk for 1 h. We determined accumulated human PrP^{res} on the basis of the specific immunoreactivity of the 3F4 monoclonal antibody (mAb) diluted 1/10,000 (Millipore; Watford, United Kingdom). We detected CWD PrP by stripping the Western blots (Thermo Fisher, Bleiswijk, Netherlands) and reprobing them with a 6H4 antibody diluted 1/40,000 (Prionics; Schlieren, Switzerland). We used ECL antimouse IgG, peroxide-linked species-specific F(ab′)2 fragment from sheep (GE Healthcare Life Sciences; Little Chalfont, United Kingdom) as a secondary antibody diluted 1/25,000. We developed membranes by chemiluminescent detection using ECL Prime (GE Healthcare Life Sciences) and acquired digital images by using an XRS Bio-Rad system (Bio-Rad Laboratories, Hercules, CA, USA) with a CCD camera.

Criteria for Positivity

The criterion for conversion was 3F4 antibody detection of a pattern of 3 clear protease-resistant bands of the expected electrophoretic mobility and ratio for PrP^{res} in the amplified sample after a defined period of Western blot image capture. The triplet pattern had to be absent from the unamplified sample tested under the same conditions analyzed in parallel.

Precipitation of Insoluble PrP

We incubated brain homogenate from white-tailed deer and reindeer CWD specimens by using 20% sarkosyl (diluted in PBS) for 10 min at room temperature. We subjected samples to centrifugation for 1 h at 100,000 × *g* at 4°C as described previously (*21*). After centrifugation, we discarded the supernatant and washed the pellet with PBS followed by a second centrifugation (100,000 × *g* for 1 h at 4°C). We resuspended the washed pellets directly in PMCA substrate before using them in a single round of amplification.

Results

Determination of Total PrP in Cervid CWD Specimens

We first characterized the CWD brain tissues for the presence of total PrP and PrP^{res} by using mAb 6H4. We detected similar levels of total PrP by using Western blotting among the elk specimens analyzed (Figure 1). However, we did not find readily detectable levels of PrP^{res} in all the samples; we detected PrP^{res} in 3 of the 5 elk specimens of the 132 MM genotype and both of the 132 ML samples (Figure 2, panels A and C). White-tailed deer samples showed similar expression levels of total PrP in the 2 specimens analyzed, but PrP^{res} levels were low (Figures 1,3). We also confirmed total PrP in the reindeer specimens, with a robust detection of PrP^{res} in 1 of the available samples (reindeer 1) but low levels in the other specimen (reindeer 2) (Figures 1,4).

In vitro Conversion of Human PrP by Cervid Prions

Elk (*C. Canadensis*)

We then performed a single round of PMCA, incubating the *PRNP* codon 132 MM elk CWD seeds in humanized transgenic 129 MM mouse brain substrate. Only those elk CWD samples that had readily detectable PrP^{res} (as detected by 6H4 mAb) were able to produce human PrP^{res} (detectable by the 3F4 mAb) after Western blot and proteinase K treatment, consistent with CWD PrP^{res} playing a direct role in the misfolding process (Figure 2, panel A).

We then addressed the role of the human *PRNP* codon 129 and the cervid *PRNP* codon 132 polymorphisms in the conversion of human PrP^{C}. To seed PMCA reactions, we

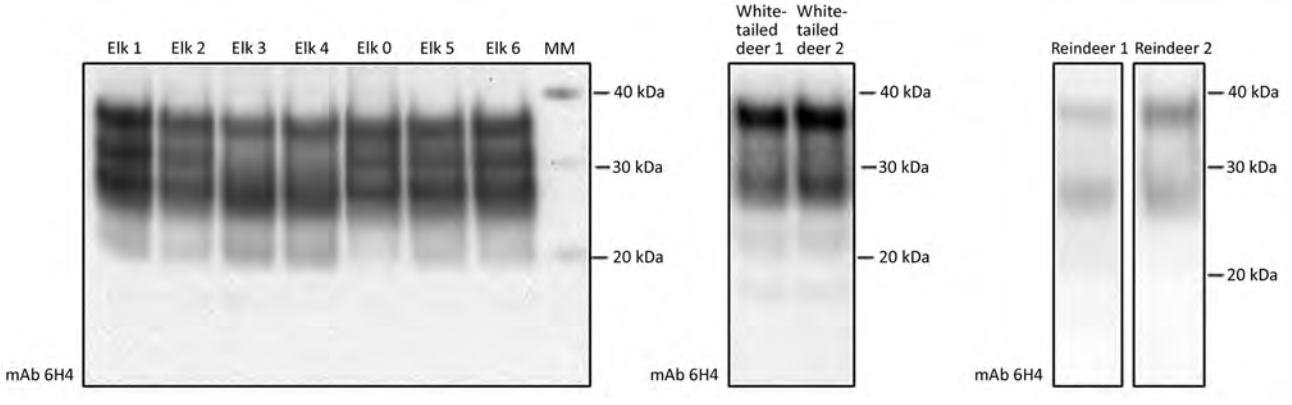

Figure 1. Western blot analysis showing detectable levels of prion protein in the chronic wasting disease–affected cervid brain specimens used to evaluate the susceptibility of the human prion protein (PrP) to conversion by chronic wasting disease prions. We analyzed brain homogenate derived from elk, white-tailed deer, and reindeer specimens by using Western blot to evaluate levels of total PrP. We subjected 2 μL of each 10% brain homogenate sample to Western blot and assessed detection of total PrP by mAb 6H4. We performed 3 technical repeats with similar results; a representative Western blot is shown. Reference molecular markers have been included. mAb, monoclonal antibody.

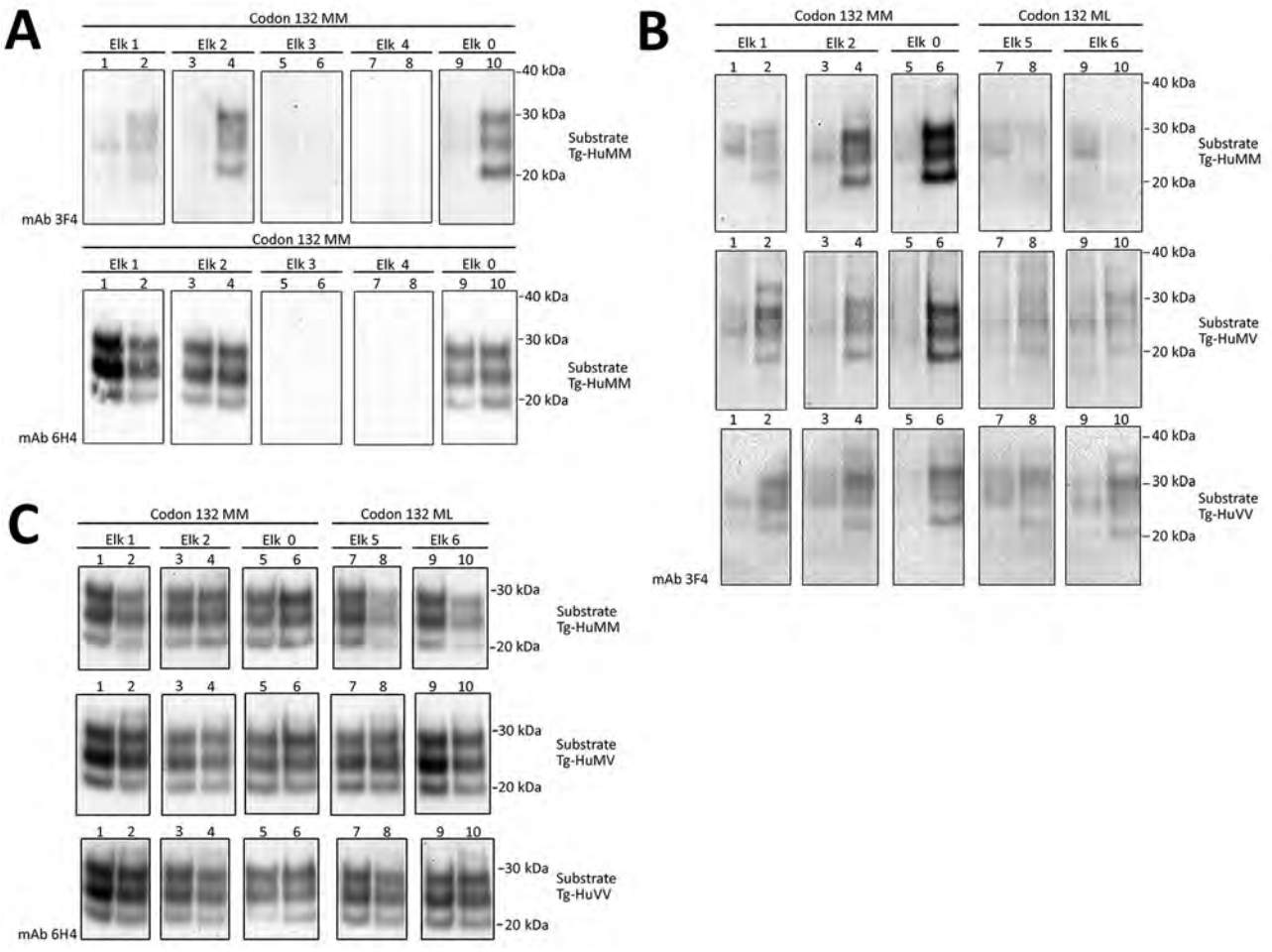

Figure 2. Evaluation of the in vitro conversion of human prion protein (PrP) seeded with the misfolded, disease-associated prion protein form present in chronic wasting disease (CWD)–affected elk brain samples. Western blot analysis for PrP with odd and even number lanes showing reaction mixtures before and after protein misfolding cyclic amplification. A) We incubated 5 elk CWD specimens (elk 0–4) homozygous for *Prnp* codon 132 methionine (MM) in Tg-HuMM brain substrate (diluted 1:3) and subjected them to a single round of protein misfolding cyclic amplification followed by proteinase K digestion. We performed Western blot analysis by using the mAb 3F4 (for the detection of human protease-resistant prion protein [PrP^{res}]) and 6H4 (detection of CWD PrP^{res} and human PrP^{res}). B) We used a panel of 3 humanized transgenic substrates (Tg-HuMM, Tg-Hu-MV, and Tg-HuVV) to evaluate the susceptibility of the human PrP to conversion. We assessed 3 CWD elk seeds of the132 MM genotype and 2 of the 132 methionine–leucine (ML) genotype. We detected conversion of the human PrP by CWD prions by using the mAb 3F4 after proteinase K digestion. C) We detected total PrP^{res} by using Western blot with mAb 6H4. The elk specimen previously reported (*15*) is designated elk 0. We performed ≥5 repeats for the amplification of elk CWD 132 MM seeds and ≥3 for the 132 ML specimens with similar results. Reference molecular markers have been included. Molecular mass of electrophoretic markers is given. mAb, monoclonal antibody; Tg-HuMM, humanized transgenic *PRNP* codon 129 homozygous methionine; Tg-HuMV, humanized transgenic methionine/valine; Tg-HuVV, humanized transgenic valine/valine.

used CWD brain homogenates from 132 MM and 132 ML elk with comparable quantities of CWD PrP^{res} (Figure 2, panel C) and then used humanized transgenic mouse substrates of the 3 possible *PRNP* codon 129 genotypes (129 MM, 129 MV, and 129VV). Each of the CWD methionine homozygous (132 MM) samples resulted in human PrP^{res} formation when used to seed the matched humanized substrate (129 MM). The heterozygous elk seeds did not result in any detectable conversion of the humanized 129 MM PrP substrate. When we incubated these same CWD brain homogenates with the heterozygous (129 MV) and homozygous (129VV) humanized substrates, we observed low levels of human PrP^{res} formation but no obvious difference in efficiency between the CWD 132MM and CWD 132ML samples in the 129VV substrate (Figure 2, panel B).

White-tailed Deer (*O. virginianus*)

We performed similar analyses to determine the competence of white-tailed deer CWD to convert the human PrP (Figure 3). To maintain consistency, we homogenized

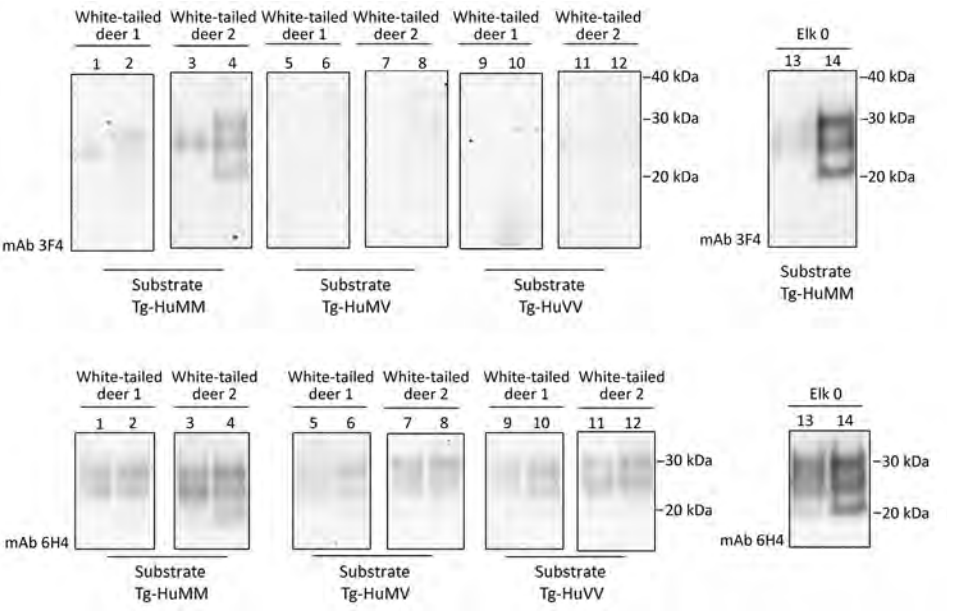

Figure 3. Evaluation of the in vitro conversion of human prion protein (PrP) seeded with the misfolded, disease-associated prion protein form present in chronic wasting disease (CWD)–affected white-tailed deer brain samples. We incubated 2 white-tailed deer CWD brain homogenates, derived from 2 affected animals (white-tailed deer 1 and 2), in a panel of 3 humanized transgenic substrates (Tg-HuMM, Tg-HuMV, and Tg-HuVV) and subjected them to a single round of protein misfolding cyclic amplification (PMCA) followed by proteinase K digestion. We diluted CWD brain homogenate 1:3 in PMCA substrate and performed Western blot analysis by using the mAb 3F4 (for the detection of human protease-resistant prion protein [PrP^{res}]) and mAb 6H4 (for detection of CWD PrP^{res} and human PrP^{res}). We incorporated the elk specimen designated elk 0 as a control. We performed ≥3 repeats for the amplification of white-tailed deer CWD 1 and 2 specimens with similar results. Reference molecular markers have been included. Molecular mass of electrophoretic markers is given. Odd and even number lanes show reaction mixtures before and after PMCA. mAb, monoclonal antibody; Tg-HuMM, humanized transgenic *PRNP* codon 129 homozygous methionine; Tg-Hu-MV, humanized transgenic methionine/valine; Tg-HuVV, humanized transgenic valine/valine.

the 2 available CWD white-tailed deer specimens at 10% (weight/volume) and then normalized by volume (seed/substrate) to seed the PMCA reactions. Detection of CWD PrP^{res} by 6H4 antibody revealed that the levels of PrP^{res} in the unamplified samples were not equivalent to the elk CWD specimens used for PMCA (Figure 2, panel C; Figure 3). However, 1 of the analyzed specimens showed some conversion of the humanized 129 MM PrP substrate, although PrP^{res} formation was undetectable in the heterozygous and valine homozygous substrate with the 3F4 antibody (Figure 3). These results suggest a higher degree of molecular compatibility of the *PRNP* codon 129 MM human genotype and CWD PrP^{Sc}, consistent with what we observed in most of the elk CWD 132 MM specimens.

Reindeer (*R. tarandus*)

Natural cases of CWD in reindeer have been detected in Norway, but North American reindeer were previously shown to be experimentally susceptible to white-tailed deer CWD by the oral route (*18*). We tested samples from these 2 experimentally infected reindeer (*18*) (Figure 4). Both reindeer specimens were capable of converting the humanized 129 MM PrP substrate, although different amounts of CWD PrP^{res} were detected by the 6H4 antibody in the frozen samples. Densitometry analysis suggested that 1 specimen (from reindeer 2) had roughly one tenth of the PrP^{res} of the other sample, showing that the reaction with small amounts of reindeer PrP^{res} are able to convert the humanized substrate. The *PRNP* codon 129 MV and VV genotype substrates also were readily susceptible to conversion by the reindeer seeds (Figure 4). Seeding efficiency of reindeer CWD was maintained when the seeding material was normalized by using semipurified PrP^{res} (Figure 5), arguing against the possibility that the apparent enhanced seeding potential of reindeer CWD simply reflects the increased abundance of PrP^{res} in reindeer samples or was a result of conversion of endogenous reindeer seed–associated PrP^{C}.

Discussion

Characterization of the transmission properties of CWD and evaluation of their zoonotic potential are important for public health purposes. Given that CWD affects several members of the family *Cervidae*, it seems reasonable to consider whether the zoonotic potential of CWD prions could be affected by factors such as CWD strain, cervid species, geographic location, and *Prnp–PRNP* polymorphic variation. We have previously used an in vitro conversion assay (PMCA) to investigate the susceptibility of the human PrP to conversion to its disease-associated form by several animal prion diseases, including CWD (*15*,*16*,*22*). The sensitivity of our molecular model for the detection of zoonotic conversion depends on the combination of 1) the action of proteinase K to degrade the abundant human PrP^{C} that constitutes the substrate while only N-terminally truncating any human PrP^{res} produced and 2) the presence of the 3F4 epitope on human but not

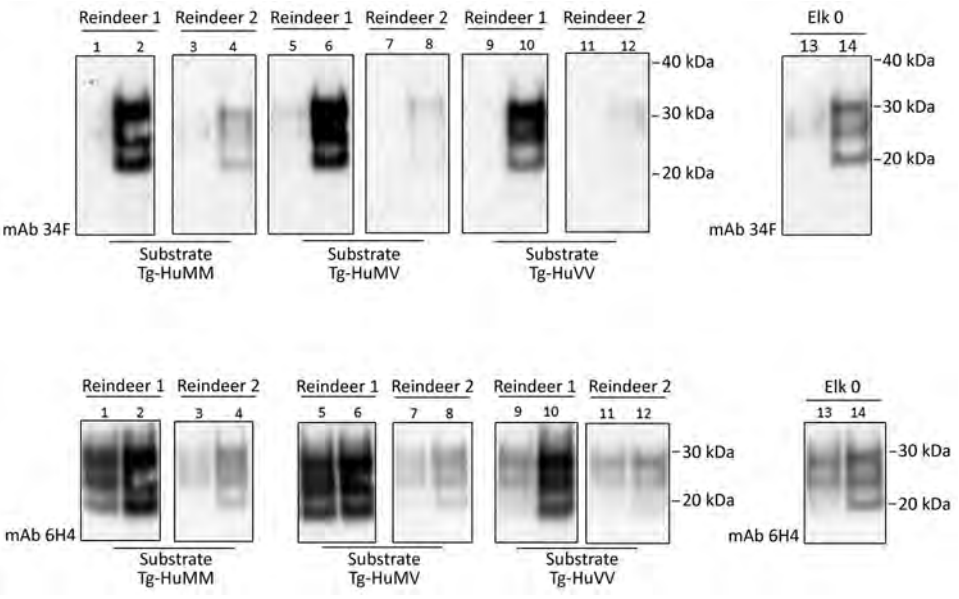

Figure 4. Evaluation of the in vitro conversion of human prion protein (PrP) seeded with the misfolded, disease-associated prion protein form present in chronic wasting disease (CWD)–affected reindeer brain samples. We incubated 2 reindeer CWD specimens (reindeer 1 and 2) in a panel of 3 humanized transgenic substrates (Tg-HuMM, Tg-HuMV, and Tg-HuVV) and subjected them to a single round of protein misfolding cyclic amplification (PMCA). We diluted PMCA seeds 3 times in fresh PMCA substrate (dilution factor 1:3) and evaluated PMCA reactions for the presence of protease-resistant prion protein (PrP^{res}) by proteinase K digestion. We performed Western blot analysis by using mAb 3F4 (for the detection of human PrP^{res}) and mAb 6H4 (for detection of CWD PrP^{res} and human PrP^{res}). We incorporated the elk specimen designated elk 0 as a control. We performed ≥5 repeats for the amplification of reindeer 1 and 2 specimens. Reference molecular markers have been included. Molecular mass of electrophoretic markers is given. Odd and even number lanes show reaction mixtures before and after PMCA. mAb, monoclonal antibody; Tg-HuMM, humanized transgenic *PRNP* codon 129 homozygous methionine; Tg-Hu-MV, humanized transgenic methionine/valine; Tg-HuVV, humanized transgenic valine/valine.

cervid PrP. In effect, this degree of sensitivity means that any human PrP^{res} formed during the PMCA reaction can be detected down to the limit of Western blot sensitivity. In contrast, if other antibodies that detect both cervid and human PrP are used, such as 6H4, then newly formed human PrP^{res} must be detected as a measurable increase in PrP^{res} over the amount remaining in the reaction product from the cervid seed. Although best known for the efficient amplification of prions in research and diagnostic contexts, the variation of the PMCA method employed in our study is optimized for the definitive detection of zoonotic reaction products of inherently inefficient conversion reactions conducted across species barriers. By using this system, we previously made and reported the novel observation that elk CWD prions could convert human PrP^{C} from human brain and could also convert recombinant human PrP^{C} expressed in transgenic mice and eukaryotic cell cultures (*15*).

A previous publication suggested that mule deer PrP^{Sc} was unable to convert humanized transgenic substrate in PMCA assays (*23*) and required a further step of in vitro conditioning in deer substrate PMCA before it was able to cross the deer–human molecular barrier (*24*). However, prions from other species, such as elk (*15*) and reindeer affected by CWD, appear to be compatible with the human protein in a single round of amplification (as shown in our study). These observations suggest that different deer species affected by CWD could present differing degrees of molecular compatibility with normal form of human PrP.

The contribution of the polymorphism at codon 129 of the human PrP gene has been extensively studied and is recognized as a risk factor for Creutzfeldt-Jakob disease (*4*). In cervids, the equivalent codon corresponds to the position 132 encoding methionine or leucine. This polymorphism in the elk gene has been shown to play an important role in CWD susceptibility (*25*,*26*). We have investigated the effect of this cervid *Prnp* polymorphism on the conversion of the humanized transgenic substrate according to the variation in the equivalent *PRNP* codon 129 polymorphism. Interestingly, only the homologs methionine homozygous seed–substrate reactions could readily convert the human PrP, whereas the heterozygous elk PrP^{Sc} was unable to do so, even though comparable amounts of PrP^{res} were used to seed the reaction. In addition, we observed only low levels of human PrP^{res} formation in the reactions seeded with the homozygous methionine (132 MM) and the heterozygous (132 ML) seeds incubated with the other 2 human polymorphic substrates (129 MV and 129 VV). The presence of the amino acid leucine at position 132 of the elk *Prnp* gene has been attributed to a lower degree of prion conversion compared with methionine on the basis of experiments in mice made transgenic for these polymorphic variants (*26*). Considering the differences observed for the amplification of the homozygous human methionine substrate by the 2 polymorphic elk seeds (MM and ML), reappraisal of the susceptibility of human PrP^{C} by the full range of cervid polymorphic variants affected by CWD in would be warranted.

In light of the recent identification of the first cases of CWD in Europe in a free-ranging reindeer (*R. tarandus*) in Norway (*2*), we also decided to evaluate the in vitro conversion potential of CWD in 2 experimentally infected reindeer (*18*). Formation of human PrP^{res} was readily detectable after a single round of PMCA, and in all 3 humanized polymorphic substrates (MM, MV, and VV). This finding suggests that CWD prions from reindeer could be more compatible with human PrP^{C} generally and might therefore present a greater risk for zoonosis than, for example, CWD prions from white-tailed deer. A more comprehensive comparison of CWD in the affected species, coupled with the polymorphic variations in the human and deer *PRNP–Prnp* genes, in vivo and in vitro, will be required before firm conclusions can be drawn. Analysis of the *Prnp* sequence of the CWD reindeer in Norway was reported to be identical to the specimens used in our study (*2*). This finding raises the possibility of a direct comparison of zoonotic potential between CWD acquired in the wild and that produced in a controlled laboratory setting. (Table).

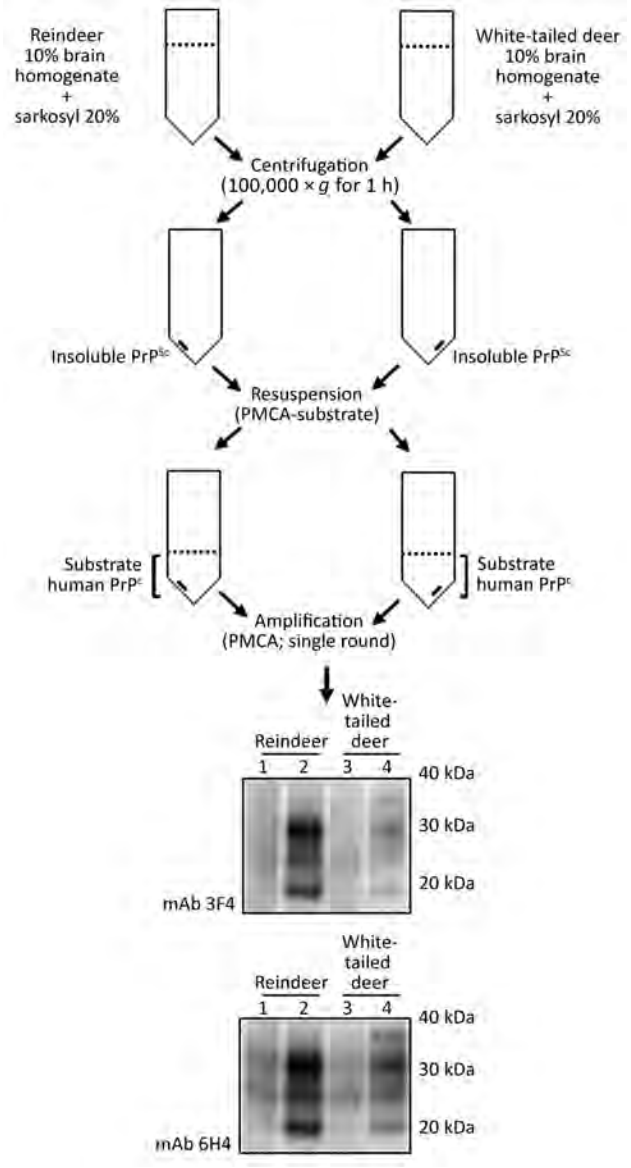

Figure 5. Schematic representation of the partial purification of misfolded, disease-associated prion protein from chronic wasting disease (CWD)–affected deer brain specimens and its continued ability to seed the conversion of human prion protein (PrP) during protein misfolding cyclic amplification (PMCA) reactions. We normalized PrP, partially purified by detergent insolubility from reindeer and white-tailed deer CWD specimens, by using protease-resistant prion protein (PrP^{res}) and subjected PrP to a single round of PMCA in humanized transgenic *PRNP* codon 129 homozygous methionine. We performed Western blot analysis by using mAb 3F4 (for detection of human PrP^{res}) and mAb 6H4 (for detection of CWD PrP^{res} and human PrP^{res}). Molecular mass of electrophoretic markers is given. Odd and even number lanes show reaction mixtures before and after PMCA. mAb, monoclonal antibody; PMCA, protein misfolding cyclic amplification; PrP^{C}, normal isoform of the prion protein; PrP^{Sc}, disease-associated isoform of the prion protein.

The prion hypothesis proposes that direct molecular interaction between PrP^{Sc} and PrP^{C} is necessary for conversion and prion replication. Accordingly, polymorphic variants of the PrP of host and agent might play a role in determining compatibility and potential zoonotic risk. In this study, we have examined the capacity of the human PrP^{C} to support in vitro conversion by elk, white-tailed deer, and reindeer CWD PrP^{Sc}. Our data confirm that elk CWD prions can convert the human PrP^{C}, at least in vitro, and show that the homologous *PRNP* polymorphisms at codon 129 and 132 in humans and cervids affect conversion efficiency. Other species affected by CWD, particularly caribou or reindeer, also seem able to convert the human PrP. It will be important to determine whether other polymorphic variants found in other CWD-affected *Cervidae* or perhaps other factors (*17*) exert similar effects on the ability to convert human PrP and thus affect their zoonotic potential.

Acknowledgments

We thank Aru Balachandran for originally providing cervid brain tissues, Abigail Diack and Jean Manson for providing mouse brain tissue, and James Ironside for his critical reading of the manuscript at an early stage.

This report is independent research commissioned and funded by the United Kingdom's Department of Health Policy Research Programme and the Government of Scotland. The views expressed in this publication are those of the authors and not necessarily those of the Department of Health or the Government of Scotland.

Author contributions: The study was conceived and designed by M.A.B. and M.W.H. The experiments were conducted by M.A.B. and A.L. Chronic wasting disease brain specimens were provided by G.M. The manuscript was written by M.A.B. and M.W.H. All authors contributed to the editing and revision of the manuscript.

About the Author

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Poverty and Community-Acquired Antimicrobial Resistance with Extended-Spectrum β-Lactamase–Producing Organisms, Hyderabad, India

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The decreasing effectiveness of antimicrobial agents is a global public health threat, yet risk factors for community-acquired antimicrobial resistance (CA-AMR) in low-income settings have not been clearly elucidated. Our aim was to identify risk factors for CA-AMR with extended-spectrum β-lactamase (ESBL)–producing organisms among urban-dwelling women in India. We collected microbiological and survey data in an observational study of primigravidae women in a public hospital in Hyderabad, India. We analyzed the data using multivariate logistic and linear regression and found that 7% of 1,836 women had bacteriuria; 48% of isolates were ESBL-producing organisms. Women in the bottom 50th percentile of income distribution were more likely to have bacteriuria (adjusted odds ratio 1.44, 95% CI 0.99–2.10) and significantly more likely to have bacteriuria with ESBL-producing organisms (adjusted odds ratio 2.04, 95% CI 1.17–3.54). Nonparametric analyses demonstrated a negative relationship between the prevalence of ESBL and income.

Antimicrobial resistance (AMR) is a growing global public health threat that could reverse decades of progress in increasing longevity around the world (*1*). The rapid pace of AMR spread coupled with a shortage of novel antimicrobial agents has led the World Health Organization (WHO) to warn of a "postantimicrobial era" (*2*). By 2050, deaths attributable to AMR could exceed 10 million per year, more than the number of deaths from cancer and traffic accidents combined, while costing the global economy US $60–$100 trillion of economic output (*3*). Most policy recommendations for curtailing AMR are tailored to wealthy nations; however, the threat of AMR is also severe for poorer countries (*4*,*5*). Infectious diseases are still common causes of illness and death in such settings, and the availability of second- or third-line therapies is limited (*6*,*7*). Yet, detailed estimates of the prevalence of AMR in developing countries are limited and often rely on samples from returning travelers or in-country patients receiving care for suspected infectious diseases. These data point to an alarming rate of community-acquired AMR (CA-AMR) in extended-spectrum β-lactamase (ESBL)–producing organisms, including in Hyderabad, India, the setting of this study (*8*–*11*). Such trends are of international concern given the possibility for AMR pathogens and genetic elements to spread across political and geographic boundaries.

Because of the lack of detailed data from developing countries, empirical work on predictors of CA-AMR is generally limited to responses from wealthy countries or cross-country comparisons. These studies generally document robust positive relationships between antimicrobial drug use, healthcare provider contacts, and the prevalence of AMR (*12*–*15*). These strong links have led to the hypothesis that growing drug resistance in developing countries is attributable to rising incomes, which increase demand for many health products, including antimicrobial drugs (*16*–*18*). Yet, other factors that place the poor at greater risk for CA-AMR might be more pronounced in developing countries. First, the poor are more likely to be exposed to infectious agents from other humans and are at higher risk for illness because of malnutrition and immunodeficiency (*4*). Second, the poor are more likely to experience subinhibitory doses of antimicrobial agents because of shorter courses

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DOI: https://doi.org/10.3201/eid2408.171030

of treatment, sharing medication, or expired or low-quality drugs (*19–22*). Third, the poor may be more likely to acquire resistant pathogens or AMR genetic elements in their food or water, leading to CA-AMR (*23–25*).

To clarify the prevalence of and risk factors for CA-AMR and the specific relationship with poverty for individual persons in a developing country context, we prospectively collected data on 1,836 primigravidae women in a large public hospital in Hyderabad, India, over 12 months. To reduce the probability that AMR was acquired through a healthcare contact or activity, our study population consisted of women who were carrying a pregnancy to term for the first time and had never been hospitalized for a pregnancy. We focused on ESBL-producing organisms because findings by other researchers demonstrated rising rates of community-acquired infections associated with these organisms (*8–11*,*15*). We complemented our microbiological sample collection with a detailed survey on sociodemographic information and assessed AMR risk factors using the US Demographic and Health Surveys tool AMR Module for Population-Based Surveys (*26*,*27*).

Methods

Study Design

The design was a cross-sectional observational study of women in Hyderabad, India, carrying a pregnancy to full term for the first time. The study was approved by the Indian Council of Medical Research (ICMR), the Institutional Review Board of Gandhi Medical College and Hospital, and the Administrative Panel for the Protection of Human Subjects (Institutional Review Board) of Stanford University.

Setting and Participants

We conducted the study at Gandhi Medical College and Hospital, a large public teaching hospital in Hyderabad that provides free healthcare for all. We surveyed first-time pregnant women seeking antenatal care from October 1, 2015, through September 29, 2016. We identified eligible patients from the outpatient clinic roster. Patients were deemed eligible if they were ≤40 years of age, pregnant for the first time, and had not been interviewed on a prior visit. After obtaining informed consent, a team member interviewed patients in a quiet research office. One woman with an incomplete survey was omitted. Results are robust to her inclusion.

Data Sources/Measurement

We performed urine culture and bacterial identification using ChromID CPS3 agar and the VITEK-2 system (both from BioMerieux, Marcy l'Etoile, France). We performed antimicrobial susceptibility testing and interpretations, including ESBL screening using theVITEK-2 ESBL test (identification and antimicrobial susceptibility pattern), in accordance with guidelines from the Clinical and Laboratory Standards Institute (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/17-1030-Techapp1.pdf). We diagnosed bacteriuria when there were $\geq 10^5$ CFU of a single bacterial strain per milliliter of urine or when ≥2 different colony types were present and 1 had a colony count of $\geq 10^5$ CFU/mL.

A trained onsite investigator conducted a structured interview at the time of the antenatal visit using a questionnaire based on the Demographic and Health Surveys tool AMR Module for Population-Based Surveys (*27*). We queried patients on their usual and current residence, occupation, husband's occupation, household income, religion, caste, education level, dietary and hygiene practices, and recent nonvitamin tablet consumption. Many of the women did not understand the original question about antimicrobial drug use, so we used ingestion of any tablet other than a vitamin as an upper bound on recent antimicrobial drug ingestion. Study staff also directed women to use the clean-catch urine sample technique. We sent all laboratory test results to the patient's healthcare provider for further action. Definitions of variables included in the analysis are provided in the online Technical Appendix.

Statistical Methods

We performed 3 analyses to identify predictors of bacteriuria or growth of an ESBL-producing organism. First, we assessed univariate relationships among sociodemographic, clinical, and environmental exposures and bacteriuria or CA-AMR caused by ESBL-producing organisms; we used *t*-tests for continuous outcomes and χ^2 tests for categorical outcomes.

Guided by the results from an unadjusted analysis, which demonstrated poverty as a significant predictor of the outcome, we used logistic regression to estimate the association between income and bacteriuria, as well as between income and bacteriuria from ESBL-producing organisms. The model also included background characteristics that might influence bacteriuria or AMR, such as respondents' education level. We included age and Hindu religion because results for both were significant in 1 of the 2 univariate analyses. We included prior hospitalization and history of abortion because these variables reflect prior exposure to the inpatient medical system, which has been shown to be a risk factor for AMR in industrialized countries (*28*). We did not include previous antimicrobial drug use in our main model, even though it is included in a related analysis (online Technical Appendix Figure 1). Because few study participants reported any tablet ingestion in the last 30 days, the confidence intervals were very large (*13*). The reported results on income were not sensitive to the inclusion of tablet ingestion.

Next, we used linear regression to assess the nonparametric relationship between income and CA-AMR from ESBL-producing organisms. Specifically, we divided income into quartiles and included these quartiles in the linear regression along with the covariates previously described and used in the logit regression. For the 59 respondents missing income information (3.2% of the sample), we generated predicted income values from husband's occupation, education level, age at marriage, religion, and season. We normalized income per 10,000 rupees for graphing purposes. We performed the same analysis with the missing income values dropped rather than substituted with predicted values (online Technical Appendix Figure 2).

Results

The average age of women in the study was 21.8 years, slightly higher than the national average age at the time of first birth, which is 19.9 years (Table 1). Approximately one third (631/1,836) of the women surveyed were anemic; 862 (46.5%) were underweight and 288 (15.6%) reported a previous abortion, although none had previously carried a pregnancy to term. Forty-seven (2.6%) reported taking any nonvitamin tablet in the last 30 days.

A total of 126 respondents had significant bacterial growth in their urine, defined as $\geq 10^5$ CFU/mL. Gram-negative rods accounted for 107/126 (85%) of the isolates, including *Escherichia coli* (n = 75), *Klebsiella* (n = 28), *Sphingomonas* (n = 2), *Enterobacter* (n = 1), and *Citrobacter* (n = 1). The remaining 19 isolates were gram-positive organisms, including *Staphylococcus*, *Streptococcus*, and *Enterococcus*; 1 organism was unknown. Of the ESBL isolates, 82% were *E. coli* and 18% were *K. pneumoniae*. At this level of prevalence, the study had 80% power to detect differences of ≈0.10 in the proportion of categorical variables and ≈0.75 in continuous variables.

Women with significant bacteriuria did not substantially differ from those without bacteriuria in terms of age, age at time of marriage, or educational background (Table 1). Prevalence of anemia, dysuria, fever, and low weight were also not statistically different between the 2 groups, nor were hospitalizations within the previous year or use of nonvitamin tablets over the previous 30 days. Hindu participants were less likely to have high levels of bacteriuria than were those from other religious groups (p = 0.02), although we did not find a significant relationship between ESBL and religion. Women with bacteriuria were significantly more likely to fall in the lower half of the income distribution: 60.0% of women with bacterial growth had household incomes below the sample median, compared with 50.2% of women with no bacterial growth (p = 0.04).

We compared women with ESBL-producing bacteria with women without these bacteria (Table 2). Similar to the findings regarding bacteriuria, the demographic and clinical characteristics of women with ESBL-producing

Table 1. Unadjusted relationship between significant bacteriuria and sociodemographic, clinical, and environmental characteristics for pregnant women in Hyderabad, India*

Characteristic	All respondents, N = 1,836	>10^5 CFU/mL bacteria in urine, n = 126	No bacteria in urine, n = 1,710	p value
Sociodemographic characteristics				
Mean age, y (SD)	21.81 (2.95)	22.05 (3.02)	21.79 (2.94)	0.35
Mean age at marriage, y (SD)	20.31 (2.87)	20.29 (3.22)	20.31 (2.84)	0.93
Low income, no. (%)†	932 (50.8)	75 (60.0)	857 (50.2)	**0.04**
Less than secondary education, no. (%)‡	322 (17.5)	23 (18.4)	299 (17.5)	0.81
Hindu, no. (%)§	1,228 (66.9)	72 (57.6)	1,156 (67.8)	**0.02**
Clinical characteristics, no. (%)				
Anemia	631 (33.6)	48 (38.4)	583 (34.2)	0.38
Low weight of mother	862 (46.5)	61 (48.8)	801 (47.0)	0.78
Previous abortion	288 (15.6)	18 (14.4)	270 (15.8)	0.80
Previous hospitalization¶	159 (8.7)	13 (10.4)	146 (8.6)	0.51
Tablet during last 30 days#	47 (2.6)	5 (4.0)	42 (2.5)	0.25
Dysuria**	220 (12.0)	16 (12.8)	204 (12.0)	0.78
Fever	84 (4.6)	5 (4.0)	79 (4.6)	1.00
Environmental and hygiene-related characteristics, no. (%)				
Household does not treat water	1,339 (72.9)	93 (74.4)	1,246 (73.0)	0.92
Household sewage not piped	90 (4.9)	8 (6.4)	82 (4.8)	0.39
Respondent strictly vegetarian	142 (7.7)	8 (6.4)	134 (7.9)	0.73
Handwashing <5 times/d	345 (18.8)	26 (20.8)	319 (18.7)	0.64

*Bold indicates statistical significance. p values were derived from *t*-test (for mean age variables) or χ^2 test (for categorical variables). CFU, colony-forming units.
†Income is total household income in previous 30 d; low income is an indicator variable for below the 50th percentile of income.
‡Less than secondary education is an indicator variable for whether the participant reported no schooling or primary-only schooling.
§Hindu is an indicator variable for Hindu religion.
¶Hospitalization in the previous 12 mo.
#Tablet means any tablet other than a vitamin taken by the respondent in the 30 d before interview. We used the term "tablet" because "antimicrobial" was unclear for many respondents.
**For dysuria, an answer of "don't know" was coded as 0 (no).

Table 2. Unadjusted relationship between community-acquired antimicrobial drug resistance caused by ESBL-producing organisms and sociodemographic, clinical, and environmental characteristics for pregnant women in Hyderabad, India*

Characteristic	All respondents, N = 1,836	ESBL present, n = 60	No ESBL present, n = 1,776	p value
Sociodemographic characteristics				
Mean age, y (SD)	21.81 (2.95)	22.65 (3.48)	21.78 (2.93)	**0.06**
Mean age at marriage, y (SD)	20.31 (2.87)	20.57 (4.19)	20.30 (2.82)	0.63
Low income, no. (%)†	932 (50.8)	40 (66.7)	892 (50.4)	**0.01**
Less than secondary education, no. (%)‡	322 (17.5)	10 (16.7)	312 (17.6)	1.00
Hindu, no. (%)§	1,228 (66.9)	36 (60.0)	1,192 (67.3)	0.27
Clinical characteristics, no. (%)				
Anemia	631 (33.6)	19 (31.7)	612 (34.6)	0.78
Low weight of mother	862 (46.5)	32 (53.3)	830 (46.9)	0.36
Previous abortion	288 (15.6)	11 (18.3)	277 (15.6)	0.59
Previous hospitalization¶	159 (8.7)	6 (10.0)	153 (8.6)	0.64
Tablet during last 30 days#	47 (2.6)	3 (5.0)	44 (2.5)	0.20
Dysuria**	220 (12.0)	7 (11.7)	213 (12.0)	1.00
Fever	84 (4.6)	2 (3.3)	82 (4.6)	1.00
Environmental and hygiene-related characteristics, no. (%)				
Household does not treat water	1,339 (72.9)	45 (75.0)	1,294 (73.1)	0.77
Household sewage not piped	90 (4.9)	4 (6.7)	86 (4.9)	0.53
Respondent strictly vegetarian	142 (7.7)	2 (3.3)	140 (7.9)	0.32
Handwashing <5 times/d	345 (18.8)	15 (25.0)	330 (18.6)	0.24

*p values were derived from *t*-test (for mean age variables) or χ^2 test (for categorical variables). Bold indicates statistical significance. ESBL, extended-spectrum β-lactamase.
†Income is total household income in previous 30 d; low income is an indicator variable for below the 50th percentile of income.
‡Less than secondary education is an indicator variable for whether the participant reported no schooling or primary-only schooling.
§Hindu is an indicator variable for Hindu religion.
¶Hospitalization in previous 12 mo.
#Tablet means any tablet other than a vitamin taken by the respondent in the 30 d before interview. We used "tablet" because "antimicrobial" was unclear for many respondents.
**For dysuria, an answer of "don't know" was coded as 0 (no).

bacteria were not substantially different from those of women without these bacteria. However, 66.7% of women with ESBL-producing bacteria had household income below the median versus 50.4% of women without these bacteria, a significant finding (p = 0.01).

Figure 1 shows the adjusted odds ratios (aOR) and 95% CIs for the relationships between respondent characteristics and the 2 study outcomes: any significant levels of bacteriuria or bacteriuria with ESBL-producing organisms. Being in the bottom half of the income distribution was associated with a greater likelihood of significant bacteriuria (aOR 1.44, 95% CI 0.99–2.10) and a greater likelihood of bacteriuria caused by ESBL-producing organisms (aOR 2.04, 95% CI 1.17–3.54). The only other predictor of ESBL that achieved statistical significance was age; higher age was positively associated with ESBL-producing organisms (aOR 1.09, 95% CI 1.02–1.18).

Nonparametric relationships between income and bacteriuria (Figure 2, panel A) and between income and ESBL (Figure 2, panel B), adjusted for the same set of covariates as in Figure 1, demonstrate a robust negative relationship between income quartile and both outcome variables. The poorest quartile had a predicted prevalence of 8.67% (95% CI 6.59%–10.73%) for significant bacteriuria and 4.68% (95% CI 3.23%–6.13%) for bacteriuria caused by ESBL organisms. When we restricted the analysis to women who had substantial bacterial growth in the urine, we observed a similar ESBL–income gradient (online Technical Appendix Figures 3, 4). This relationship did not occur between income and other potentially correlated outcome variables, such as hospitalization, past abortion, and tablet consumption (online Technical Appendix Figure 5).

We estimated a model that included a first- and second-order term in income to investigate nonlinearities in the relationship between income and CA-AMR (online Technical Appendix Figure 6). This approach was motivated by the observation of a slightly greater prevalence of ESBL bacteruria in the fourth versus the third income quartile (Figure 2, panel B). The coefficient on income was negative and significant (p = 0.001), consistent with the results from the other models. The coefficient on income squared was positive and significant (p<0.001), which implies a U-shaped function relating AMR and income. However, we calculated that the lowest ESBL risk occurs at an income of 24,000 rupees, which corresponds to roughly the 95th percentile of income in our sample. This finding implies that, for most women we surveyed, the probability of ESBL declines as household income rises.

Discussion

This study produced 4 main results. First, 7% of women (126/1,836) had bacteriuria and ≈48% of their urine isolates were ESBL. This finding is striking for 2 reasons: almost none of these women reported recent antimicrobial

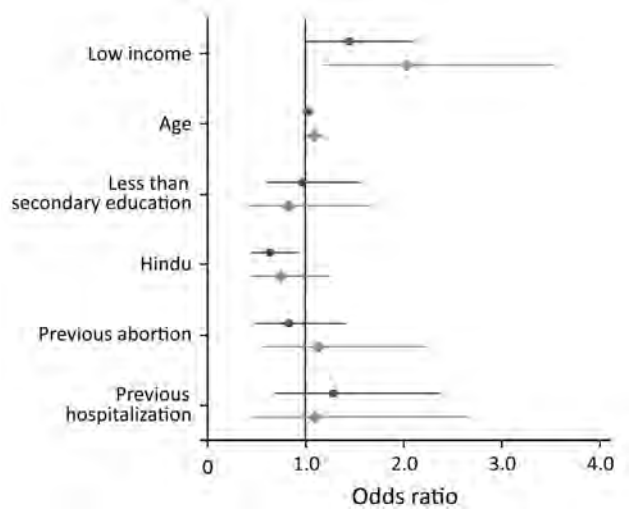

Figure 1. Adjusted odds ratios of bacteriuria and community-acquired antimicrobial resistance with ESBL-producing organisms by selected predictive variables for pregnant women in Hyderabad, India. Black dots represent odds ratios for bacterial growth in urine culture; lines indicate 95% CIs. Gray diamonds represent odds ratios for ESBL-producing organisms; lines indicate 95% CIs. The vertical line shows odds ratio = 1.0. ESBL, extended-spectrum β-lactamase.

drug use, and the sample (women carrying pregnancies to full term for the first time) was specifically chosen to mitigate healthcare-associated exposures that increase the risk for multidrug-resistant organisms. Second, women who had ESBL-producing organisms were less likely than those without these organisms to have been hospitalized recently or to report recent ingestion of nonvitamin tablets, thereby excluding some of the major correlates of AMR among wealthier populations. Third, the most robust predictor of whether women had clinically significant bacteriuria, including with ESBL-producing organisms, was household income. Outside of the top 5% of incomes, this relationship was negative and dose-responsive: the poorer the respondent, the higher her risk for CA-AMR. Fourth, if bacteriuria was present, income was still a robust predictor of ESBL, suggesting that the income–AMR gradient is not driven exclusively by living conditions that would place the poor at higher risk for any bacteriuria; rather, the poor are at higher risk specifically for CA-ESBL.

Theoretical arguments could be made for a link between higher income, antimicrobial consumption, and AMR. Yet, in the context of this study, when comparing across patients within a given public healthcare system that serves all regardless of their ability to pay, we found the opposite: a negative relationship between income and CA-AMR. There are several possible explanations for this finding. First, the poor might not be able to afford the highest quality antimicrobial drugs and may rely instead on expired pills or counterfeit brands, thereby increasing exposure to subinhibitory concentrations of antimicrobial drugs that fuel the emergence of drug-resistant strains (*29*). Second, impoverished persons are more likely to be poorly nourished and thus exposed to infectious diseases, increasing their demand for antimicrobial drugs relative to the wealthy. Given the low prevalence of nonvitamin tablet ingestion (an upper bound on use of oral antimicrobial drugs) in our data, however, these hypotheses seem less likely. Third, the higher prevalence of AMR among poorer persons could be due to contamination. This possibility also seems an unlikely explanation because the isolates we would consider to be members of the skin microbiota (e.g., staphylococcal and streptococcal species) were distributed evenly among the high- and low-income brackets.

The most likely explanation, therefore, appears to be that the poor are exposed to an environmental source of antimicrobial drugs that is placing them at higher risk for CA-AMR than their wealthier peers, resulting in the CA-AMR wealth gradient that we observed. In Hyderabad, where we conducted this study, other researchers have noted levels of many antimicrobial drugs in wastewater treatment plants and treatment plant effluents, including ciprofloxacin, that are several-fold higher than maximal therapeutic plasma levels (*25*,*30*,*31*).

Hindu religion was also marginally significant and protective in some of our specifications, but because our income measures are noisy, this variable may also be picking up relative socioeconomic status; in our sample, Hindu women reported higher mean incomes and higher education levels than women of other religious backgrounds. The differences seem unlikely to be related to diet because strict vegetarianism was not protective.

This study had some limitations. We gathered data at a single hospital in Hyderabad, India; results may differ in other impoverished communities with different environmental exposures and in wealthier populations. In addition, survey responses were self-reported and therefore subject to measurement error and surveyor demand bias; however, it is unclear how this fact might affect the relationship between income and AMR if women did not know their urine results when they answered the survey questions. Future research should attempt to verify some of the self-reported replies and use household consumption survey data in addition to estimates of income to measure poverty (*32*). Further, for reasons related to cultural sensitivity and logistical feasibility, we did not collect fecal samples, and such samples might have revealed different relationships than the urine samples did. In addition, because the study was performed at a large public hospital, we observed only the lower tail of the income distribution. Data from private hospitals that cater to the wealthy might show different patterns and, combined with our data from the public sector,

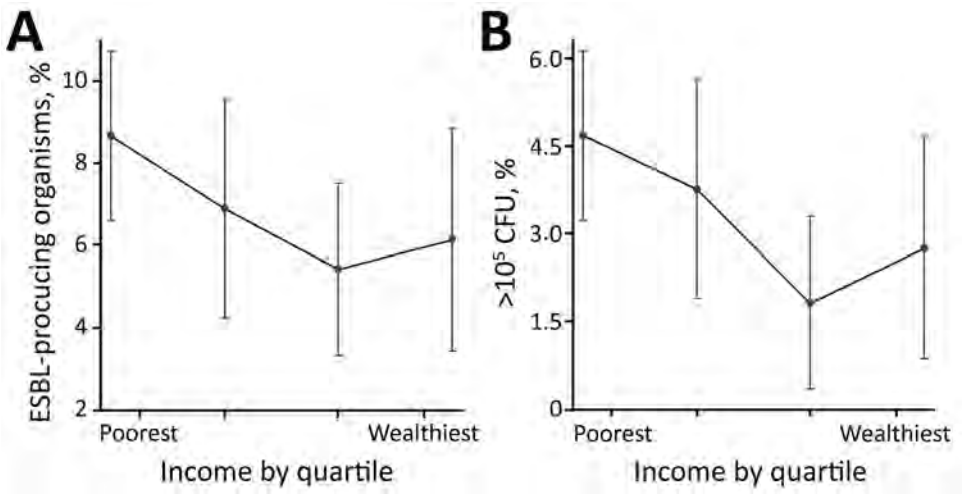

Figure 2. Nonparametric relationships between significant bacterial growth in urine culture and income (A) and between community-acquired antimicrobial resistance with ESBL and income (B) for pregnant women in Hyderabad, India, adjusted for respondent age, education level, income, religious background, hospitalization in previous 12 months, and previous abortion. Dots indicate adjusted mean predicted outcome; error bars indicate 95% CIs. Tick marks along baselines indicate quartiles of income. ESBL, extended-spectrum β-lactamase.

might reveal a more robust U-shaped relationship between income and AMR in India. Finally, we identified 2 isolates of *Sphingomonas* spp., organisms commonly found in a variety of nonhost environments and occasionally identified as nosocomial pathogens (*2*) or as pathogens in pregnant women (*33*). One of the isolates was highly resistant to antimicrobial drugs, including all carbapenems in the study. The isolates did not have the same resistance pattern, however, so it is unlikely that these 2 cases were linked.

Our study has several implications for policy. To date, recommendations for reducing CA-AMR often focus on reducing selective pressure for AMR emergence, including limiting outpatient antimicrobial prescriptions, in accordance with well-established research demonstrating the link between antimicrobial drug use and AMR (*34*,*35*). Our findings suggest that this recommendation may be insufficient when applied to the poorest of the poor in urban settings in developing countries. If the factors correlated with poverty, including environmental antimicrobial drug exposures, increase risk for AMR in these women, then a policy response should focus on identifying and mitigating such exposures. Future research should seek confirmation of our results in other community-dwelling populations, mapping potential hotspots of CA-AMR among the urban poor and identifying causative factors.

M.A. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Design and conduct of the study: M.A., A.K., N.K., J.L., D.R., and D.K.O. Collection, management, analysis, and interpretation of the data: M.A., A.K., N.K., D.K.O., D.A.R., and J.L. Preparation, review, and approval of the manuscript: all authors.

Financial support for this research was provided by the Global Development and Poverty Initiative of the Stanford Freeman Spogli Institute for International Studies and the Graduate School of Business. The funding source had no role in the design, analyses, interpretation of the data, or decision to publish this study. D.A.R. is supported by the Thomas C. and Joan M. Merigan Endowment at Stanford University and the Chan-Zuckerberg Biohub.

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Toxoplasmosis in Transplant Recipients, Europe, 2010–2014

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Transplantation activity is increasing, leading to a growing number of patients at risk for toxoplasmosis. We reviewed toxoplasmosis prevention practices, prevalence, and outcomes for hematopoietic stem cell transplant (HSCT) and solid organ transplant (SOT; heart, kidney, or liver) patients in Europe. We collected electronic data on the transplant population and prevention guidelines/regulations and clinical data on toxoplasmosis cases diagnosed during 2010–2014. Serologic pretransplant screening of allo-hematopoietic stem cell donors was performed in 80% of countries, screening of organ donors in 100%. SOT recipients were systematically screened in 6 countries. Targeted anti-*Toxoplasma* chemoprophylaxis was heterogeneous. A total of 87 toxoplasmosis cases were recorded (58 allo-HSCTs, 29 SOTs). The 6-month survival rate was lower among *Toxoplasma*-seropositive recipients and among allo-hematopoietic stem cell and liver recipients. Chemoprophylaxis improved outcomes for SOT recipients. Toxoplasmosis remains associated with high mortality rates among transplant recipients. Guidelines are urgently needed to standardize prophylactic regimens and optimize patient management.

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DOI: https://doi.org/10.3201/eid2408.180045

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Toxoplasmosis is a zoonosis that infects humans and other warm-blooded animals worldwide; prevalence and clinical severity vary by geographic area (*1*). After primary infection, the parasite persists lifelong within dormant tissue cysts. Transmission to humans mainly occurs by ingestion of food or water contaminated with oocysts from feces of infected felids or undercooked meat containing cysts (*2*). Although largely asymptomatic in adults, toxoplasmosis is a life-threatening opportunistic infection in immunocompromised patients of all ages. Similar to *Pneumocystis* pneumonia, toxoplasmosis has become more frequently diagnosed for patients receiving immunosuppressive therapy than for patients with HIV infection (*3*,*4*). The growing number of grafts makes transplant patients a population at increasing risk. In transplant recipients (solid organ transplant [SOT] or hematopoietic stem cell transplant [HSCT]), disease can result from reactivation of past latent infection or from primary infection acquired through contaminated food or through a transplanted organ containing latent cysts (*5*). In contrast to incidence among HIV-infected patients, the incidence of toxoplasmosis among transplant recipients is poorly documented; published studies reporting patient series are scarce (*6*–*8*), and the literature consists mostly of case reports (*2*,*9*–*12*).

The risk for reactivation of chronic infection varies according to the immunosuppressive protocol and therefore according to the type of graft (*13*); risk is highest for seropositive allo-HSCT recipients receiving a seronegative graft. Among SOT recipients, the risk of a seronegative recipient (R–) acquiring infection from a seropositive donor (D+) organ (D+/R–) depends on the organ type; risk is highest for heart transplant recipients. Prevention measures rely on pretransplant serologic screening of donor, recipient, or both and on chemoprophylaxis; however, guidelines and regulations differ largely among countries. Regarding chemoprophylaxis, a multicenter study in France revealed variable practices in terms of regimen and duration of treatment (*4*). Some experts have proposed a tight clinical and molecular follow-up protocol for HSCT patients, aiming at early diagnosis of *Toxoplasma* reactivation to improve survival rates (*14*–*16*), but the cost:benefit ratio of this strategy is still under debate. We reviewed prevention practices implemented in European countries and evaluated the burden of toxoplasmosis among HSCT and SOT recipients.

Methods

Participating Centers

We recruited applicants through 2 study groups of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID; the European Study Group on Clinical Parasitology and the European Study Group on Immunocompromised Hosts) and through the Spanish Transplantation Infection Study Group, the Italian Society of Clinical Microbiology Infections and Transplant Working Group, and the Swiss Transplant Cohort Study. For each country, a local coordinator was identified and was in charge of contacting investigators from transplantation or infectious diseases units from representative centers.

Data Collection

Participants were invited to answer a detailed questionnaire adapted to the type of graft and designed to collect the following information: *Toxoplasma* seroprevalence in the country (documented by articles or recent surveys); implementation of a case reporting system for toxoplasmosis in transplant recipients; annual number of transplant procedures for each organ type in the participating center and in the whole country; pretransplant serologic screening policy for recipients and donors; implementation of recipient monitoring after transplantation and methods used (PCR, serology); and chemoprophylaxis regimen and duration according to organ type (if cotrimoxazole was given primarily for preventing *Pneumocystis* pneumonia, this use was recorded) and according to the recipient serologic results (primary or secondary prophylaxis, whether chemoprophylaxis was given to seronegative recipients, seropositive recipients, or both). When official national guidelines were lacking, to obtain representative data, we collected information about local practices in several transplant centers whenever possible.

As a second step, we sent an electronic case reporting form to all voluntary participating centers, which retrospectively recorded the number of cases of toxoplasmosis diagnosed per center over a 5-year period (2010–2014). The form collected the following information: patient age and sex; date of transplantation and type of graft; *Toxoplasma* serologic status of recipient and donor; date of toxoplasmosis diagnosis; site of infection (cerebral, ocular, disseminated); tools contributing to diagnosis (serology, molecular diagnosis, pathology, direct examination, imaging); chemoprophylaxis type, date of initiation, and duration; patient survival at 2 and 6 months; and date of death, if applicable. From each center and for each organ type, we also collected the mean patient age and the mean percentage of the whole transplant patient population surviving at 2 months and at 6 months. The number of cases and clinical data were retrieved from hospital medical or laboratory databases or from local or national databases, if existing. Participants were invited to send only aggregated data generated automatically by the database. The study was approved by the Ethics Committee of the University Hospital of Rennes, France (approval no. 15.12).

Statistical Analyses

Descriptive statistics are expressed as frequency (percentage) or mean ± SE. Comparison of qualitative data between

groups was based on exact χ^2 tests for equal proportions or Fisher exact tests if single table values were <5; quantitative data were compared by using analysis of variance or *t*-test (nonparametric test). We computed data by using SAS software version 9.4 (SAS Institute, Cary, NC, USA).

Results

Participating Centers, Transplantation Activity, and Case Notification

Overall, 46 centers from 11 countries (1–10 centers/country) participated in the survey; countries represented were France, Germany, Greece, Italy, Romania, Serbia, Slovakia, Spain, Switzerland, Turkey, and the United Kingdom (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/18-0045-Techapp1.pdf). Responses indicated that 5 countries (Switzerland, Slovakia, Turkey, Greece and the United Kingdom) report toxoplasmosis cases in a national database.

During 2010–2014, the mean annual number (range) of allo-HSCT procedures reported per country was 1,016 (13–1,900) and of auto-HSCT was 1,524 (14–3,078) (online Technical Appendix). Regarding SOT recipients, the mean annual number (range) of transplantations was 155 (10–420) for heart, 1,286 (55–3,074) for kidney, and 622 (35–1,241) for liver. The cumulative annual transplant activity among the responding centers reached a total of 1,089 allo-HSCT and 1,168 auto-HSCT (26 centers) and, for SOT, 394 heart (26 centers), 2,566 kidney (35 centers), and 1,455 liver (26 centers) transplants.

Pretransplant Serologic Screening for Toxoplasmosis

Although serologic screening of HSCT donors is not mandatory, all responding countries reported that they were performing this screening. Screening of allo-HSCT recipients was performed in all countries except Slovakia (mandatory in 4 countries), whereas screening of auto-HSCT recipients was performed regularly (4 countries), inconstantly (5 countries), or not at all (1 country). Overall, of 26 responding centers, 24 centers screened allo-HSCT and 17 screened auto-HSCT recipients for *Toxoplasma* antibodies before transplantation.

Serologic screening of solid organ donors (heart, kidney, or liver) was performed in all countries, although screening was reportedly mandatory in only 7 countries (France, Greece, Italy, Romania, Slovakia, Switzerland, and Turkey). At most centers, SOT recipients were screened (24/26 liver, 31/35 kidney, and 25/26 heart).

Anti-*Toxoplasma* Chemoprophylaxis Practices and Follow-up

Virtually all allo-HSCT recipients received cotrimoxazole chemoprophylaxis, whether primarily targeting *Pneumocystis* or *Toxoplasma*. At the 24 responding centers, cotrimoxazole was usually prescribed for ≥6 months despite the lack of official guidelines at 11 (46%) centers. The preferred regimen at 60% of centers was 960 mg 3 times a week but ranged from 480 mg 2 times a week to 1,920 mg 3 times a week. Auto-HSCT patients at 73% of centers received cotrimoxazole, administered mostly for 3 or 6 months. Serologic follow-up was reported by 2 allo-HSCT centers and PCR-based follow-up by 4.

For heart transplant recipients, 24 (92%) of 26 centers stated that they gave cotrimoxazole prophylaxis (3 months to lifelong), and 10 (43%) of 23 centers implemented serologic follow-up for *Toxoplasma* 2 and 4 times per year, particularly in cases of serologic mismatch (D+/R–). The most frequently prescribed regimen was 960 mg of cotrimoxazole 3 times a week or 480 mg daily. Although anti-*Pneumocystis* prophylaxis was implemented at 29 (83%) of 35 kidney and 17 (65%) of 26 liver transplant centers for 3–12 months, specific recommendations regarding toxoplasmosis chemoprophylaxis in this population were reported by only 4 countries (France, Greece, Spain, Turkey). The most frequently used regimen was cotrimoxazole at 480 mg daily (50% of kidney and 40% of liver transplant centers). Serologic monitoring of D+/R– patients was reportedly performed at 6 kidney and 5 liver transplant centers.

Incidence and Clinical Presentation of Toxoplasmosis

Overall, during the 5-year study period, 87 cases of *Toxoplasma* infection in transplant patients (58 HSCT, 29 SOT) were reported from 15 centers in 8 countries (online Technical Appendix). Severe manifestations (cerebral toxoplasmosis, disseminated toxoplasmosis, pulmonary toxoplasmosis) were more frequently observed (42 [48%] patients) than were mild manifestations (ocular toxoplasmosis, fever; 14 [16%] patients). A total of 31 (36%) patients had no apparent clinical signs. Asymptomatic episodes occurred mainly among HSCT recipients (81%) and were diagnosed mostly on the basis of a positive PCR (84%). Symptomatic HSCT recipients most often had disseminated (10/33, 30%) or cerebral (11/33, 33%) toxoplasmosis; these cases accounted for 60% of all cases of disseminated and 85% of cerebral toxoplasmosis (Table 1).

For the 87 reported cases, PCR was the most helpful diagnostic tool (77 [89%] cases), followed by imaging (32 [37%] cases) and serology (28 [32%] cases) (Table 1). PCR was reportedly positive for 100% of patients with cerebral and 90% with pulmonary toxoplasmosis (Table 1).

Pretransplantation *Toxoplasma* serologic test results for donor and recipient were available for 70 of the 87 patients (46 HSCT and 24 SOT). Toxoplasmosis occurred in the main groups at risk: in 35 (76%) of 46 D–/R+ HSCT recipients and 11 (46%) of 24 D+/R– SOT recipients (Table 2). Overall, 35 patients (18 HSCT and 17 SOT recipients) received chemoprophylaxis (Table 3). Only 4 (36%) of 11

Table 1. Characteristics of 87 transplant patients with toxoplasmosis, according to clinical presentation, Europe, 2010–2014*

Variables	Clinical type						p value
	Cerebral	Ocular	Disseminated	Pulmonary	Fever alone	No signs	
No. (%) patients	13 (15)	4 (5)	19 (22)	10 (11)	10 (11)	31 (36)	
Patient age, y, mean ± SE	37.0 ± 7.7	60.7 ± 0.8	47.8 ± 5.6	53.1 ± 4.8	35.5 ±4.4	46.4 ± 4.2	<0.0001
Time graft/diagnosis, wk, mean ± SE	123 ± 151	313 ± 175	163 ± 124	19 ± 11	73 ± 43	99 ± 51	<0.05
Diagnosis by, no. (%)							
PCR	13 (100)	3 (75)	17 (89)	9 (90)	9 (90)	26 (84)	<0.001
Serology	3 (23)	3 (75)	9 (47)	2 (20)	5 (50)	5 (16)	0.2278
Imaging	12 (92)	3 (75)	8 (42)	7 (70)	0	2 (6)	<0.01
Microscopy	1 (8)	0	6 (32)	1 (10)	0	0	<0.01
Graft type, no. (%)							<0.05
Liver, n = 8	1 (8)	1 (25)	3 (16)	2 (20)	0	1 (3)	
Kidney, n = 9	1 (8)	1 (25)	1 (5)	2 (20)	3 (30)	1 (3)†	
Heart, n = 12	0	1 (25)	5 (26)	0	2 (20)	4 (13)‡	
Allo-HSC, n = 58	11 (85)	1 (25)	10 (53)	6 (60)	5 (50)	25 (81)§	
No. with mismatch, n = 11	0	1 (25)	5 (26)	1 (10)	4 (40)	0	<0.05
Survival, no. (%)							
2 mo	5 (38)	4 (100)	13 (68)	5 (50)	10 (100)	24 (77)	<0.0001
6 mo	2 (15)	4 (100)	10 (53)	5 (50)	7 (70)	18 (58)	<0.001

*HSC, hematopoietic stem cell.
†This patient was receiving chemoprophylaxis.
‡1 patient was receiving chemoprophylaxis.
§11 patients were receiving chemoprophylaxis.

D+/R– SOT recipients received chemoprophylaxis, but for all of them toxoplasmosis occurred after discontinuation of prophylaxis (data not shown). Overall, toxoplasmosis was diagnosed after the end of prophylaxis for 17 recipients (9 HSCT and 8 SOT). For 9 HSCT and 5 SOT recipients, toxoplasmosis occurred during chemoprophylaxis (Table 3). Of these, 13 (93%) were asymptomatic: 1 kidney, 1 heart, and 11 HSC transplant recipients (Table 1). The proportion of mismatched cases (D+/R–) did not differ according to organ type (Table 4).

The mean time between transplantation and toxoplasmosis diagnosis was shorter among patients with pulmonary toxoplasmosis ($p<0.05$) (Table 1) than among patients with other types of disease manifestation. For seropositive recipients, the mean time to toxoplasmosis onset was short (<4 months after transplantation) compared with that for seronegative recipients (>4 years) (Table 2). Furthermore, the time to disease onset after transplantation was shorter among HSCT patients than SOT recipients ($p<0.0001$) (Table 4). The incidence of toxoplasmosis differed among the responding countries but seemed to not be linked to the seroprevalence in the country (online Technical Appendix).

Risk Factors for Death

Survival rates differed significantly between HSCT and SOT recipients ($p<0.001$) (Table 5). The 2-month survival rate was significantly poorer for patients with cerebral (38%) or pulmonary (50%) toxoplasmosis ($p<0.001$) (Table 1). Survival rates were also poorer for seropositive patients ($p<0.05$ at 2 months and $p<0.001$ at 6 months) (Table 5), mainly consisting of HSCT patients (Table 2). Of note, the percentage of asymptomatic patients who survived 6 months (58%) was similar to that of patients with pulmonary (50%) or disseminated (53%) toxoplasmosis (Table 1). A lower percentage of HSCT and liver transplant recipients survived at 2 and 6 months after diagnosis; deep site–associated toxoplasmosis was diagnosed for only half of them (Table 4). The survival rates for HSCT (38%) and liver transplant (50%) recipients with toxoplasmosis were significantly lower than those for the general HSC (84%) and liver transplant (75%) populations ($p<0.05$) (Table 4).

Transplant recipients in whom toxoplasmosis developed were less likely to survive if they were not receiving chemoprophylaxis before or at onset of disease ($p<0.05$ at 2 months and $p<0.01$ at 6 months after disease onset) (Table

Table 2. Characteristics of transplant donors and recipients at transplantation, according to *Toxoplasma* serologic status, Europe, 2010–2014*

Serologic status of donor/recipient†	Prophylaxis, no. (%)	Graft type, no.				Survived 6 mo, no. (%)	Wks between diagnosis and graft, mean ± SE
		Liver	Kidney	Heart	HSC		
Positive/positive, n = 9‡	5 (56)	2	0	1	6	3 (33)	21 ± 9
Positive/negative, n = 11§	4 (36)	3	4	4	0	9 (82)	309 ± 275
Negative/positive, n = 36¶	12 (33)	0	0	1	35	12 (33)	15 ± 3
Negative/negative, n = 14	9 (64)	2	2	5	5	11 (79)	123 ± 31
p value	0.1975	NA	NA	NA	NA	<0.01 (0.0029)	<0.05

*HSC, hematopoietic stem cell; NA, not applicable.
†Missing data for 17 patients.
‡2 liver transplant recipients died.
§Group in which solid organ transplant patients are most at risk for toxoplasmosis.
¶Group in which HSC transplant patients are most at risk for toxoplasmosis.

Table 3. Toxoplasmosis occurrence and outcomes for HSCT and SOT patients, according to prophylaxis, Europe, 2010–2014*

Characteristic	HSCT, no. (%), n = 58†	SOT, no. (%), n = 29‡	p value
Seropositive before transplantation	41/46 (89)§	4/24 (17)¶	<0.0001
Diagnosis during chemoprophylaxis	9/50 (18)	5/28 (18)	NS
Diagnosis after chemoprophylaxis	9/50 (18)	8/28 (29)	NS
2-mo survival rate			
With prophylaxis	13/18 (72)	17/17 (100)	<0.05
Without prophylaxis	18/32 (56)	9/11 (82)	0.1657
6-mo survival rate			
With prophylaxis	9/18 (50)	17/17 (100)	0.01
Without prophylaxis	9/32 (28)	7/11 (64)#	0.0679

*HSCT, hematopoietic stem cell transplant; NS, not significant; SOT, solid organ transplant.
†Prophylaxiis data missing for 8 patients.
‡Prophylaxis data missing for 1 patient; incomplete information regarding dates of onset and/or stop of cotrimoxazole for 4 patients.
§Serology data missing data for 4 patients.
¶Serology data missing data for 4 patients.
#p<0.05 between SOT with or without chemoprophylaxis.

5); this finding was particularly common among SOT recipients (p<0.05) (Table 3). However, despite chemoprophylaxis, the outcome remained poorer for HSCT patients than for SOT patients (Tables 3, 5).

Discussion

We provide an overview of practices used to prevent toxoplasmosis in transplant patients in Europe. Despite the well-recognized risk linked to either endogenous reactivation or to transplantation of a cyst-containing organ, prevention policies seem heterogeneous among countries. Serologic screening of solid organ or hematopoietic stem cell donors for *Toxoplasma*, although not mandatory in all countries, seems to be general practice, probably as a result of recommendations of national societies of transplantation, and is mandatory when organs are exchanged between countries. Similarly, pretransplant serologic screening of recipients, although also not mandatory in all countries, was reportedly performed by nearly all responding centers. However, for 17 cases, the serologic status of the recipient or donor was not available in medical charts. Management practices regarding chemoprophylaxis based on donor and recipient serologic results vary substantially, particularly for kidney and liver transplant patients. Indeed, only 35 (50%) of 70 recipients had received chemoprophylaxis, although it was indicated either because of *Toxoplasma* mismatch (SOT) or seropositivity (HSCT). Only 4 (36%) of 11 SOT patients with D+/R– serologic results had received chemoprophylaxis. These 4 patients were all alive 6 months after transplantation. However, our study did not address long-term survival, which at 5 years after transplantation was reportedly poorer for D+/R– than for D–/R– heart transplant recipients (*17*). In that study, Chehrazi-Raffle et al. (*17*) did not record the duration of

Table 4. Characteristics of transplant patients with toxoplasmosis, according to graft type and comparison to overall graft population, Europe, 2010–2014*

	Allo-HSC		Kidney		Liver		Heart		
Characteristics	Case-patients	All TP	Case-patients	All TP	Case-patients	All TP	Case-patients	All TP	p value
Patients, no.	58	4,108	9	6,507	8	2,983	12	998	NA
Age, y, mean ± SE	46.8 ± 5.3	50.7	44.6 ± 5.9	50.9	55.1 ± 1.5	51.6	44.4 ± 6.4	47.5	NA
Female sex, %	43	38	44	63	38	30	17	22	NA
Male sex, %	57	62	56	37	62	70	83	78	NA
Mean time diagnosis/graft, wk, mean ± SE	20.6 ± 4.6	NA	198 ± 68	ND	152 ± 144	ND	441 ± 155	ND	<0.0001
Mean time diagnosis/death, d, mean ± SE	47 ± 18	NA	33	ND	38 ± 17	ND	NA	ND	0.8595
Mismatched serologic results (D+/R–), no. (%)	0	NA	4 (33)	NA	3 (38)	NA	4 (33)	NA	0.8923
2-mo survival, no. (%)	36 (62)	ND	8 (89)	ND	5 (63)	ND	12 (100)	ND	<0.05
Deep site involvement	12 (43)	ND	3 (60)	ND	4 (57)	ND	6 (100)	ND	0.0513
Fever only	5 (100)	ND	3 (100)	ND	0	ND	2 (100)	ND	1
No clinical signs	18 (72)		1 (100)		1 (100)		4 (100)		0.1407
6-mo survival, %†	38	84‡	89	72	50	75§	100	60	<0.0001
Deep site involvement	25	NA	60	NA	28	NA	100	NA	<0.01
Fever only	40	NA	100	NA	0	NA	100	NA	0.2083

*D+, donor positive; NA, not applicable; ND, not determined; NS, not significant; R–, recipient negative; TP, transplant patients.
†The survival rate for the general TP population was calculated at a time similar to the mean time of diagnosis of toxoplasmosis after graft in case-patients.
‡p<0.01 compared with case-patients.
§p<0.05 compared with case-patients.

Table 5. Survival among transplant patients with toxoplasmosis, according to patients' characteristics, Europe, 2010–2014

Characteristic	2-mo survival		6-mo survival	
	No. patients/no. survived (%)	p value*	No. patients/no. survived (%)	p value*
All patients	61/87 (70)	Not applicable	46/87 (53)	Not applicable
Chemoprophylaxis				
Yes	30/35 (86)	<0.05	26/35 (74)	<0.01
No	27/43 (63)		16/43 (37)	
Recipient serologic status				
Positive	27/45 (60)	<0.05	15/45 (33)	<0.001
Negative	22/25 (88)		20/25 (80)	
Type of graft				
Hematopoietic stem cell	36/58 (62)	<0.05	22/58 (38)	<0.001
Solid organ	25/29 (86)		24/29 (83)	

*Exact χ^2 test.

chemoprophylaxis, a parameter that could be of greater interest. Similarly, only 18 HSCT patients received chemoprophylaxis, although 45 were known to be seropositive (Table 3). Our study also did not address long-term disabilities resulting from toxoplasmosis.

Even with this limited number of cases reported by the participating centers, our study provides some helpful insights and useful data. From a diagnosis point of view, our findings confirm that PCR has become an essential microbiological tool for investigating active infection, as already emphasized in previous studies (*18*,*19*). Indeed, we can confirm that 9 (10.3%) of the 87 cases of toxoplasmosis were diagnosed by PCR in patients with fever only; thus, earlier treatment could be commenced before more serious complications developed; these patients were mostly HSCT recipients. PCRs on blood from 26 patients with no obvious clinical signs were also positive. This finding is consistent with previously reported findings for allo-HSCT patients in centers where routine monitoring by PCR of blood is conducted for several months after transplantation (*14*–*16*,*20*,*21*). Martino et al. (*22*) concluded that clinical toxoplasmosis evolved in about one third of these patients and that early treatment increased survival rates. In our study, survival rates were poor among patients who were asymptomatic at the time of diagnosis (58%) (Table 1), probably because as allo-HSCT patients they were at high risk for death from other causes. Our study did not record what treatment decisions were taken as a direct result of PCR results, and so a more detailed future study of treatment regimens and how quickly they were initiated may provide further valuable insights into factors affecting mortality rates in this clinical group.

Not surprisingly, among the 87 patients, the proportion with disseminated and pulmonary toxoplasmosis was high; this clinical picture is known to be frequent among transplant patients (*4*,*23*,*24*). The high frequency (100%) of positive PCR results among patients with cerebral toxoplasmosis differs from previous estimates of sensitivity in this clinical setting (*2*), suggesting high circulating parasite loads, late diagnoses, or both, which could account for the unusually high mortality rate (85%) among patients with cerebral toxoplasmosis in this study. Another explanation is that diagnostic sensitivity of molecular diagnosis has been mainly evaluated in HIV-infected patients, a patient population that differs from transplant recipients and experiences more severe disease with rapid dissemination of the parasites. On the other hand, ocular toxoplasmosis, a mild form of the disease, occurred mostly after the first year after transplantation, when immune suppressive therapy is usually reduced, thus explaining the 100% survival rate, probably resulting from confinement of parasites in the ocular compartment (*25*).

In HSCT patients, *Toxoplasma* reactivation predominantly occurred within several months (20.6 ± 4.6 weeks) after engraftment, which might suggest that chemoprophylaxis was stopped too early. Indeed, toxoplasmosis was diagnosed for 9 HSCT patients after chemoprophylaxis was stopped (Table 3); this finding is consistent with the policy at 9 centers of discontinuing chemoprophylaxis at 6 months. These data support the practice of monitoring CD4+ T-cell counts to guide chemoprophylaxis discontinuation, as suggested by others (*13*). However, toxoplasmosis was also diagnosed during chemoprophylaxis for 9 additional HSCT and 5 SOT patients, which might be related to inadequate regimens of cotrimoxazole or poor observance. A recent systematic review (*13*) reported that breakthrough toxoplasmosis in HSCT patients was observed when cotrimoxazole was given only 2 times per week at a dosage of 960 mg (57% of cases) or 480 mg daily (18%).

A major finding of this study is the observation that life-threatening toxoplasmosis can occur in HSCT and SOT patients after chemoprophylaxis is stopped. However, in SOT patients, the rather late occurrence after transplant (>3 years) and the high survival rates suggest that infection acquired long after transplantation is usually mild and the source is probably contaminated food. Conversely, life-threatening early infection was associated with a high mortality rate and was mostly observed in liver transplant patients, suggesting that serologic results might not have been taken into account to guide chemoprophylaxis.

Overall, prognosis of *Toxoplasma* infection was good for SOT patients; the all-cause mortality rate of 17% was similar to that reported from Spain (13.6%), where 17 of 22 patients had a primary-acquired infection (*6*). Higher prevalence and severity of disease was confirmed among HSCT patients; survival rate was only 38% at 6 months, similar to mean survival rates recently reported (*13*). We assume that death was attributable to toxoplasmosis in deceased HSCT and liver transplant patients because their 6-month survival rate was significantly poorer than that of their counterparts without toxoplasmosis ($p<0.01$). A similar effect of toxoplasmosis on survival of HSCT patients has been recently demonstrated in a case–control study (*26*). However, whether chemoprophylaxis positively influences outcome remains unclear. Indeed, overall survival rates were better among patients who received cotrimoxazole than among those who received no treatment; but when considering HSCT and SOT patients separately, survival rates remained significantly better for SOT patients only. This finding raises the question of the effectiveness of prophylaxis, in terms of regimen and duration.

This study has several limitations. First, we used aggregated data, so individual analyses or modifications of the analysis plan were not possible after data collection. Therefore, individual data such as immunosuppressive regimen, graft versus host disease, or simultaneous infections were not recorded, and multivariate analyses to further explore mortality rates were not possible. The number of participating centers per country varied, and for some countries (particularly Germany and Turkey), these centers accounted for a small proportion of the transplantation activity in the whole country (online Technical Appendix); thus, we cannot be sure that the data collected were representative for the whole country. The absence of correlation between seroprevalence and the number of cases reported among countries may be attributed to several confounding factors, such as 1) good management of prevention in countries where seroprevalence is high, 2) lack of awareness and possible underdiagnosis of *Toxoplasma*-associated risk in countries where seroprevalence or transplantation activity is low, 3) underreporting because of lack of follow-up, 4) overdiagnosis because of systematic screening (asymptomatic cases), or 5) migration of patients from eastern Europe (higher seroprevalence) to western Europe for transplantation (*27*).

Overall, this study confirms that toxoplasmosis in transplant recipients is a clinical problem throughout Europe, regardless of local seroprevalence. This finding suggests that substantial health gains may be achieved by the development and adoption of common prevention guidelines based on best practice. Whether chemoprophylaxis duration should be extended and for what duration remains to be determined. Nevertheless, our results suggest that to prevent late onset of toxoplasmosis, cotrimoxazole should be given for ≥6 months. In case of drug intolerance, low dosage, or discontinuation, follow-up by regular PCR of blood could help guide preemptive treatment. In SOT patients with *Toxoplasma* mismatch (D+/R–), cotrimoxazole prophylaxis should be given for ≥1 year. Last, recommendations associated with hygiene, similar to those provided to seronegative pregnant women to avoid contamination, should be extended to all seronegative transplant patients.

Acknowledgments

We thank Emmanuel Oger and Frédéric Balusson for their assistance with data processing. We also thank the Spanish Transplantation Infection Study Group from the Spanish Network for Research in Infectious Diseases; the Swiss Transplant Cohort Study; and the many practitioners from transplantation units, microbiology laboratories, and infectious diseases units who participated in data collection: Eric Levesque, Marie Matignon, Christine Robin, Aude Boignard, Marie-Noëlle Hilleret, Bénédicte Janbon, Anne Thiebaut-Bertrand, Pascale Boissonnat, Anne Conrad, Sophie Ducastelle-Leprêtre, Claire Pouteil-Noble, Méja Rabodonirina, Sylvie Radenne, Ana Roussoulières, Gilles Salles, Laurent Sebbag, Martine Wallon, Frédérique Foudrinier, Christophe Camus, Bernard Lelong, Tony Marchand, Cécile Vigneau, Nunzia Decembrino, Angela di Matteo, Alessandra Sensini, Luscalov Dan, Luscalov Simona, Ľubomír Beňa, Ivana Dedinská, Zuzana Žilinská, Eva Goncalvesová, František Ondriska, Arancha Bermudez, Manuel Cobo-Belaustegui, Carlota Gudiol, Núria Sabé, Erden Atilla, Ayse Çiftçioglu, Bilgi Baca, and Tonguc Utku Yilmaz.

This work was supported by a grant (P307-09) from ESCMID and involved the ESCMID Study Group on Clinical Parasitology and the ESCMID Study Group on Immunocompromised Hosts.

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Novel *Enterobacter* Lineage as Leading Cause of Nosocomial Outbreak Involving Carbapenemase–Producing Strains

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We investigated unusual carbapenemase-producing *Enterobacter cloacae* complex isolates (n = 8) in the novel sequence type (ST) 873, which caused nosocomial infections in 2 hospitals in France. Whole-genome sequence typing showed the 1-year persistence of the epidemic strain, which harbored a bla_{VIM-4} ST1-IncHI2 plasmid, in 1 health institution and 2 closely related strains harboring $bla_{CTX-M-15}$ in the other. These isolates formed a new subgroup in the *E. hormaechei* metacluster, according to their *hsp60* sequences and phylogenomic analysis. The average nucleotide identities, specific biochemical properties, and pangenomic and functional investigations of isolates suggested isolates of a novel species that had acquired genes associated with adhesion and mobility. The emergence of this novel *Enterobacter* phylogenetic lineage within hospitals should be closely monitored because of its ability to persist and spread.

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DOI: https://doi.org/10.3201/eid2408.180151

Controlling the dissemination of carbapenemase-producing *Enterobacteriaceae* (CPE) is challenging because carbapenems are among the few antimicrobial drugs that can be used to treat severe infections in this family (*1*,*2*). Tzouvelekis et al. calculated the mortality rate of primary bacteremia involving CPEs without active therapy to be 54% (*3*). Thus, CPEs may carry the threat of a return to the pre–antimicrobial drug era.

The *Enterobacter cloacae* complex (ECC) has become the third most common species among CPEs in France (*4*). ECCs are not dominated by any single genotype (*5*), and only certain subspecies/species have previously been associated with infections and nosocomial outbreaks (*6*–*8*). The accurate identification of species and subspecies within the ECC is therefore needed to monitor outbreaks and infections.

The identification of species and subspecies within the ECC is challenging, and even more problematic because routine bacterial identification methods based on biochemical tests or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry are yet unable to distinguish between them (*9*–*13*). In a seminal work, Hoffmann and Roggenkamp defined 13 genetic clusters (I –XIII) of the ECC on the basis of *hsp60* gene sequences and assigned them to species and subspecies (*14*). Recently, Chavda et al. extended the number of clusters in the ECC to 18 phylogenomic groups (A–R) by analyzing core single-nucleotide polymorphisms (SNPs) in 390 whole genomes (*15*).

Using whole-genome sequencing (WGS) approaches, we investigated a cluster of nosocomial carbapenemase-producing ECC isolates collected over a 13-month period in a university hospital in France. The results suggest a double-string diffusion mechanism involving the emergence of both a carbapenemase-encoding plasmid and an ECC phylogenetic lineage not previously described.

[1]These authors contributed equally to this article.

Materials and Methods

Clinical and Epidemiologic Survey

The patients were admitted to the Edouard Herriot Hospital in Lyon, France, where a prospective surveillance of CPEs has been implemented since 2012. Case-patients were defined as persons hospitalized after CPE was diagnosed in ≥1 clinical sample during January 12, 2014–December 31, 2015 (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/8/18-0151-Techapp1.pdf). We investigated contact patients and performed an environmental study (online Technical Appendix).

Bacteria Isolation and Phenotypic Characterization

We isolated CPE on a chromogenic medium, chromID CARBA (bioMérieux, Marcy l'Etoile, France). We preincubated environmental CPE specimens for 24 h at 36°C in Trypticase soy broth (TSB; bioMérieux) supplemented with 0.5 mg/L ertapenem. The clinical (n = 7) and environmental (n = 2) isolates were identified by mass spectrometry (VitekMS; bioMérieux). We used the API 50CH system (bioMérieux) for biochemical testing and assessed antimicrobial susceptibility according to the EUCAST guidelines (http://www.eucast.org/). We detected carbapenemase by using the RAPIDEC CARBA-NP test (bioMérieux) (*16*). As previously described, we determined the sequence of $bla_{\text{VIM-4}}$ by using the Sanger method (*17*). We performed conjugation experiments at 25°C as previously described (*18*) and plasmid size determination by pulsed-field gel electrophoresis (*19*). Biofilm formation and cell adhesion assays are described in the Technical Appendix.

WGS and Genome Assembly

We determined the whole-genome sequences of strains C45 and C309 by using a hybrid de novo assembly of 2 × 150-bp paired-end reads generated by using sequencing technology by Illumina (San Diego, CA, USA) and long reads generated by using Pacific Biosciences technology (Menlo Park, CA, USA). We determined WGS of the other strains by using a de novo assembly of 2 × 150-bp paired-end reads. We performed the assemblies by using SPAdes (*20*), mapped the reads by using the Burrows-Wheeler aligner (BWA) (*21*), and polished the assembly by using Pilon (*22*). The raw data were deposited in EMBL as project PRJEB22398 and the assemblies as LT991954–60. We report further analysis processes in the online Technical Appendix.

Results

Emergence of Carbapenemase-Producing ECC strains

During January 12, 2014–December 31, 2015, a total of 320 positive cultures for ECC with antibiogram were identified in the Edouard Herriot Hospital in Lyon. Each of 7 (2.2%) ECCs recovered from 7 patients (designated P1–7) produced a carbapenemase (Table 1). These isolates were resistant to penicillins and combinations of penicillins, reacting by releasing β-lactamase inhibitors, oxyimino cephalosporins, and ertapenem (Table 2). PCR and sequencing showed the presence of the carbapenemase-encoding gene $bla_{\text{VIM-4}}$ in the 7 isolates. During January 11, 2013–November 30, 2014, no positive cultures for ECC with antibiogram showed $bla_{\text{VIM-4}}$. These results suggest an epidemic spread of VIM-4–producing ECC strains in the hospital.

Temporal and Spatial Links of the Carbapenemase-Encoding ECC Cases

The attack rate of CPE was 0.7/10,000 hospital stays during January 12, 2014–December 31, 2015, compared with 0.0/10,000 hospital stays during January 11, 2013–November 30, 2014 (p = 0.008; Figure 1). The all-causes crude mortality rate among patients with ECC isolates was 43% (n = 3). We provide additional clinical data in the online Technical Appendix. None of the patients had a recent history of travel or hospitalization in foreign countries. Only 2 patients (P6 and P7) were hospitalized in the same unit at the same time. Five patients (P1, P2, P3, P4, and P7) had undergone surgery in the same operating room but at different dates and with different operating teams. We identified CPE in urine samples of 5 patients (P1–P5) that were drawn from the patients' urinary catheters. CPE was isolated from a skin sample that we excised from a necrotic ulcer from P6 and in a sample of operative peritoneal fluid from P7. Patient P1 was simply colonized, whereas patients P2–P7 were infected. All patients had ≥1 negative samples from the same clinical site during hospitalization before colonization/infection by ECC (Table 1). These findings showed no clear-cut epidemiologic or temporal links between the VIM-4 ECC case-patients, except that surgical procedures were performed in the same operating room on 5 of the 7 patients.

Environmental Investigations

Putative sources previously described in other settings, such as handwashing sinks (*23*) and endoscopes (*24*), were not assumed to be a source of $bla_{\text{VIM-4}}$ ECC because surveillance samples were negative during the study period. In addition to the 102 contact patients, we screened 65 persons during the first 5 episodes (illnesses in P1–P5) without identifying any secondary cases. For the last episode, which involved P6 and P7, we screened 125 of 160 contact patients; all were negative. These findings support a key role for 1 or multiple environmental reservoirs in the nosocomial transmission of $bla_{\text{VIM-4}}$ ECC to patients. However, transmission by healthcare workers (HCWs) cannot be completely ruled out. We did not screen HCWs

Table 1. Case descriptions of VIM-4–producing *Enterobacter cloacae* complex nosocomial infections in outbreak involving carbapenemase-producing strains, Lyon, France, January 12, 2014–December 31, 2015*

Characteristic	Patient no.						
	P1	P2	P3	P4	P5	P6	P7
Patient age, y/sex	67/F	72/M	64/F	69/M	87/M	84/F	82/M
Hospitalization duration	10 d	106 d	52 d	26 d	6 d	69 d	36 d
Purpose of hospitalization	Kidney transplant	Peripheral arterial disease	Septic shock	Kidney transplant	Consciousness disorder	Necrotic purpura	Vesical lithotrity
Type of ward	Transplant, medicine	Surgery, medical, ICU	Medical, ICU	Transplant, medical	Medical, ICU	Medical	Surgery, medical, ICU
Antimicrobial therapy before diagnosis†	VAN, OFL, AMX	PTZ, VAN, AMI, MEM,† CTR	CTR, VAN, TZP, MET, AMI, CLI, IMP†	AMX, VAN	AMC	CTR, GEN	CTX, OFL, AMX, PTZ, GEN
Urinary catheter	Yes	Yes	Yes	Yes	Yes	No	Yes
Intubation	No	Yes	Yes	No	No	No	No
Central venous catheter	Yes	Yes	Yes	Yes	No	No	No
In-hospital death: delay from admission, d, and etiology	No	Yes: 106, septic shock from respiratory system	Yes: 52, septic shock, undetermined origin	No	No	No	Yes: 36, septic shock, peritonitis with hemorrhage
Last negative sample;‡ delay from admission, d	Urinary; 7	Urinary; 61	Urinary; 1	Urinary; 20	Rectal swab; 2	Necrotic skin; 39	Urinary; 2
First positive sample	Urinary	Urinary	Urinary	Urinary	Urinary	Necrotic Skin	Peritoneal fluid
Delay from admission, d	32	76	41	29	20	49	28
Delay from last negative sample, d	25	15	40	9	18	10	26
ST	ST873	ST873	ST118	ST873	ST118	ST873	ST110
Isolate identification	C45	C46	C47	C48	C308	C310	C309

*AMI, amikacin; AMX, amoxicillin; AMC, amoxicillin-clavulanate; CLI, clindamycin; CTR, ceftriaxone; CTX, cefotaxime; GEN, gentamicin; ICU, intensive care unit; ICU, intensive care unit; IMP, imipenem; MET, metronidazole; MEM, meropenem; OFL, ofloxacin; PTZ, piperacillin/tazobactam; ST, sequence type; VAN, vancomycin.
†Carbapenems.
‡For detection of VIM-4–producing *Enterobacter cloacae* complex.

for possible CPE carriage, but no particular HCW was involved in care of all CPE cases.

The data, including the temporal distribution of the cases over 13 months and the molecular characterization of the isolates, suggested intermittent transmission of human or environmental origin. We therefore implemented environmental screening in 3 rooms occupied by patients P3 and P7, including the beds, mattress covers, and shared equipment, in June and August 2016. These rooms were investigated because they had been occupied ≥1 time by patients with VIM-4–producing ECC (data not shown). Analysis of the environmental samples after the discharge of patients with VIM-4–producing ECC showed that those collected from a radiator and the mattress cover in 1 patient's room (P7) were contaminated by VIM-4–producing ECC isolates (E14 and E16). The antibedsore mattresses were used in different rooms for several patients. The incidence of CPE was reduced by discarding the mattress covers after the patients known to be VIM-4–producing ECC carriers were discharged from the hospital (data not

Table 2. Key features of clinical VIM-4–producing *Enterobacter cloacae* complex isolates in nosocomial outbreak involving carbapenamase-producing strains, Lyon, France, January 12, 2014–December 31, 2015*

Isolate	Species	ST	hsp60 cluster (phylogenome)	Size of assembled genomes, bp	MICs, mg/L						
					ETP	IPM	MEM	CAZ	CTX	FEP	ATM
C45	*E. cloacae* complex	873	NA (S)	5,290,194	2	2	1	32	>32	4	4
C46	*E. cloacae* complex	873	NA (S)	5,257,311	2	1	0.5	24	>32	2	4
C48	*E. cloacae* complex	873	NA (S)	5,260,873	2	2	0.5	24	>32	2	4
C310	*E. cloacae* complex	873	NA (S)	5,254,482	2	2	1	24	>32	2	2
E14	*E. cloacae* complex	873	NA (S)	5,251,662	2	2	1	32	>32	4	4
E16	*E. cloacae* complex	873	NA (S)	5,250,845	2	2	1	32	>32	4	4
C47	*E. cloacae* cluster III	118	III (D)	5,083,854	2	2	0.5	16	>32	2	0.25
C308	*E. cloacae* cluster III	118	III (D)	4,998,377	2	1	0.25	32	>32	2	0.5
C309	*E. hormaechei steigerwaltii*	110	VIII (B)	5,200,769	4	2	0.5	96	>32	8	32

*ATM, aztreonam; CAZ, ceftazidime; CTX, cefotaxime; ETP, ertapenem; FEP; IPM, imipenem; MEM, meropenem; NA, not applicable; ST, sequence type.

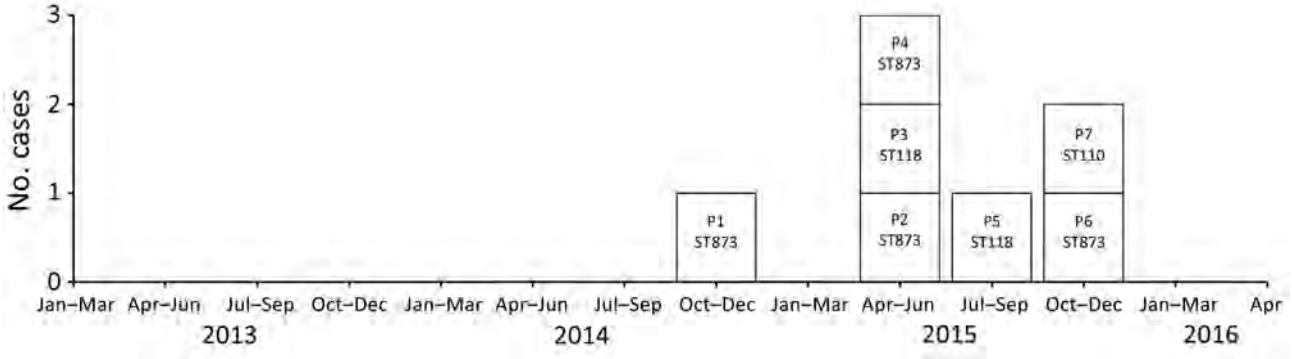

Figure 1. Epidemic curve of VIM-4–producing *Enterobacter cloacae* complex isolates (n = 7) in nosocomial outbreak involving carbapenamase-producing *Enterobacter* strains, Lyon, France, January 12, 2014–December 31, 2015. The attack rate was 0.7/10,000 hospital stays during the study period versus 0.0/10,000 hospital stays during January 11, 2013–November 30, 2014 (p = 0.008). The patients (P1–7) are labeled according to the ST of isolate with which they were infected or colonized. ST, sequence type.

shown). These data suggest that the transmission of ECCs by contact with mattress covers could be 1 of the key causative factors, especially for the last episode (P6–P7).

Multiclonal Spread of the VIM-4 Carbapenemase

We sequenced the genomes of isolates to assess the molecular links between the VIM-4 ECC cases (Table 2). Six isolates (C45, C46, C48, C310, E14, and E16) formed a clonal cluster designated clone A, in which core genome SNP analysis showed a diversion of <10 SNPs and wgMLST. (Figure 2).

Isolate C309 belonged to ST110, isolates C47 and C308 to ST118, and the 6 isolates of clone A to ST873 (*dnaA*:85/*fusA*:63/*gyrB*:101/*leuS*:103/*pyrG*:96/*rplB*:6/*rpoB*:53). By screening a collection of 30 ECCs isolated in France during the same period, we identified 2 ST873 isolates (CNR1568 and CNR1569) containing the extended-spectrum β-lactamase–encoding gene $bla_{\text{CTX-M-15}}$ in the teaching hospital of Caen. These related isolates differed by 28 core genome SNPs and 27 loci (Figure 2). The clustering of the ECC isolates was also apparent in the analysis of the antimicrobial resistance gene contents (Figure 3). Overall, these data show a multiclonal spread of $bla_{\text{VIM-4}}$ ECCs, the predominance of ECC genotype ST873 among $bla_{\text{VIM-4}}$ ECC, and the interregional spread of this ECC lineage.

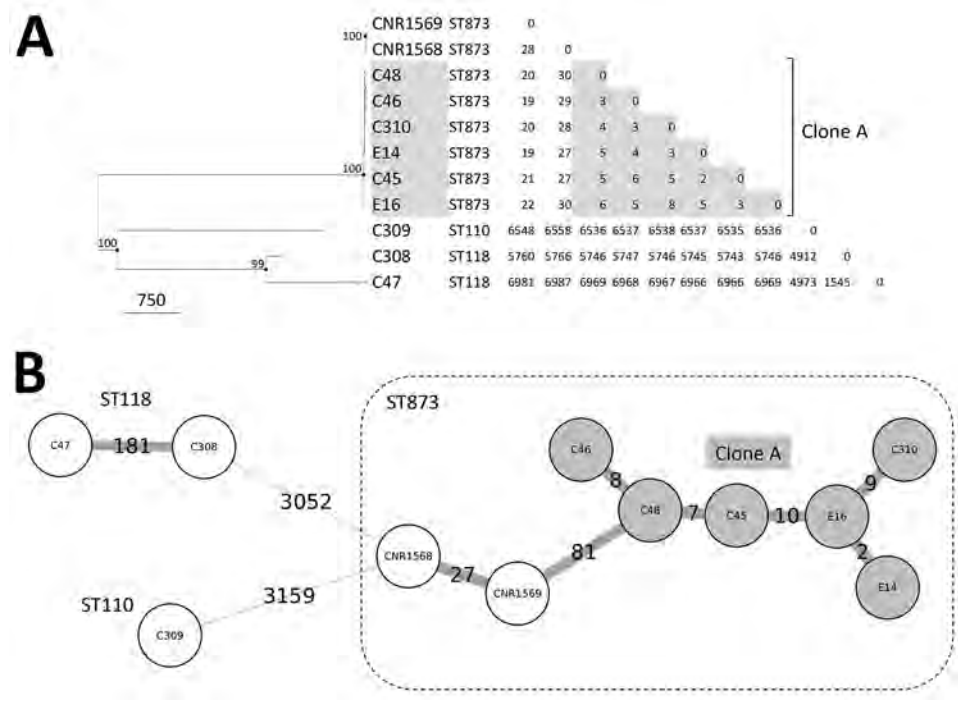

Figure 2. Whole-genome typing of *Enterobacter cloacae* complex isolates from nosocomial outbreak involving carbapenamase-producing *Enterobacter* strains, Lyon, France, January 12, 2014–December 31, 2015. A) Dendrogram inferred by the maximum-likelihood method on the basis of core genome SNPs. The node sizes are proportional to the bootstrap values; values >80 are indicated. Scale bar indicates SNPs. The relatedness of the strains was determined by using <15 variant sites as clonality criteria. B) Minimum-spanning tree based on a whole-genome multilocus sequence typing approach, combining the analysis of core genome loci and the presence or absence of accessory genes. Labels on branches indicate the absolute number of variant loci (clonality threshold ≤10 variant loci). SNP, single-nucleotide polymorphism; ST, sequence type.

VIM-4–Encoding Plasmids

To explore possible links between clone A and the other VIM-4–encoding isolates, we investigated the plasmid contents and the transferability of bla_{VIM-4} by conjugation. The transfer of ertapenem resistance into *Escherichia coli* C600 was successful; all of the 9 isolates were at room temperature. Hybridization of plasmids with a specific probe revealed the location of bla_{VIM-4} on conjugative plasmids of ≈300 kb (n = 6) in the clone A isolates, C47, and C308 and of ≈245 kb (n = 1) in isolate C309. In all isolates, analysis of the assembled genomes showed the presence of an ST1-IncHI2 replicon encoding bla_{VIM-4}.

The plasmid from isolate C45 designated pC45-VIM4 formed a circular 299,117-bp sequence (Figure 4, panel A). The antimicrobial drug resistance genes were located in a ≈50-kb region (bases 97,253–154,784); bla_{VIM-4} gene was included in a 16-kb Tn*21*-like transposon designated Tn*6540* (bases 97,253–113,368). Tn*6540* comprised a class 1 integron including bla_{VIM-4} as the first gene cassette, followed by *aac*(*6′*)-*Il*, *dfrA1b*, Δant(*3″*), and *smr2*. Seven heavy metal resistance loci were also encoded by pC45-VIM4: the tellurite resistance genes *terY3Y2XY1W* (bases 65,568–69,439) and *terZABCDEF* (bases 76,028–82,281); the cobalt-zinc-cadmium-resistance protein (bases 118,576 to 119,544); operon *copS*/*copE* (bases 165,340 to 167,337); the efflux system *rcnR*/*rcnA* (bases 167,595 to 169,105); the arsenic resistance genes *arsCBRH* (bases 181,666–184,550 bp); and 1 complete mercury resistance operon, *merRTCADE* (base 134,519–138,533).

We identified a similar organization in the other bla_{VIM-4} isolates (Figure 4, panel B). However, in C309, the VIM-4–encoding plasmid designated pC309-VIM4 (254,277-bp) differed by 2 deletion sites (≈24 kb and ≈21 kb) flanked by mobile elements (IS*26* and IS*Pa21*). These deletions resulted in the loss of resistance genes *aac*(*6′*)-*Ib7*, *tetA*, bla_{TEM-1}, *ant*(*3″*), and *ant*(*2″*)-Ia. These results suggest the horizontal transfer of the same bla_{VIM-4}–encoding plasmid in several lineages of ECC.

Three related IncHI2 plasmids encoding bla_{VIM-1} were identified in GenBank (Figure 4, panel C). Except in the region encoding resistance genes, pC45-VIM4 shared 94% of its sequence and most of gene synteny with pMRVIM0813; pRH-R27 and pRH-R178 reported from Germany are more distantly related (85% and 60% of overlap).

A New Cluster in the Phylogenomic Tree of the ECC

Because specific subgroups within the ECC are more prone to cause nosocomial infections or outbreaks, we characterized the isolates at the species and subspecies levels as described by Hoffmann and Roggenkamp (*14*) and Chavda et al. (*15*). In the *hsp60*-based neighbor-joining tree comprising 52 representative reference and type strains (online Technical Appendix Figure 1), the sequences of C47 and C308 co-localized with *hsp*60 cluster III and that of C309 localized with *hsp*60 cluster VIII; both had ≥99.3% identity within the clusters. The *hsp60* sequences of the ST873 isolates formed a new cluster that shared only 96.7%–97.1% identity with the closest related sequences.

To confirm that the ST873 isolates formed a new subgroup, we performed a phylogenomic analysis with 398 ECC genomes downloaded from GenBank. In the resulting

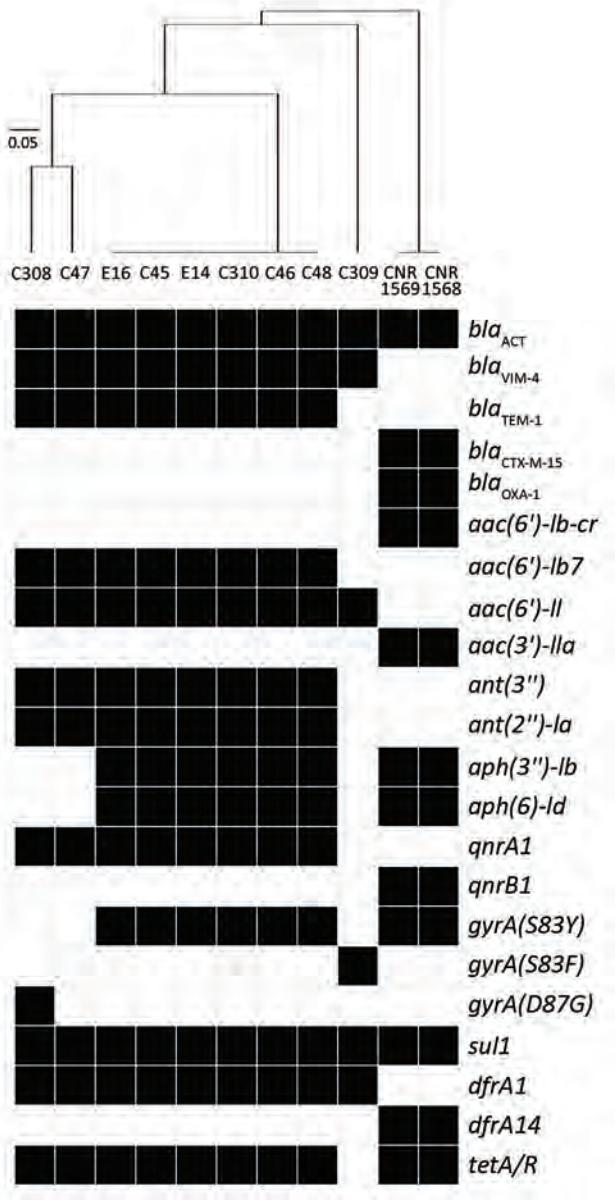

Figure 3. Genetic resistance determinants in *Enterobacter cloacae* complex isolates from nosocomial outbreak involving carbapenamase-producing *Enterobacter* strains, Lyon, France, January 12, 2014–December 31, 2015. Black cells indicate presence and white cells absence of resistance determinants. The isolates were classified according to the content in resistance determinants by using a binary distance matrix and UPGMA clustering method. Scale bar indicates the dissimilarity in resistance gene content.

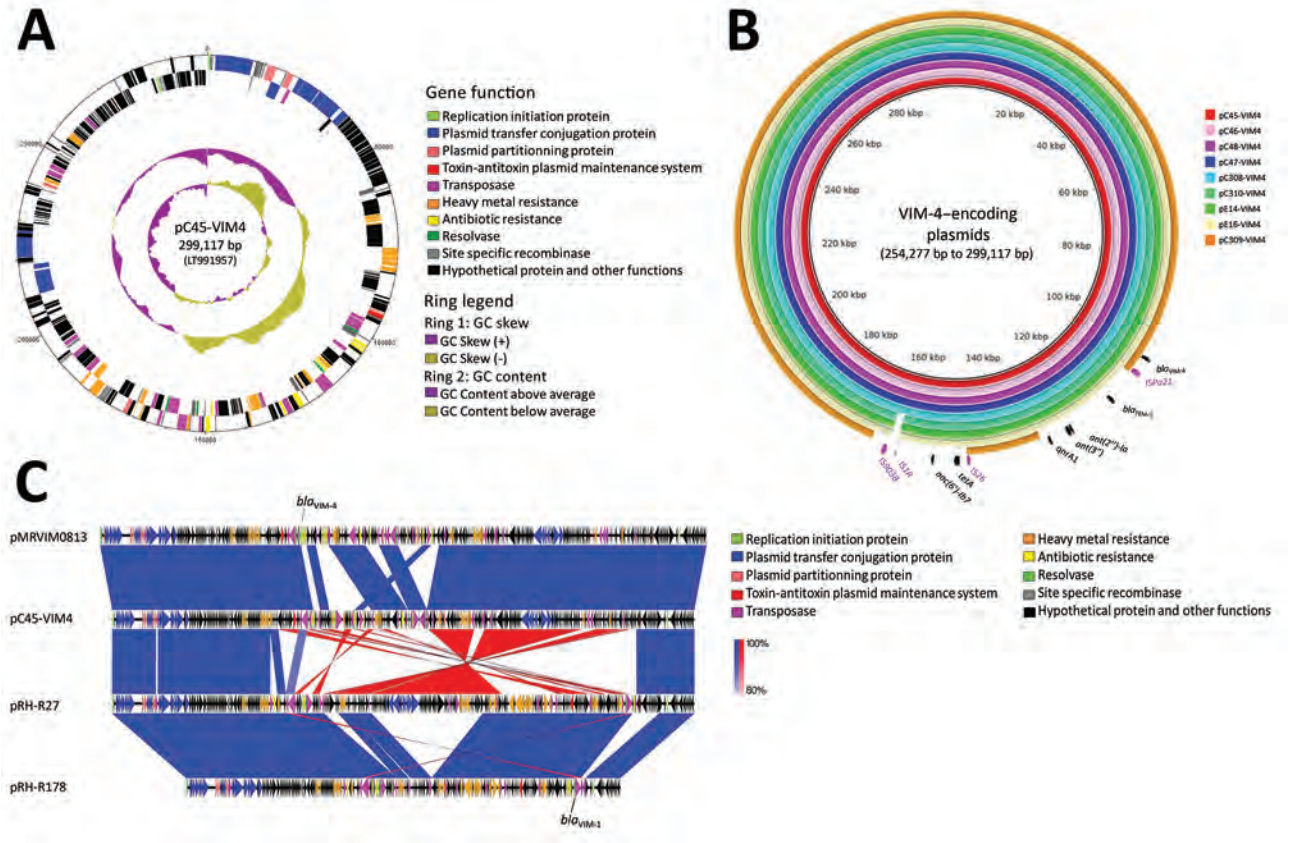

Figure 4. Analysis of *bla*$_{VIM-4}$–encoding plasmids from study of nosocomial outbreak involving carbapenamase-producing *Enterobacter* strains, Lyon, France, January 12, 2014–December 31, 2015. A) Schematic representation of ST1-IncHI2 plasmid pC45-VIM4. The first ring indicates the coordinates of the complete plasmid circle. The 2 outer rings represent the forward and reverse open reading frames, respectively. B) Comparative sequence analysis of ST1-IncHI2 *bla*$_{VIM-4}$–encoding plasmids from this study. The plasmids of isolates C45, C46, C47, C48, C308, C309, C310, E14, and E16 are designated pC45-VIM4, pC46-VIM4, pC47-VIM4, pC48-VIM4, pC308-VIM4, pC310-VIM4, pE14-VIM4, pE16-VIM4, and pC309-VIM4, respectively. C) Comparative sequence analysis of *bla*$_{VIM-4}$–encoding plasmid pC45-VIM4 to the related *bla*$_{VIM-1}$–encoding IncHI2 plasmids pMRVIM0813 (GenBank accession no. KP975077), pRH-R27 (GenBank accession no. LN555650), and pRH-R178 (GenBank accession no. HG530658). Vertical blocks between sequences indicate regions of shared similarity shaded according to blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Blue indicates matches in the same direction; red indicates inverted matches.

phylogenomic tree (Figure 5), the genomes were distributed in 2 major branches corresponding to the *E. hormaechei* and *E. cloacae* metaclusters as previously reported (*14*,*15*). The *E. hormaechei* metacluster comprised 6 branches corresponding to Chavda's phylogenomic groups A–E, and a new phylogenomic group, designated S, comprising solely the ST873 isolates. As expected, the C47, C308, and C309 isolates clustered in Chavda's phylogenomic groups D and B, which correspond to Hoffmann's *hsp60* clusters III and VIII (Figure 5). These findings fortify the hypothesis that the ST873 isolates could be a new species or subspecies in the *E. hormaechei* metacluster.

A New Species in the *E. hormaechei* Metacluster

Average nucleotide identity (ANI) and percentage of conserved DNA (PCD) can accurately replace DNA–DNA hybridization values for species delineation by using 0.95 and 0.69 as ANI and PCD thresholds, respectively (*25*,*26*). We therefore calculated ANIs and PCDs for the ST873 isolates against 398 ECC genomes by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The PCD values were high enough (>0.69) within the *E. hormaechei* and *E. cloacae* metaclusters for the delineation of species and subspecies by ANI calculations (online Technical Appendix Figure 2). Genomes within the same phylogenomic group shared ANI mean values ≥98% (Figure 6). In the *E. cloacae* metacluster, the ANI values supported the designation of the phylogenomic groups as different species (ANI, 0.87–0.94), except for *E. cloacae* subsp. *cloacae* and *E. cloacae* subsp. *dissolvens* (ANI, 0.95). In the *E. hormaechei* group, most phylogenomic groups shared ANI values 0.96–0.98, supporting the split of the *E. hormaechei* metacluster into different subspecies. As expected, isolates C47 and C308 clustered in the *E. hormaechei*

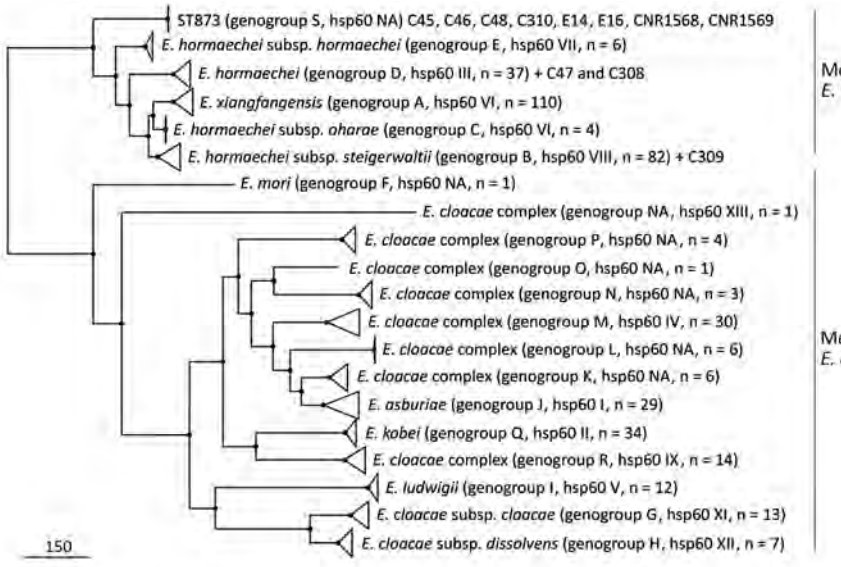

Figure 5. Approximately maximum-likelihood phylogenetic trees based on recombination free core single-nucleotide polymorphisms (SNPs) inferred from ST873, ST110 and ST118 genomes and 398 representative genomes of *Enterobacter cloacae* complex strains in study of nosocomial outbreak involving carbapenamase-producing *Enterobacter* strains, Lyon, France, January 12, 2014–December 31, 2015. All nodes are supported by Shimodaira-Hasegawa test values ≥97%. Scale bar indicates SNPs. NA, nonattributed; ST, sequence type.

phylogenomic group D and isolate C309 in the phylogenomic group B (also designated *E. hormaechei* subsp. *steigerwaltii*). The new phylogenomic group S, comprising the ST873 isolates, had ANI values below the species cutoff (<0.95) against all groups, including those in the *E. hormaechei* metacluster. We obtained similar results by using the MUMmer-based approach (online Technical Appendix Figures 3, 4), suggesting that the ST-873 isolates are a new species of the *E. hormaechei* metacluster.

The 3 subspecies *E. hormaechei* subsp. *hormaechei*, *E. hormaechei* subsp. *oharae*, and *E. hormaechei* subsp. *steigerwaltii* can be differentiated by using D-adonitol, D-arabitol, D-sorbitol, and D-melibiose fermentation tests (bioMérieux). By using the API 50CH system, we found that the biochemical characterization of our isolates yielded results compatible with *E. hormaechei* subsp. *steigerwaltii* (*hsp60* cluster VIII and phylogenomic group B), as expected (online Technical Appendix Tables 1, 2). Of interest, the ST873 isolates produced a distinguishable biochemical phenotype in the *E. hormaechei* metacluster by growing on only D-melibiose as the sole carbon source (online Technical Appendix Table 2). Overall, our molecular and biochemical data agree with those of previous studies in the field and suggest that the ST873 isolates are a new species of the *E. hormaechei* metacluster.

Functional Genomics in the *E. hormaechei* Metacluster

To investigate the functional features of the ST873 isolates and other phylogenomic subgroups in the *E. hormaechei* metacluster, we constructed a pangenome by using 245 strains including our isolates. The pangenome was divided into 3 sections: 1) the core genome (the set of genes shared by 99% of strains), 2) the accessory genome (the set of genes present in some but not all representatives), and 3) the unique genome (genes unique to individual strains). The 245 strains examined yielded a pangenome of 25,221 genes. On the basis of this dataset, the core genome is composed of 2,575 genes, the accessory genome of 14,849 genes, and the unique genome of 7,797 genes (Figure 7, panel A). Functional annotation of the pangenome on the COG database showed an overrepresentation of genes belonging to the groups with housekeeping functions (COG categories C, G, E, F, I, P, H, J, O, D, and T) in the core genome (Figure 7, panel B). The accessory genome and the unique genes had a similar distribution of functional annotations with an overrepresentation of genes involved in DNA recombination (genes encoding integrases, transposases, or resolvases) and defense mechanisms, as well as those belonging to the mobilome (COG categories L, U, V, and X), as expected.

The distribution of variable genome annotations among the phylogenomic groups showed closely related distributions of functional annotations (Figure 7, panel C), except for cell mobility annotations (COG category N, comprising the flagellar- and adhesion-related functions), which were overrepresented in phylogenomic group B and the ST873 isolates (phylogenomic group S), and mobilome annotations (COG category X), which were overrepresented in phylogenomic group A. The differences in functional distribution were enhanced by the analysis of variable genes specific to phylogenomic groups (Figure 7, panel D). Phylogenomic group B (*hsp60* group VIII), the most prevalent subgroup in human infections, and the new epidemic phylogenomic group S (ST873) had an overrepresentation of genes involved in cell motility (COG category N), as previously observed among variable genes, showing that these functions are overrepresented and based on specific genes in

Metacluster	Name (no.)	hsp60 cluster	Genomic cluster	A	B	C	D	E	S	F	G	H	I	J	K	L	M	N	O	P	Q	R	NA
E. hormaechei	*E. xiangfangensis* (110)	VI	A	0.99																			
	E. hormaechei steigerwaltii (83)	VIII	B	0.97	0.99																		
	E. hormaechei oharae (4)	VI	C	0.97	0.98	1.00																	
	E. hormaechei (37)	III	D	0.96	0.96	0.96	0.99																
	E. hormaechei hormaechei (6)	VII	E	0.95	0.95	0.95	0.94	1.00															
	ST873 (8)	NA	S	0.93	0.94	0.94	0.94	0.93	1.00														
E. cloacae	*E. mori* (1)	NA	F	0.88	0.88	0.88	0.88	0.88	0.88	1.00													
	E. cloacae cloacae (13)	XI	G	0.87	0.87	0.87	0.87	0.87	0.87	0.88	0.99												
	E. cloacae dissolvens (7)	XII	H	0.87	0.87	0.87	0.87	0.87	0.87	0.88	0.95	0.99											
	E. ludwigii (12)	NA	I	0.86	0.86	0.86	0.86	0.86	0.86	0.87	0.88	0.88	0.99										
	E. asburiae (29)	I	J	0.88	0.88	0.88	0.88	0.88	0.88	0.90	0.89	0.89	0.88	0.98									
	E. cloacae complex (6)	NA	K	0.88	0.88	0.88	0.87	0.88	0.88	0.89	0.88	0.89	0.88	0.94	0.99								
	E. cloacae complex (6)	NA	L	0.88	0.88	0.88	0.88	0.88	0.88	0.89	0.88	0.88	0.88	0.93	0.93	1.00							
	E. cloacae complex (30)	NA	M	0.88	0.88	0.88	0.88	0.88	0.88	0.89	0.88	0.89	0.88	0.93	0.94	0.93	0.98						
	E. cloacae complex (3)	NA	N	0.87	0.87	0.87	0.87	0.87	0.87	0.88	0.89	0.89	0.88	0.91	0.91	0.91	0.92	0.99					
	E. cloacae complex (1)	NA	O	0.87	0.87	0.87	0.87	0.87	0.87	0.89	0.89	0.89	0.88	0.92	0.92	0.91	0.92	0.92	1.00				
	E. cloacae complex (4)	NA	P	0.87	0.87	0.87	0.87	0.87	0.87	0.88	0.88	0.88	0.88	0.91	0.90	0.90	0.90	0.90	0.90	0.99			
	E. kobei (34)	II	Q	0.87	0.87	0.88	0.87	0.87	0.87	0.88	0.88	0.88	0.88	0.91	0.90	0.91	0.90	0.90	0.90	0.89	0.99		
	E. cloacae complex (14)	IX	R	0.88	0.88	0.88	0.88	0.88	0.89	0.89	0.88	0.88	0.88	0.91	0.91	0.91	0.91	0.90	0.91	0.89	0.91	0.99	
	E. cloacae complex (1)	XIII	NA	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	0.86	1.00

Figure 6. Average nucleotide identity calculated from BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) pairwise comparisons of ST873 genomes and 398 *Enterobacter cloacae* complex genomes in study of nosocomial outbreak involving carbapenamase-producing *Enterobacter* strains, Lyon, France, January 12, 2014–December 31, 2015. NA, nonattributed.

these ECC lineages. The ST873 group also exhibited enrichment in specific genes related to signal transduction (COG category T), and phylogenomic group A exhibited enrichment in genes related to recombination and the mobilome (COG categories L and X). We also observed other differences in genes related to metabolic functions. Phylogenomic group A demonstrated an enrichment in specific genes involved in lipid metabolism (COG category I), and phylogenomic group D has specific genes involved in coenzyme, nucleotide, and secondary metabolism (COG categories F, H and Q); phylogenomic group B accumulated specific genes involved in energy production and carbohydrate, amino acid, and ion metabolism (COG categories C, G, E and P). Overall, these data suggest that there exist distinct lifestyles in ECCs explaining varied abilities to colonize the hospital environment and to induce nosocomial infections.

Adhesion to Abiotic Surface and Epithelial Cells

To investigate the overrepresentation of mobility/adhesion functions in the ST873 isolates in the context of their prolonged persistence in hospital, we compared the ability of our isolates to initiate biofilm formation on an abiotic surface and their adhesion to intestinal epithelial cells. The ST873 isolates had greater ability to initiate biofilm on PVC than did isolates of ST118 and ST110 (online Technical Appendix Figure 5, panel A). We also observed slight differences of adhesion to HT29 intestinal epithelial cells (online Technical Appendix Figure 5, panelB). These results suggest that ST873 isolates have original adhesion features, as suggested by the pangenomic analysis.

Discussion

One major issue regarding CPEs is whether the main driver of the spread of carbapenemases is the transmission of successful clonal lineages or the horizontal transfer of carbapenemase genes by mobile genetic elements such as plasmids. Our study provides evidence for the spread of an epidemic VIM-4–encoding IncHI2 plasmid in distinct lineages of the ECC and the 1-year persistence of an epidemic strain ST-873, suggesting a double-string diffusion mechanism involving the emergence of both a VIM-4–encoding plasmid and a persistent ECC phylogenetic lineage.

ECC accounted for 9.4% and VIM-4–producing ECC for 3.9% of CPEs during 2015 in France (*27*). Only 16 VIM-4–producing *Enterobacteriaceae* cases were diagnosed in France during the same period; the 7 ECC cases included in this study, 6 cases of *E. coli*, and 1 additional case of a VIM-4–producing ECC, in the Paris area, and 2 cases of *Citrobacter freundii* in northern France. During this period, 6 other cases of CPE were identified in the same hospital: 5 produced oxacillinase 48 carbapenemases, and 1 produced New Delhi metallo-β-lactamase 1; these cases, in line with common observations in France and other countries in Europe, were imported (*4*). Our investigation was therefore prompted by the contrast between the low incidence of VIM-4–producing ECC cases in France and our case series, which suggested a new carbapenemase-spreading factor.

All the VIM-4 isolates we found harbored a similar $bla_{\text{VIM-4}}$–encoding, self-conjugative plasmid. The $bla_{\text{VIM-4}}$ gene has previously been reported only in IncA/C plasmids in a neighboring country (Italy) (*28*). The epidemic plasmid we identified belongs to the ST1-IncHI2 incompatibility

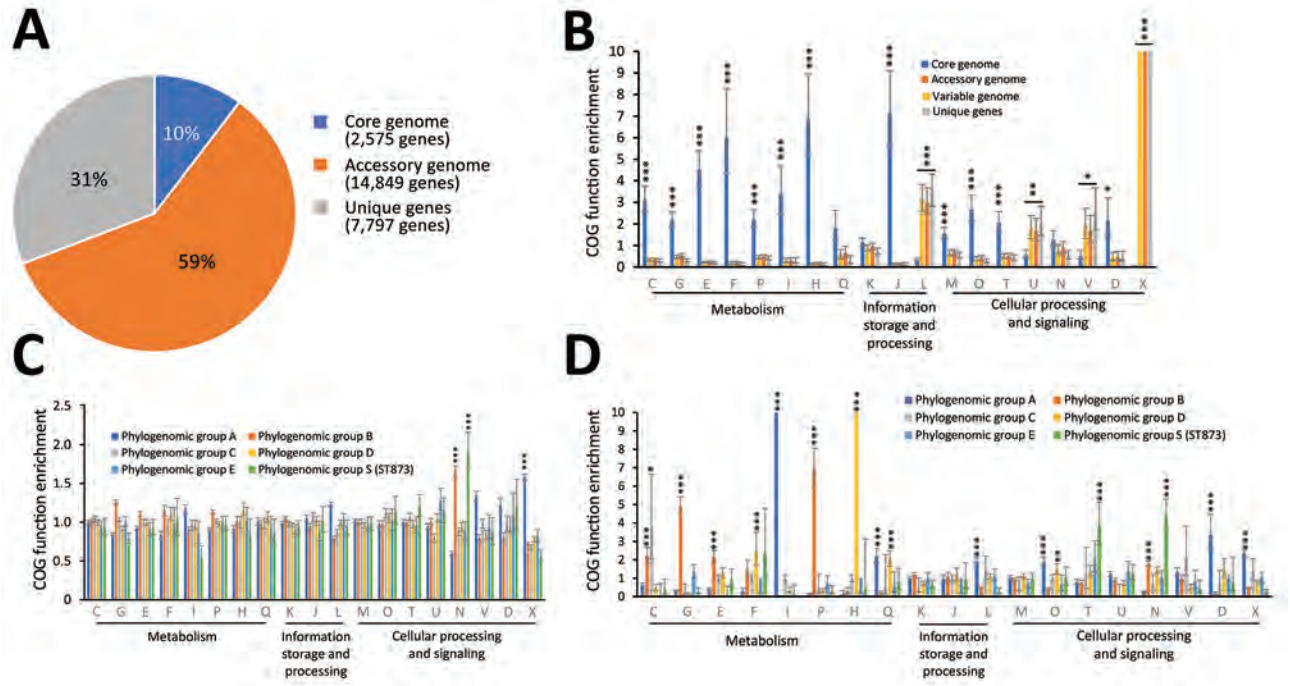

Figure 7. Pangenome analysis of metacluster *Enterobacter hormaechei* in study of nosocomial outbreak involving carbapenamase-producing *Enterobacter* strains, Lyon, France, January 12, 2014–December 31, 2015. A) Distribution of COGs); B) functional annotations in the pangenome; C) functional annotations in the variable genome (accessory genome + unique genes); and D) functional annotations for specific genes. Bar charts show the enrichment of COG categories as odds ratios; error bars indicate 95% CIs. Asterisks indicate certain COG categories that are significantly enriched: *p<0.05; **p<0.01;***p<0.001, all by Fisher exact test. Each COG category is identified by a 1-letter abbreviation: C, energy production and conversion; D, cell cycle control and mitosis; E, amino acid metabolism and transport; F, nucleotide metabolism and transport; G, carbohydrate metabolism and transport; H, coenzyme metabolism; I, lipid metabolism; J, translation; K, transcription; L, replication, recombination and repair; M, cell wall/membrane/envelope biogenesis; N, cell motility; O, post-translational modification, protein turnover, and chaperone functions; P, inorganic ion transport and metabolism; Q, secondary metabolism; T, signal transduction; U, intracellular trafficking and secretion; V, defense mechanisms; and X, mobilome. COG, clusters of orthologous groups.

group. IncHI2 plasmids are frequent among the ECCs and are often associated with the dissemination of genes encoding extended-spectrum β-lactamases and, at least to some extent, bla_{VIM-1} (*29*). Although bla_{VIM-4} differs from bla_{VIM-1} by a point mutation, the IncHI2 bla_{VIM-4} plasmids we identified substantially diverge from previously reported bla_{VIM-1} plasmids by the ≈50kb region encoding the associated resistance genes.

The VIM-4 epidemic strain ST873 persisted in the hospital for ≈1 year despite the application of specific isolation precautions for patients colonized or infected. During the investigation of another nosocomial outbreak that occurred in 2016 in Caen University Hospital (692 km from Lyon), we identified 2 CTX-M-15–encoding ECC strains belonging to ST873, which suggests that the spread of ST873 is not geographically limited. These strains had traits of speciation and specific genes related to signal transduction, cell motility, and adhesion. These functions have a crucial role in the initiation of biofilm formation (*30*), which was enhanced in the ST873 isolates compared with the other VIM-4 isolates in our study. Biofilm formation, a key function for host–pathogen interactions and environmental survival, may explain the successful persistence of ST873 isolates in the hospital settings of this study.

Nosocomial infections mediated by *Enterobacteriaceae* can be transmitted to patients in medical settings by HCWs, patient-to-patient spread, or environmental sources (*31*). Our findings emphasize the need to consider clinical circumstances such as bed contamination, as previously observed (*32*). In addition, the VIM-4–encoding plasmid pECC-VIM4 transferred to a bacterial recipient at room temperature, which could explain why successful horizontal transfer into ECC multiclonal isolates occurred in the hospital environment and provided support for the crucial importance of environmental reservoirs in the transmission of nosocomial pathogens. Hence, the threat of outbreaks can be limited by high-quality cleaning and disinfection of patient-care areas and the regular replacement of equipment such as mattress covers.

In conclusion, we report a nosocomial outbreak of multiclonal VIM-4–producing ECC that originated from contamination in the hospital environment. The

predominant clone belongs to a new lineage in the ECC and should be closely monitored in the context of nosocomial infections caused by its apparent ability to maintain and spread in a hospital setting. Our study also delineated the multifactorial spread of the VIM-4 carbapenemase and emphasizes the usefulness of ECC phylogenomic typing in the investigation of outbreaks.

Acknowledgments

We thank Laurent Argaud, Thomas Rimmele, Jacques Ninet, Laurent Juillard, Michel Chuzeville, Olivier Monneuse, Patrick Lermusiaux, Lionel Badet, Bernard Floccard, and their staff for patients' care and Laurent Guillouard and Alexis Pontvianne for technical assistance.

This work was supported by INSERM, INRA, the Université Clermont Auvergne, and the Centre Hospitalier Universitaire de Clermont-Ferrand.

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Therapeutic and Transmission-Blocking Efficacy of Dihydroartemisinin/Piperaquine and Chloroquine against *Plasmodium vivax* Malaria, Cambodia

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We assessed the efficacy of standard 3-day courses of chloroquine and dihydroartemisinin/piperaquine against *Plasmodium vivax* malaria. Compared with chloroquine, dihydroartemisinin/piperaquine was faster in clearing asexual *P. vivax* parasites and blocking human-to-mosquito transmission. This drug combination was also more effective in preventing potential recurrences for ≥2 months.

Plasmodium vivax is the most widespread human malaria parasite. Almost 2.5 billion persons are at risk for infection in >90 countries (*1*,*2*). Since the 1950s–1960s, Southeast Asia has been the cradle of emergence and spread of *P. falciparum* antimalarial drug resistance, a major obstacle for malaria control. Over the past decade, control efforts in Cambodia have led to an impressive decrease in malaria burden, with a slower decrease of *P. vivax* than for *P. falciparum* (*3*).

P. vivax resistance to chloroquine has emerged more recently; the first cases were observed in 2009 in Rattanakiri Province in northeastern Cambodia (17.4% treatment failures after 28 days of follow-up), which led to withdrawal of chloroquine and use of dihydroartemisinin/piperaquine (DHA/PPQ) as first-line therapy for uncomplicated *P. vivax* malaria in 2012 (*4*). We assessed the efficacy of standard 3-day courses of chloroquine and DHA/PPQ for treating *P. vivax* malaria, preventing recurrences, and blocking human-to-mosquito transmission.

The Study

We conducted an open-label, randomized, control trial in June–December 2014 in Rattanakiri Province, Cambodia. Febrile patients or patients with a history of fever in the previous 48 h who sought treatment in health facilities and had positive results by rapid diagnostic test (CareStart Malaria HRP2/pLDH Pf/PAN Combo; Access Bio, Inc., Somerset, NJ, USA) for non–*P. falciparum* malaria were offered participation in the study. Pregnant or lactating women and patients with signs of severe malaria, other known illnesses, or inability to provide informed consent were excluded. Patients with *P. vivax* monoinfection confirmed by PCR were eligible for the study (*5*).

At enrollment, after we obtained written informed consent, patients were randomized to receive supervised standard 3-day courses of DHA/PPQ (Duo-Cotecxin; Zhejiang Holley Nanhu Pharmaceutical Co., Ltd., Jiaxing, China) or chloroquine (Nivaquine; Sanofi-Aventis, Paris, France). For each participant, medical histories were obtained and clinical and biological examinations performed. We followed up with patients according to an extended World Health Organization protocol on days 1, 2, 3, 5, and 7 and then weekly until day 63. At each visit, we performed clinical examinations and obtained an axillary temperature and a capillary blood sample.

Malaria parasites were detected by microscopy (Giemsa-stained blood films) and PCR as described (*5*). Chloroquine resistance was ruled out for patients if no parasites were detected by microscopy on day 28 or, in case of recurrence, if the chloroquine blood concentration on the day of recurrence did not exceed >100 ng/mL (*6*,*7*). We measured chloroquine blood concentrations by using liquid chromatography–tandem mass spectrometry for 50-μL samples of whole blood.

During January–March 2016, we conducted an additional study at the same site to evaluate the infectivity of *P. vivax* from blood of symptomatic patients to *Anopheles dirus* mosquito vectors; we tested pretreatment and posttreatment blood samples by using membrane feeding assays without serum replacement (*8*). Any febrile patients seeking antimalarial treatment with similar inclusion/exclusion criteria described previously were enrolled in this study.

After we obtained written informed consent, we fed batches of 50 *An. dirus* mosquitoes with blood collected from these patients on 3 occasions: 1) before the first dose of DHA/PPQ or chloroquine, 2) on the same day at 9:00 PM (i.e., 2–11 h after treatment), and 3) at 24 h posttreatment for patients treated with chloroquine. We performed statistical analyses by using GraphPad Prism 5 (GraphPad, San Diego,

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DOI: https://doi.org/10.3201/eid2408.170768

CA, USA) and R software (*9*). Both studies were approved by the Cambodian National Ethic Committee (038 NECHR, 2/24/2014 and 475 NECHR, 12/28/2015).

For the drug comparison study, we enrolled 50 patients (25 in each study arm); in each arm, 5 patients were lost to follow-up during days 2–35. A total of 40 patients (20 in each

Table 1. Baseline characteristics of *Plasmodium vivax*–infected patients in a clinical drug trial and a human-to-mosquito transmission study, Cambodia*

Characteristic	Chloroquine	DHA/PPQ	p value
Clinical drug trial study, June–December 2014			
No. patients followed up until day 63 (% Male)	20 (80)	20 (80)	1.00†
Patient age, y	26.5 (18.5–35)	28.5 (21.5–46)	0.11‡
Patient weight, kg	56.0 (50–59)	51.0 (49.5–53)	0.14‡
Parasites/μL of blood	5,000 (1,850–8,350)	8,900 (3,500–17,500)	0.051‡
Gametocytes/μL of blood	108 (58–200)	245 (105–745)	0.12‡
Proportion with G6PD deficiency by spot test and PCR§	2/20 (Viangchan variant)	1/20 (Canton variant)	1.00†
Leukocytes, $\times 10^9$ cells/L	7.7 (6.2–9.2)	6.9 (5.2–8.6)	0.38‡
Erythrocytes, $\times 10^{12}$ cells/L	5.01 (4.53–5.26)	5.10 (4.69–5.65)	0.42‡
Hemoglobin, g/dL	11.2 (10.3–13.8)	12.3 (11.4–13.1)	0.43‡
Hematocrit, %	37 (34–43)	40 (37–43)	0.49‡
Human-to-mosquito transmission study, January–March 2016¶			
No. (%) male patients	9 (100)	10 (70)	0.21†
Patient age, y	13.0 (12.7–38.5)	24.5 (19.0–29.0)	0.68‡
Patient weight, kg	37.0 (28.7–52.2)	53.5 (42.0–60.0)	0.09‡
Parasites/μL of blood	4,565 (3,462–6,184)	9,069 (6,833–11,591)	**0.01‡**
Gametocytes/μL of blood	221 (74.2–381.5)	1,915 (693–2,729)	**0.001‡**
Proportion of infectious patients before treatment; feeding assay before first dose of treatment#	8/9 (89)	9/10 (90)	1.00†
Proportion of infected mosquitoes before treatment; feeding assay before first dose of treatment	69.6 (26.2–84.9)	72.9 (29.75–92.7)	0.71‡
Average no. oocysts in infected mosquitoes before treatment; feeding assay before first dose of treatment	12.2 (2.4–29.8)	12.2 (2.4–40.6)	0.74‡

*Values are median (IQR) or no. positive/no. tested (%) unless otherwise indicated. Bold indicates statistical significance (p<0.05). DHA/PPQ, dihydroartemisinin/piperaquine; G6PD, glucose-6-phosphate dehydrogenase; IQR, interquartile range.
†By Ficher exact test.
‡By Mann-Whitney U test.
§For details, see Khim et al. (*10*).
¶Mosquitoes were 6–8 days old and were allowed to feed for 20 min. Feeding was conducted at the same place immediately after obtaining blood from patients. Dissections were performed 6 days after the blood meal. Midguts were dissected in 1% mercurochrome stain, and the presence and number of oocysts were determined by microscopy (×20 magnification).
#Patients were defined as being infectious when ≥1 mosquito became infected with oocysts.

Table 2. *Plasmodium vivax* clearance among infected patients, time to malaria recurrence, and vector transmission results, by allocated antimalarial drug treatment, Cambodia*

Characteristic	Chloroquine	DHA/PPQ	p value
Clinical drug trial study, June–December 2014			
Proportion of patients parasitemic at day 1 by microscopy	17/20 (85)	6/20 (30)	**0.001†**
Parasites/μL of blood at day 1	180 (80–600)	0 (0–57)	**0.0002‡**
Parasite reduction ratio at day	96.2 (68.5–98.6)	100 (99.4–100)	**0.0002‡**
Proportion of patients parasitemic at day 2 by microscopy	5/20 (25)	0/20 (0)	**0.047†**
Parasites/μL of blood at day 2	0 (0–15)	0	**0.03‡**
Parasite reduction ratio at day 2	100 (99.8–100)	100	**0.03‡**
Proportion of patients parasitemic at day 3 by microscopy	0/20 (0)	0/20 (0)	1.00†
Proportion of patients with recurrence detected by PCR	12/20 (60)	4/20 (20)	**0.02†**
Time to recurrence, d	49 (42–49)	56 (52.5–56)	**0.04‡**
Human-to-mosquito transmission study, January–March 2016			
Proportion of infectious patients after first dose of treatment; feeding assay at 9:00 PM	8/9 (89)	1/10 (10)	**0.001†**
Proportion of infected mosquitoes after first dose of treatment; feeding assay at 9:00 PM	60.7 (23.7–78.6)	0	**0.004‡**
Average no. of oocysts in infected mosquitoes after first dose of treatment; feeding assay at 9:00 PM	9.9 (4.1–25.7)	356.4	0.22‡
Parasite transmissibility reduction ratio (%) at 9:00 PM	19 (−13.8 to 62.7)	100	**0.003‡**
Proportion of infectious patient 24 h after first dose of chloroquine	2/9 (22)	ND	ND
Average no. of oocysts in infected mosquitoes after first dose of treatment for 2 infectious patients; feeding assay 24 h after first dose of chloroquine	1.3 and 2.4	ND	ND
Proportion of infected mosquitoes 24 h after first dose of chloroquine	0 (0–4.4)	ND	ND

*Values are no. positive/no. tested (%) or median (IQR) unless otherwise indicated. Bold indicates statistical significance (p<0.05). DHA/PPQ, dihydroartemisinin/piperaquine; IQR, interquartile range; ND, no data.
†By Fisher exact test.
‡By Mann-Whitney U test.

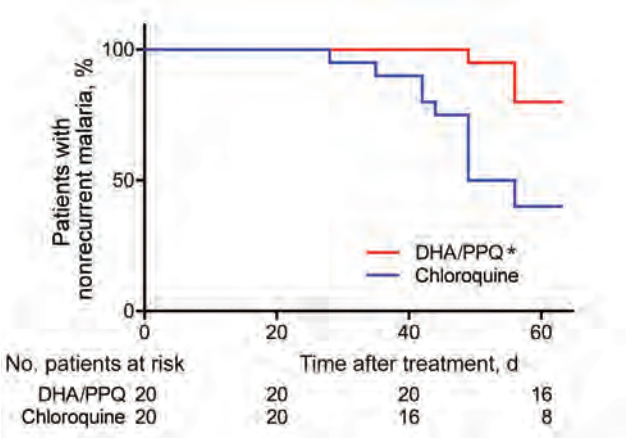

Figure 1. Cumulative proportion of patients with nonrecurrent *Plasmodium vivax* malaria given a 3-day course of DHA/PPQ and chloroquine detected by PCR within 63 days of follow-up, Cambodia. *p<0.01, by log-rank test during Kaplan-Meier survival analysis. DHA/PPQ, dihydroartemisinin/piperaquine.

study arm) were followed up until day 63. Baseline patient characteristics were similar for both patient groups (Table 1). We did not observe any adverse events or early clinical failures. The proportion of patients still parasitemic on days 1 and 2 (detected by microscopy) was lower for the DHA/PPQ–treated group than for the chloroquine-treated group (Table 2). Medians of the parasite reduction ratio recorded on days 1 and 2 were higher for the DHA/PPQ–treated patient group (Table 2). All patients, regardless of their treatment, were microscopically parasite free at day 3.

Within 2 months of follow up, there were fewer patients with a recurrence (detected by PCR) in the DHA/PPQ-treated group than in the chloroquine-treated group (odds ratio 0.17, 95% CI 0.05–0.66; p<0.05, by log-rank test, p<0.01 by Kaplan-Meier survival analysis) (Table 2; Figure 1). Median time to recurrence after treatments was also delayed in patients given DHA/PPQ (56 days) compared with those patients given chloroquine (49 days) (Table 2). No recurrence occurred before day 28 in either study arm, which is suggestive of relapse or reinfection, rather than recrudescence of drug-resistant parasites (*6*,*7*). In the chloroquine-treated group, 12/20 patients with recurrence had a chloroquine blood concentration <100 ng/mL on the day of recurrence (chloroquine + desethyl chloroquine: median 55.6 ng/mL, interquartile range 40.0–61.7 ng/mL); these results excluded likely chloroquine resistance (*6*,*7*).

For the mosquito-to-human transmission study, we enrolled 19 patients (9 given chloroquine and 10 given DHA/PPQ). Baseline patient characteristics were similar in both patient groups, except for day 0 parasitemia and gametocytemia, which were higher for the DHA/PPQ-treated group (Table 1). The proportion of infectious blood from *P. vivax*–infected patients and the median proportion of infected mosquitoes fed on blood collected before the

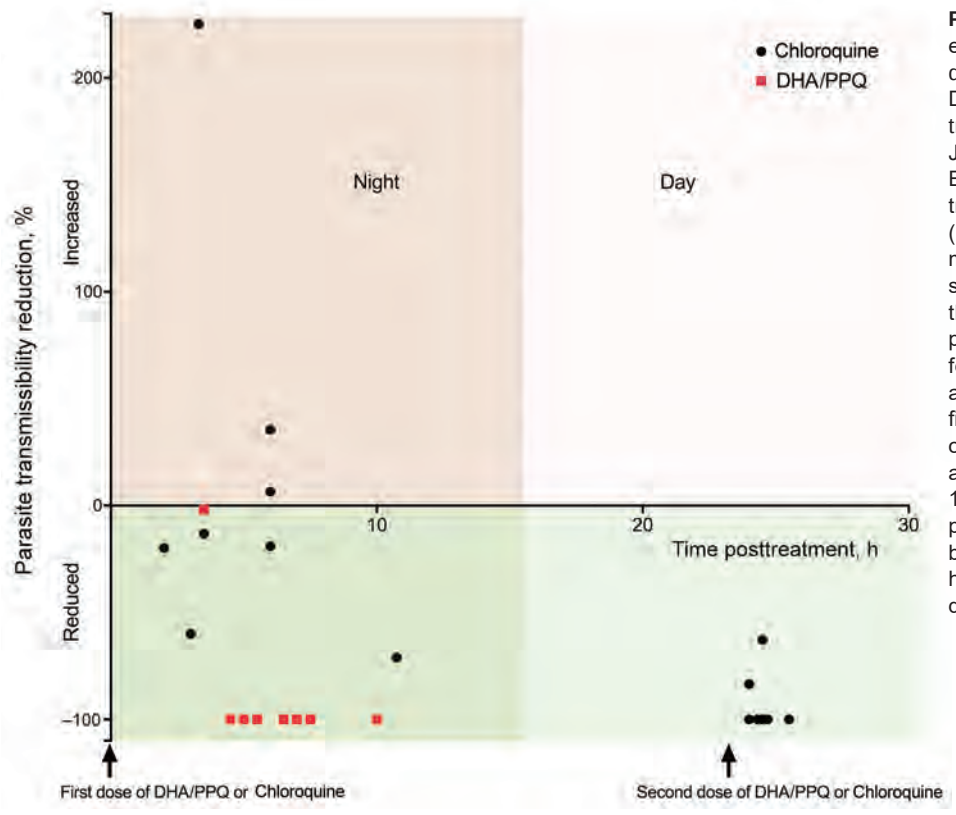

Figure 2. Transmission-blocking efficacy of allocated antimalarial drug treatment (chloroquine and DHA/PPQ) on human-to-mosquito transmission of *Plasmodium vivax*, January–March 2016, Cambodia. Each dot represents the parasite transmissibility reduction ratio (i.e., 100 – [proportion of infected mosquitoes fed with blood samples collected at 9:00 PM after the first dose of treatment × 100/ proportion of infected mosquitoes fed with blood samples collected at patient enrollment before the first dose of treatment]). Only data of infectious patients at enrollment are shown (17/19 patients; Table 1). For the chloroquine-treated patient group, a second mosquito blood feeding was performed 24 h after the first dose. DHA/PPQ, dihydroartemisinin/piperaquine.

first dose of DHA/PPQ or chloroquine were similar for both patient groups (Table 1). Despite an initial higher day 0 gametocytemia for the DHA/PPQ-treated group, the proportions of infectious *P. vivax* blood collected at 9:00 PM after the first dose of DHA/PPQ or chloroquine and the median proportion of infected mosquitoes were lower for the DHA/PPQ-treated group than for the chloroquine-treated group (Table 2). Overall, DHA/PPQ acted faster than chloroquine in decreasing over time the proportion of infectious patients (generalized linear mixed model time for drug interaction, $\chi_1^2 = 113.1$, $p < 0.0001$) (Figure 2). For the group given chloroquine, 2 (22%) of 9 blood samples were still infectious 24 hours after the first dose (Table 2).

Conclusions

We confirm that DHA/PPQ acts faster (≤48 h) than chloroquine (≈72 h) in eliminating sexual and asexual *P. vivax* parasites and that DHA/PPQ provides an excellent post-exposure prophylaxis against potential recurrences for ≥2 months (*11*). This benefit relies on the combination of artemisinin derivatives (DHA), which are fast-acting drugs capable of eliminating any *P. vivax* blood stages, and a long-lasting partner drug (PPQ), which has a long terminal elimination half-life and is highly effective in preventing *P. vivax* recurrence for up to 56 days. Although the number of patients enrolled was small, we demonstrated that DHA/PPQ also acts faster (<5 h) than chloroquine in killing *P. vivax* sexual stages and thus prevents the risk for transmission of parasites to the mosquito vector the night after uptake of the first dose. This rapid clearance of gametocytes is a major benefit of DHA/PPQ in comparison with chloroquine, given that *P. vivax* gametocytes appear early in the course of disease and must be eliminated as soon as possible to limit risk of transmission (*12*,*13*).

In summary, our findings support the recommendation of DHA/PPQ as first-line treatment for *P. falciparum* and *P. vivax* uncomplicated malaria in regions to which these species are co-endemic. These findings apply to areas in which chloroquine is still effective and no *P. falciparum* resistance to PPQ has been observed.

Acknowledgments

We thank all patients for participating in this study.

This study was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health (grant R01 434 A103328) and the Institut Pasteur (grant PTR 2014–490). A.V. was supported by a Calmette and Yersin fellowship from the Institut Pasteur International Network.

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Dual-Genotype *Orientia tsutsugamushi* Infection in Patient with Rash and Eschar, Vietnam, 2016

Nhiem Le-Viet, Duc-Tuan Phan, Nho Le-Viet, Sinh Trinh, Muoi To, Didier Raoult, Philippe Parola

We report a dual-genotype *Orientia tsutsugamushi* infection in Vietnam in 2016. The patient had fever, rash, and an eschar. The Kawasaki genotype was identified in the eschar specimen and Karp genotype in the whole blood specimen. The genotype co-infection rate for scrub typhus is unknown and should be further evaluated.

Scrub typhus is an acute febrile zoonosis caused by *Orientia tsutsugamushi* that is transmitted by larval trombiculid mites in rural areas (*1*). Scrub typhus is a major public health issue in the Asia-Pacific region and might also be present in Africa (*2*) and South America (*3*). Clinical manifestations can vary from mild symptoms to fatal disease in the absence of appropriate antimicrobial drugs (*4*). Many antigenic variants of *O. tsutsugamushi* exist, including Gilliam, Kato, Karp, Kawasaki, and Kuroki. This antigenic variation depends largely on the immune-dominant 56-kDa type-specific antigen (TSA) located on the surface of the bacteria membrane. Genotypes of *O. tsutsugamushi* are based on the 56-kDa TSA gene, which are commonly used to identify the diverse strains present in endemic countries (*4*–*6*). We report a case of a patient infected with 2 distinct genotypes of *O. tsutsugamushi*.

The Study

A 35-year-old man, who often harvested agarwood in the forests of central Vietnam, had a fever and headache during a harvesting trip. On the seventh day of illness, he sought treatment at Quang Nam Northern Mountainous Region General Hospital (Quang Nam, Vietnam). He had continuous high fever, rigor, increasing headache, and muscle pain but no nausea, vomiting, or abdominal pain. A rash developed 1 day after the fever. During his time working in the forest, the man took 3 pills of acetaminophen per day to treat his symptoms, but this medicine did not improve his condition. On examination, the patient was highly febrile (39°C–40°C) and had an oval-shaped, painless eschar measuring 8 × 10 mm on his right anterior neck (Figure 1). He also had a rash covering his whole body (Figure 2). Swollen lymph nodes were observed along the mid-jugular chain of the right neck and right armpit; the largest lymph node was 10 mm in diameter, mobile, and painless. Results of routine laboratory tests for serum aspartate aminotransferase, alanine aminotransferase, and creatinine and complete blood cell counts were within reference ranges. Oral doxycycline (100 mg), prescribed immediately after scrub typhus was suspected, was administered twice a day starting on the first day of hospitalization. Defervescence occurred 24 hours after the first dose of doxycycline, and the man completely recovered.

On the day of admission, we collected a sample of the eschar by rotating a swab vigorously at the eschar base after the crust was removed, as previously described (*7*). We also took 500 μL of whole blood and 500 μL of acute-phase plasma and stored these specimens at –20°C. Specimens were transported to Marseille, France, for molecular biology and serologic testing.

We isolated DNA from the eschar and whole blood specimens and performed a real-time quantitative PCR (qPCR) specific for the periplasmic serine protease gene of *O. tsutsugamushi*; qPCR results showed these specimens were positive for the protease gene (*6*). Then, we subjected the DNA to a conventional PCR targeting the *O. tsutsugamushi* 56-kDa TSA gene using appropriate negative controls and sequenced the PCR products. We analyzed the partial 56-kDa TSA gene sequences from our patient with ABI PRISM DNA Sequencing Analysis software version 3.0 (Applied Biosystems, Foster City, CA, USA) and compared them with those available in the GenBank database using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The DNA sequence obtained from the eschar specimen (GenBank accession no. MF769529) was closely related to the Kawasaki genotype, showing 97.2% identity to the reference strain TPC0707a (GenBank accession no. GQ332758) (*8*). The eschar specimen sequence was even

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DOI: https://doi.org/10.3201/eid2408.171622

more similar to a Kawasaki isolate previously detected in a patient in Quang Nam Province, Vietnam (GenBank accession no. KU871388, 98.8% identity) (*6*). The DNA sequence obtained from the whole blood specimen (GenBank accession no. MF769530) demonstrated 98.8% identity to that of a Karp isolate in Cambodia (GenBank accession no. HQ718422) (*4*). The whole blood specimen sequence was also highly similar (98.4% identity) to the sequences of 2 Karp-related isolates previously detected in patients in Quang Nam Province (GenBank accession nos. KU871384 and KU871378) (*6*). We tested the acute-phase plasma sample collected from the patient on the seventh day of illness using a micro-immunofluorescence assay (IFA) that included whole-cell antigens of *O. tsutsugamushi* serotypes Karp, Kato, and Gilliam. However, results were negative for specific antibodies to these antigens.

We tested for dengue virus, *Plasmodium* spp., and *Leptospira* spp. by qPCR using the whole blood specimens; negative results led to the exclusion of these pathogens as the causative agent of the fever. We also tested for other rickettsiae (*Rickettsia typhi*, *R. felis*, *R. conorii* and other spotted fever group rickettsiae, and *Coxiella burnetii*) using specific qPCRs and IFAs and excluded these pathogens as well.

Because this patient's signs and symptoms included eschar and rash, his clinical presentation was typical for scrub typhus. A rash is observed in ≈50% of patients infected with scrub typhus (*9*), the percentage varying depending on the *O. tsutsugamushi* genotype (*6*). Eschar is associated with many rickettsial diseases and is present in 7%–80% of scrub typhus patients (*10*). Eschar is associated with severe renal, hematologic, respiratory, and circulatory manifestations; long hospital stay; and high mortality rate (*11*). The eschar is the preferred sampling site for *O. tsutsugamushi* detection and isolation. Eschar swabs are preferred over eschar biopsy and blood samples because swabbing eschars is noninvasive, easy, and painless and PCRs of DNA from eschar swab samples are highly sensitive and specific (*6*,*12*).

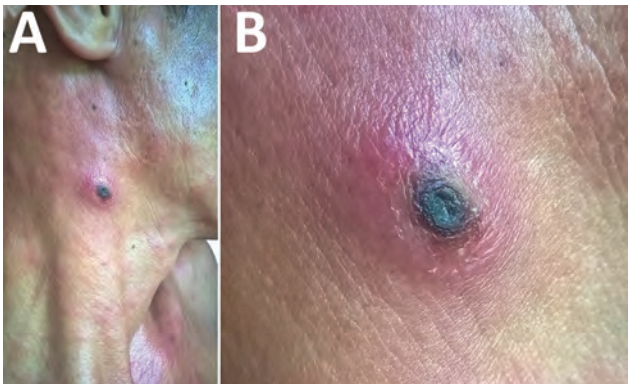

Figure 1. Eschar on right anterior neck of patient with dual genotype *Orientia tsutsugamushi* infection, Vietnam. A) Eschar location; B) enlarged view.

In 2014, *O. tsutsugamushi* genotype co-infections were reported in rodents and wild chiggers, as well as in naturally infected and laboratory-reared mites, in Thailand; the Karp genotype was detected in all mites examined, and Gilliam and UT302 were the co-infecting genotypes (*13*). Sonthayanon et al. tested whether scrub

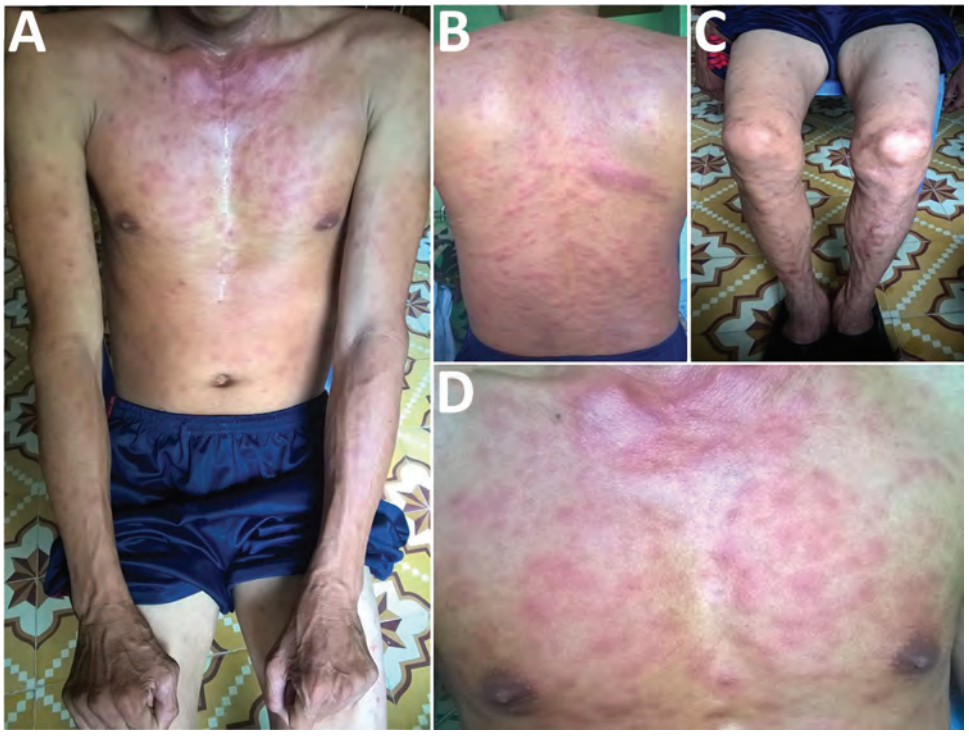

Figure 2. Rash on patient with dual-genotype *Orientia tsutsugamushi* infection, Vietnam. A) Trunk and arms; B) back; C) legs; D) chest.

typhus patients were simultaneously infected with multiple *O. tsutsugamushi* genotypes using multilocus sequence typing (*14*); however, the specific genotypes could not be identified in their study.

Conclusions

We demonstrated the coexistence of 2 different *O. tsutsugamushi* genotypes in this patient. The negative *O. tsutsugamushi* IFA results could have been a result of early stage plasma collection, on the seventh day of illness. For efficient serologic diagnosis, convalescent-phase plasma or serum is needed. Unfortunately, we could not obtain this type of sample from this patient because he returned to work in the forest after 5 days of treatment. Because serologic results can be negative for over a week after scrub typhus disease onset, we stress use of molecular assays rather than serologic tests for diagnosis.

We used appropriate controls and obtained sequences that had not been obtained before in our laboratory, making contamination a highly unlikely explanation for our results. However, in vitro isolation would have been stronger evidence for a dual *O. tsutsugamushi* infection.

Because this patient often worked in the forest, his dual infection could have been caused by bites from several mites, but bites from a single mite infected with multiple genotypes could have also happened (*13*,*14*). Although detecting both genotypes in the eschar sample would have been expected if he was bitten by a mite infected with multiple genotypes, 1 genotype might have predominated. Furthermore, a person bitten by multiple mites might have multiple eschars, but eschar formation varies, depending on the *O. tsutsugamushi* genotype (*15*). Wearing adequate personal protective gear when in wooded areas can help minimize exposure to mites and prevent scrub typhus. However, when a patient such as the one we describe seeks treatment, clinicians should administer doxycycline immediately.

Acknowledgments

We thank the staff of the Department of Internal Medicine II of Quang Nam Northern Mountainous Region General Hospital, Quang Nam, Vietnam, for their support with sample collection. We also thank the biomol team of Institut Hospitalo-Universitaire Méditerranée Infection, Marseille, France, for their support with serologic testing.

Sample collection in this study was funded by Quang Nam Provincial People's Committee under Provincial Scientific and Technological Tasks (reference no. 19/HD-SKHCN/2016) managed by Quang Nam Department of Science and Technology. Laboratory analyses and publication were funded by the government of France under the Investments of Futures Program (reference no. Méditerranée Infection 10-IAHU-03) managed by the National Agency for Research.

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Hospitalized Patient as Source of *Aspergillus fumigatus*, 2015

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Hospital-acquired aspergillosis is usually associated with environmental contamination. In 2015, continuous monitoring of airborne fungi and multilocus variable-number tandem-repeat analysis identified the source of *Aspergillus fumigatus* as the airway of a patient. Therefore, patients colonized with *Aspergillus* spp. should be treated in airborne infection isolation rooms.

Pulmonary aspergillosis is acquired by inhalation of airborne spores in the environment. Hospital-acquired aspergillosis is usually associated with airborne fungal contamination of the hospital environment, especially after building construction events. A previous report described *Aspergillus fumigatus* transmission from an intensive care unit (ICU) patient with a sporulating liver-transplant surgical site infection to 2 other patients (secondary pulmonary aspergillosis) (*1*). We describe a patient whose respiratory tract was colonized by *A. fumigatus*; we found no reports of hospital environment contamination by this organism. Continuous monitoring of indoor airborne fungal contamination with electrostatic dustfall collectors (EDCs) (*2*–*5*) demonstrated that the airway of a patient was the point source of airborne *A. fumigatus* contamination in the ICU.

The Case

On July 29, 2015, a 61-year-old man was hospitalized in the ICU at the University Hospital of Marseille (Marseille, France) with acute respiratory distress syndrome as a complication of a lung abscess. The patient had a history of smoking tobacco (1 pack/d for 25 y), a nonrepaired herniated lumbar disk, and recent periodontitis. At the time of admission, the microbiological workup results, including bacteriological and mycological culture (of blood, urine, bronchial aspirate, and superficial swab sample assessment for possible fungal colonization) and HIV serology, were negative. Chest radiographs showed bilateral interstitial pneumonitis and abscessation of the right lower lung lobe. Bronchial fibroscopy showed inflammatory mucosa. The following were initiated: mechanical ventilation with a system equipped with antimicrobial filters, extra corporeal membrane oxygenation, and antibacterial therapy (imipenem, ciprofloxacin, and vancomycin). Anemia, hypoalbuminemia, and a clotting disorder subsequently developed. On day 20, a tracheotomy was performed. The patient's airways were moisturized by water vapor from a humidification system without a filter that allowed airflow between room air and the patient's airways. No filamentous fungus was detected after culture of 9 bronchoalveolar lavage and 11 bronchial aspirate samples. The following bacteria were isolated: *Porphyromonas endodontalis* (day 5), *Klebsiella pneumoniae* (day 10), and *Pseudomonas aeruginosa* showing intermediate resistance to imipenem (day 36). Antibacterial therapy was modified to intravenous meropenem plus intrabronchial colistin and then adjusted to tazocillin and tobramycin. On days 52 and 55, *Candida albicans* was isolated from 2 blood cultures and a double-J ureteric stent sample. Caspofungin treatment was started and continued for 2 weeks. No respiratory samples were analyzed during the following months.

On day 114, the patient experienced hemoptysis. A diagnosis of pulmonary aspergillosis was based on the following criteria: positive culture, *A. fumigatus* detection in a sputum sample culture, positive galactomannan antigen (index = 4.27 in respiratory samples), and bilateral nodules visible on chest computed tomography image. During the following 47 days, 5 bronchial aspirate samples grew *A. fumigatus* on culture, and 4 of 6 samples were positive for galactomannan antigen. Seven serum samples were negative for galactomannan antigen.

Because severe liver dysfunction contraindicated the use of triazoles, 3 mg/kg/d of anti-*Aspergillus* liposomal amphotericin B was initiated on day 116. On day 134, a chest computed tomography image showed bilateral pulmonary nodules, some of which displayed a halo sign. The liposomal amphotericin B dosage was then increased to 5 mg/kg/d. Of note, during days 118–160, the patient's

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\DOI: https://doi.org/10.3201/eid2408.171865

airways were directly connected to his environment during several attempts to wean him off mechanical ventilation. The patient continued receiving liposomal amphotericin B without substantial clinical improvement. Bacteremia with *Staphylococcus aureus* and *Bacteroides thetaiotaomicron* developed and was treated with vancomycin and imipenem. The patient died on day 169.

EDCs were used to continuously monitor fungal airborne contamination in the ICU for 244 weeks; each EDC was exposed for 14 days. EDCs have been used in various indoor environments to detect and quantify cultivable microorganisms and a wide range of airborne analytes, including endotoxins, allergens, β-glucans, and microbial DNA (*2*–*4*). EDC-based measurements provide an accurate qualitative and quantitative profile of the cultivable airborne fungal communities present during a given sampling period (*5*). The patient's first room (room A) remained free of airborne *A. fumigatus* contamination from the day the patient entered the room to day 62 (Figure 1). *A. fumigatus* airborne contamination was detected during the 2-week periods of days 62–76 (2 CFU) and days 104–118 (15 CFU). On day 128, the patient was transferred from room A to room B. Room A was disinfected and thereafter remained free of airborne fungal contamination. Fifteen days after the room change, airborne *A. fumigatus* contamination was detected in room B.

All 39 *A. fumigatus* isolates collected in the ICU since the beginning of 2015 were genotyped by using a variable number of tandem repeats assay as previously described (*6*). Genotyping revealed that the *A. fumigatus* strain isolated from the sputum sample on day 116 and from the air sample collected from room A by EDC during days 62–76 was the same (Figure 2). Because no respiratory samples were collected during days 62–116, concomitant *A. fumigatus* colonization of the patient was not documented. Subsequently, the same strain was isolated from the EDC in room A during days 104–118 and several times from the EDCs placed in room B after the patient was transferred (Figures 1, 2). In summary, the 6 *A. fumigatus* strains isolated from the patient's airway and the 8 air samples from the patient's room shared the same multilocus genotype. In contrast, 2 *A. fumigatus* strains isolated during routine monitoring of the ICU for airborne fungal contamination before the patient was admitted to the ICU (the first was isolated 7 weeks earlier in room A; the second, 3 weeks earlier in room B) had 0/9 and 1/9 alleles, respectively, in common with the strain from the patient. Airborne *A. fumigatus* contamination was not detectable in room B after the patient died, although no disinfection had been performed.

Conclusions

Continuous monitoring of airborne fungal contamination by using EDCs combined with multilocus variable-

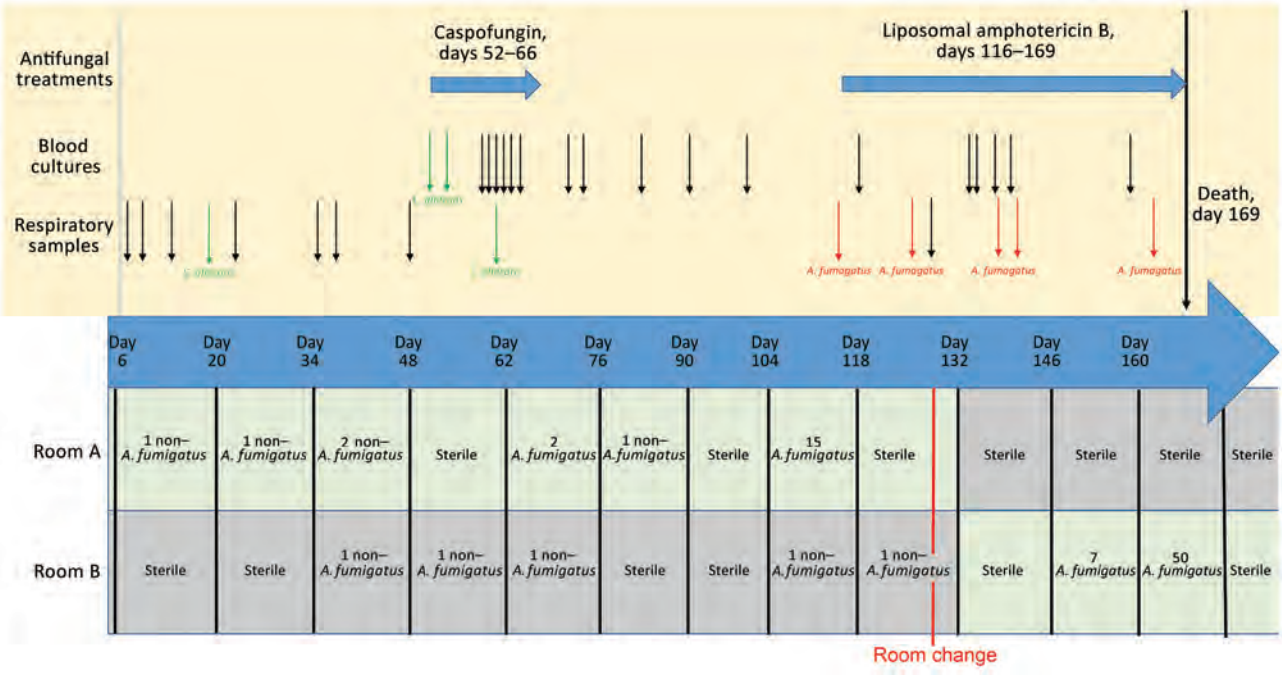

Figure 1. Timeline summarizing the antifungal treatments, patient blood and respiratory sample mycology culture results, and filamentous fungi culture results of the electrostatic dustfall collectors used for continuous monitoring of airborne fungal contamination in the intensive care unit rooms where *Aspergillus fumigatus* was found during hospital stay of colonized patient, France, 2015. *C. albicans*, *Candida albicans*.

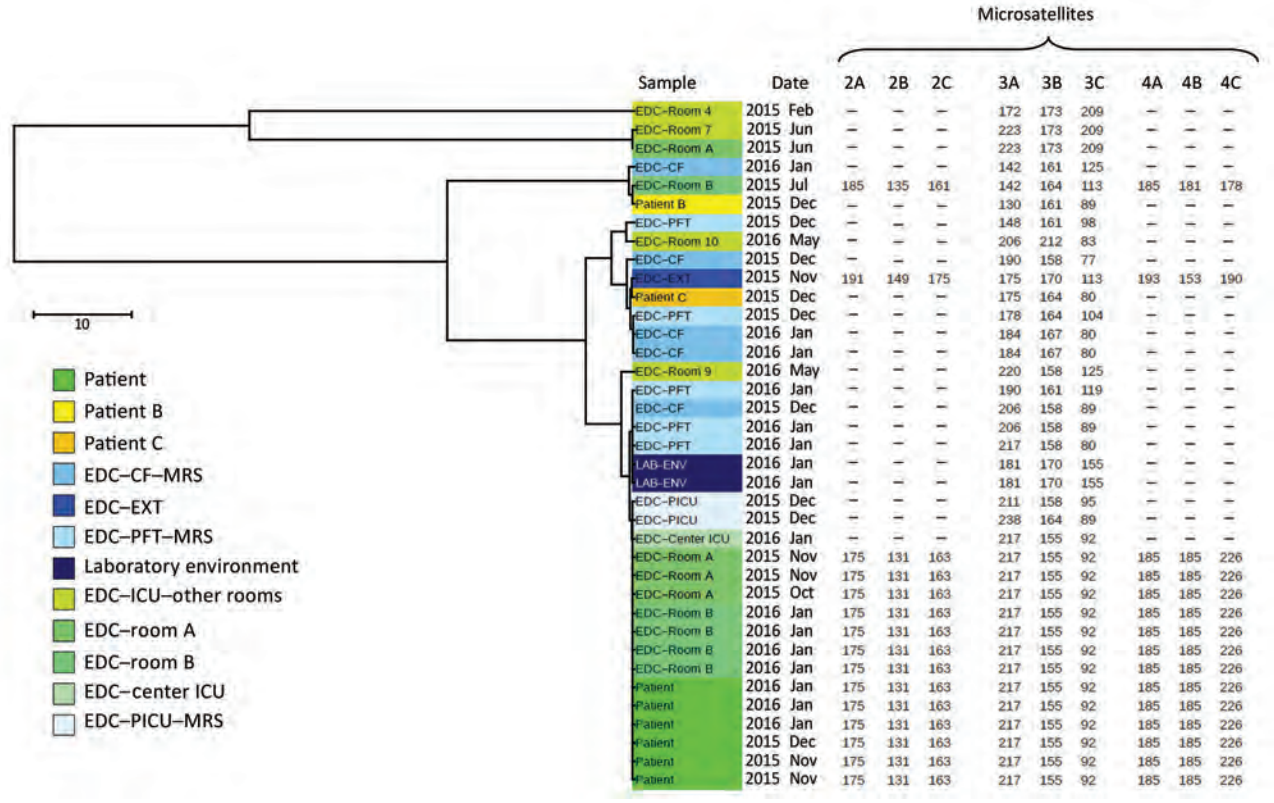

Figure 2. Multilocus variable-number tandem-repeat analysis genotyping results for *Aspergillus fumigatus* found during hospital stay of colonized patient, France, 2015. The distance tree was plotted by using iTOL version 4.2 (https://itol.embl.de/), taking into account the 3 microsatellite markers that were obtained from each *A. fumigatus* isolate sampled from the various study sites. The length-polymorphisms of 9 microsatellite markers were obtained for the 14 isolates sampled at the ICU during the patient's stay and 1 control. ICU room locations are detailed in the online Technical Appendix (https://wwwnc.cdc.gov/EID/article/24/8/17-1865-Techapp1.pdf). CF, cystic fibrosis center; EDC, electrostatic dustfall collector; EXT, a school in northeastern France; ICU, intensive care unit; MRS, Marseille; PFT, pulmonary function testing center; PICU, pediatric intensive care unit. Scale bar indicates nucleotide substitutions per site.

number tandem-repeat analysis genotyping traced the source of *A. fumigatus* contamination in this case. By using these 2 complementary approaches, we showed that the *A. fumigatus* strain had not been detected in room A before the patient's admittance. Although the patient might have been carrying this *A. fumigatus* strain before ICU admittance, it is more likely that it was hospital acquired because *A. fumigatus* was not detected in the first 9 respiratory samples tested. No respiratory samples were tested during the 6 weeks after the first positive EDC culture; thus, the time when the *A. fumigatus* colonization of the patient's airway became detectable could not be ascertained. Nevertheless, the same strain further colonized the patient's airway before he was transferred to room B, where the level of airborne contamination became highly significant; up to 50 CFU were cultured from 1 EDC in room B. Furthermore, in the absence of concomitant construction work or other known risk factors for hospital fungal contamination, we found increased contamination levels just before the patient died. Taken together, these observations support the hypothesis that the patient acquired the infection during his stay in room A and his airway then became the point source of airborne *Aspergillus* contamination of room B. The genotyping results further support the hypothesis that the patient's airway was the point source of airborne *Aspergillus* contamination of room B. Overall, these findings strongly argue that patients colonized with *Aspergillus* spp. should undergo treatment exclusively in airborne-infection isolation rooms, especially when patients at risk for aspergillosis are hospitalized in the same unit.

Acknowledgment

We thank Sandra Moore for English editing of the manuscript.

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Anncaliia algerae Microsporidial Myositis, New South Wales, Australia

Gaurav Sutrave, Adam Maundrell, Caitlin Keighley, Zoe Jennings, Susan Brammah, Min-Xia Wang, Roger Pamphlett, Cameron E. Webb, Damien Stark, Helen Englert, David Gottlieb, Ian Bilmon, Matthew R. Watts

We describe the successful management of *Anncaliia algerae* microsporidial myositis in a man with graft versus host disease after hemopoietic stem cell transplantation. We also summarize clinical presentation and management approaches and discuss the importance of research into the acquisition of this infection and strategies for prevention.

Anncaliia algerae is a microsporidian parasite that infects insects, including mosquitoes, and was first reported as a cause of fatal myositis in 2004 (*1*,*2*). Transmission occurs through contact with spores that are found in water, although the exact mechanism of transmission to humans is unknown (*2*). Myositis has been described in case-patients who were immunosuppressed because of rheumatoid arthritis, solid organ transplantation, and hematologic malignancy (*1*–*5*). It is currently unclear why 4 of the 6 previously published cases have originated in New South Wales, Australia, and the 2 other cases originated in North America (*1*–*5*). We document successful treatment of *A. algerae* infection after hemopoietic stem cell transplantation, provide an update on clinical features and management, and discuss possible routes of transmission and risk-mitigation strategies.

Case Report

A 66-year-old man sought care at a hospital, reporting a 5-week history of progressive myalgias, fatigue, and weakness. He also had a 3-week episode of nonbloody diarrhea that had resolved a week earlier. He reported no fevers, weight loss, dysphagia, or additional neurologic symptoms. He had chronic graft versus host disease (GVHD) with skin and pulmonary involvement treated with prednisone (25 mg/d orally), methotrexate (15 mg/wk orally), tacrolimus (1 mg 2×/d orally), and fluticasone/salmeterol (250 μg/50 μg 2×/d inhaled). GVHD occurred after a matched unrelated donor, allogeneic bone marrow transplant for acute myeloid leukemia. Before having acute myeloid leukemia, the patient received 6 cycles of combination chemotherapy (rituximab, cyclophosphamide, doxorubicin, vincristine, etoposide, and prednisone) to treat high-grade diffuse large B cell lymphoma.

The patient lived in a semirural area surrounded by woodland in the Blue Mountains, New South Wales, Australia. His residence had an aboveground molded-plastic rainwater tank that was fed from roof guttering through polyvinyl chloride piping, with an outlet over a mesh-covered opening in the tank cover. Water entering the tank passed through a 5–7-cm layer of decaying plant material and other debris. The tank was periodically used as a source of showering and drinking water.

On examination the patient was afebrile and had exquisite muscle tenderness and edema of the upper and lower limbs. Power was reduced in the upper and lower limb muscles (Medical Research Council grade 3–4 out of 5). Other neurologic findings were unremarkable.

Serum creatine kinase peaked at 858 U/L (reference range 55–150 U/L). On full blood count, hemoglobin was 126 g/L (reference range 130–180 g/L), and lymphocyte count was 0.9×10^9 cells/L (reference range $1.0–4.0 \times 10^9$ cells/L). C-reactive protein was 75 mg/L (reference range ≤3 mg/L), and erythrocyte sedimentation rate was 53 mm/hr (reference range 1–20 mm/hr). Alanine aminotransferase was 163 U/L and aspartate aminotransferase 235 U/L (reference range ≤40 U/L for both). Serum albumin nadir was 23 g/L (reference range 35–50 g/L). Serum creatinine, urinary albumin, and urinary protein levels were not elevated. Results of stool microscopy performed using Ryan's modified trichrome stain were negative for microsporidia.

Results of nerve conduction studies and electromyography were consistent with myopathy and axonal neuropathy. Magnetic resonance imaging of the lower limbs demonstrated myofascial edema. Light microscopy of a vastus

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DOI: https://doi.org/10.3201/eid2408.172002

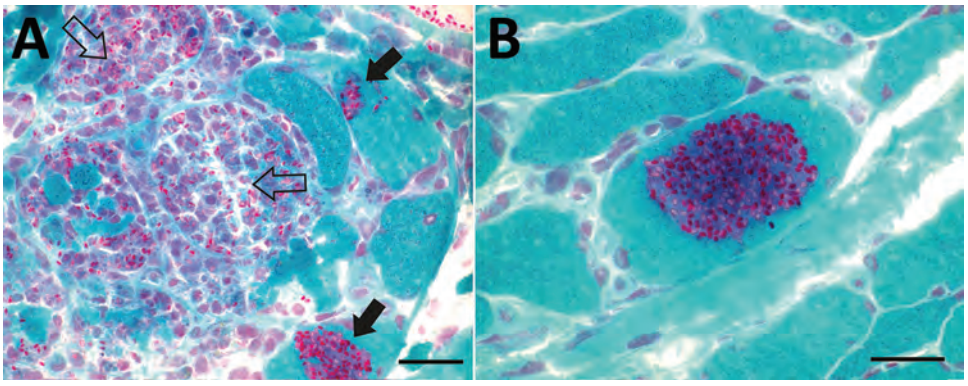

Figure 1. Light micrographs of Gomori trichrome–stained frozen sections of vastus lateralis muscle from a 66-year-old man with *Anncaliia algerae* microsporidial myositis, New South Wales, Australia. A) Necrotising myositis with red-stained, ovoid spores in green-staining viable myocytes (solid arrows) and within macrophages invading necrotic myocytes (open arrows). B) A cluster of red stained, 2–3-μm spores within a viable myocyte. Scale bars indicate 25 μm.

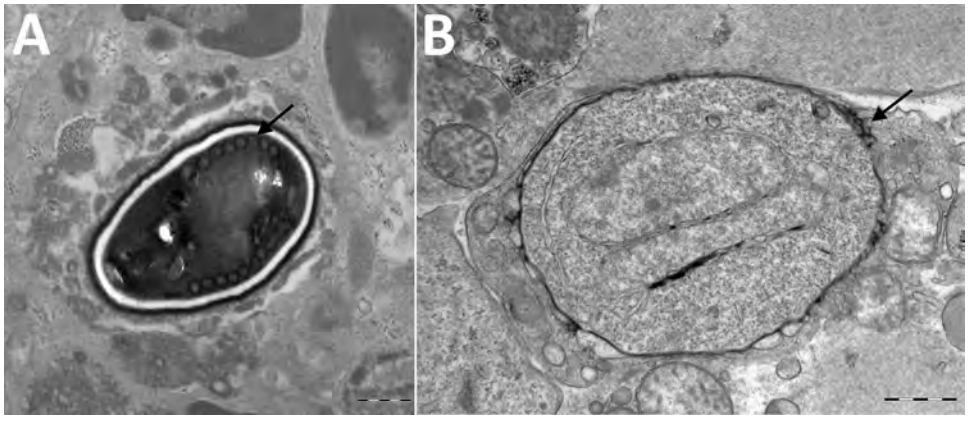

Figure 2. Transmission electron micrographs of vastus lateralis muscle from a 66 year-old man with *Anncaliia algerae* microsporidial myositis, New South Wales, Australia. A) Mature spore with 11 polar tubule coils (arrow) in a single row. Dense exospore and pale endospore. B) Binucleate, proliferative phase meront with characteristic vesicotubular appendages (arrow). Scale bars indicate 500 nm.

lateralis biopsy demonstrated ovoid organisms either free in the endomysium, within the myofiber sarcoplasm, or within macrophages in myofibers (Figure 1). Electron microscopy revealed microsporidia of the *Anncaliia* genus (Figure 2). We confirmed *A. algerae* by using PCR DNA amplification and sequence analysis.

The patient was started on albendazole (400 mg 2×/d orally) and cyclosporine (100 mg 2x/d orally); tacrolimus and methotrexate were ceased, and the prednisone dosage was reduced. Within 3 weeks, serum creatine kinase had normalized; muscle tenderness and peripheral edema had been reduced, and power increased. The patient had onset of limb contractures. Because of the ongoing immunosuppression required to manage GVHD, albendazole was continued for ≈9 months. Seven months after the patient's initial examination, a repeat muscle biopsy indicated no evidence of infection.

Discussion

Our review of published case reports and patient records indicated that systemic *A. algerae* infection has manifested as a skeletal muscle myositis (Table 1), with central nervous system and cardiac involvement documented in some cases (*1*–*5*). Dysphagia caused by bulbar muscle weakness is a particular concern because it has led to aspiration pneumonia (*2*). Limb contractures have not previously been described and, in the case of our patient, might have been related to GVHD.

Investigation findings in published case reports and patient records are summarized in Table 2 (*1*–*5*; Table 2). Muscle biopsies led to the diagnoses (*1*–*5*). Although Warthin-Starry and Gomori trichrome stains have been optimal for light microscopy, the spores can be confused with yeast cells because of their appearance (*2*,*3*; Figure 1). The features on transmission electron microscopy that allowed identification to the genus level include

Table 1. Clinical features of 7 case-patients with *Anncaliia algerae* microsporidial myositis from North America and New South Wales, Australia

Clinical feature	No. cases
Weakness	7
Muscle pain	7
Fever	6
Fatigue	6
Peripheral edema	6
Weight loss	5
Dysphagia	4
Glossitis	4
Diarrhea	4
Delirium	3
Congestive cardiac failure	1

*In 2 cases the clinical features were only sourced from published reports (*1*,*5*) rather than patient records (*2*–*4*).

Table 2. Serologic and laboratory test results for 7 case-patients with *Anncaliia algerae* microsporidial myositis from North America and New South Wales, Australia

Test	Abnormal result	No. cases
Serum creatine kinase	Elevated	7
Cardiac troponin	Elevated	2
Erythrocyte sedimentation rate and C-reactive protein	Elevated	5
Full blood count	Lymphocytopenia	6
Serum albumin	Decreased	5
Alanine aminotransferase and aspartate aminotransferase	Elevated	5
Serum creatinine	Elevated	2
Urinary protein	Elevated	3
Nerve conduction studies, electromyography	Myopathy, axonal neuropathy	6
Brain radiologic imaging	Cerebral lesions	2
Cardiac magnetic resonance imaging	Biventricular dysfunction	1
Small subunit rRNA gene PCR, muscle	*A. algerae* DNA	7
Small subunit rRNA gene PCR, cerebrospinal fluid	*A. algerae* DNA	1

*In 2 cases test results were only sourced from published reports (*1*,*5*) rather than patient records (*2*–*4*).

diplokaryotic nuclei, the absence of a parasitophorous vacuole, vesicotubular appendages, and 8–11 polar tubule coils (*1*–*6*; Figure 2). Species identification has been made with PCR amplification of the small subunit ribosomal RNA gene and sequence analysis by using DNA extracted from muscle and cerebrospinal fluid (*1*–*5*).

Successful management of *A. algerae* infection requires minimizing immunosuppression, avoiding complications such as aspiration pneumonia, and starting treatment based on albendazole (*2*). A β-tubulin sequence analysis and in vitro assays were consistent with *A. algerae* sensitivity to albendazole, although some viable spores remained in cell cultures after treatment (*7*). In a case of severe illness where substantial immunosuppression and treatment failure of albendazole monotherapy were factors, the addition of fumagillin was effective (*5*). The fumagillin, for which supplies were restricted, was obtained from the manufacturer in France through the Health Canada Special Access Program (*5*). Supply is also restricted in other jurisdictions, including the United States, where an Emergency Investigational New Drug application is required. In the case of the patient we describe, a management strategy was to change the calcineurin inhibitor from tacrolimus to cyclosporine, in light of in vitro evidence that cyclosporine chemosensitized *Encephalitozoon* spp. to the effect of albendazole (*8*).

A. algerae infects the aquatic stages of mosquitoes when larvae ingest the spores or hatch from contaminated eggs (*9*). Attempts to infect athymic mice by intravenous, oral, and intranasal routes were unsuccessful; however, direct injection of spores into the tail and feet led to infection of myocytes, neural tissue, connective tissue, and bone marrow (*10*). Ingestion, inhalation, and direct inoculation are also possible routes of human infection. A diarrheal illness before hospitalization might indicate a gastrointestinal source, but stool microscopy and gut biopsies have been negative (*2*–*4*). The 2 infected lung transplant recipients described in the literature might have been susceptible to inhaled infection (*3*,*4*). Infection through a mosquito bite is regarded as less likely because the organism has not been found in the saliva of feeding mosquitoes, and exposure to water substantially increased the rate of germination in spores from mosquito tissue (*10*,*11*). Previous patients have resided near sources of environmental water, such as golf courses and woodlands (*2*). The case-patient we describe lived adjacent to a eucalypt forest environment and drank and showered with water from a rainwater tank system that might have contained mosquito larvae or had inflow from water-filled roof gutters containing mosquito larvae. Immunocompromised persons are advised to seek medical guidance before the consumption of rainwater tank water, and until further information regarding transmission is available, other sources of untreated water should be also avoided (*12*).

Clinical case reports lead to a greater understanding about the epidemiology, pathogenesis, and management of *A. algerae* myositis. Considering the widespread use of immunosuppressive therapies and the need to minimize the risk for infection, other priorities for research include the environmental biology of this pathogen and clarification of the transmission route to humans.

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Dr. Sutrave is a hematologist with an interest in bone marrow transplantation. He is currently undertaking a PhD in evaluating adoptive cellular therapies for infections in immunocompromised patients and is working with the Cellular Therapies Group at the Westmead Institute for Medical Research, University of Sydney, Westmead, New South Wales, Australia.

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Outbreak of *Trichinella* T9 Infections Associated with Consumption of Bear Meat, Japan

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An outbreak of trichinellosis occurred in Japan in December 2016. All case-patients had eaten undercooked bear meat, from which *Trichinella* larvae were subsequently isolated. DNA sequencing analysis of the mitochondrial genes cytochrome *c*-oxidase subunit 1 and internal transcribed spacer 2 confirmed that *Trichinella* T9 had caused the outbreak.

Trichinellosis is a parasitic disease caused by the *Trichinella* spp. nematode that is contracted by eating raw or undercooked meat from infected animals. Approximately 100 species of animals, including humans, can be infected (*1*). The most common source of human trichinellosis is meat from pigs or wild boar. A total of 65,818 human cases were reported from 41 countries during 1986–2009 (*2*).

In Japan, trichinellosis is rarely encountered in the clinical setting, and only 5 imported cases (1 in 1998 [*3*], 1 in 1999 [*4*], 1 in 2003 [*5*], 2 in 2009 [*6*]) have been reported during the past few decades. Three outbreaks of domestically acquired trichinellosis have been reported since 1975 (*7*), the last reported outbreak occurring in 1981; all were associated with bear meat consumption, but the etiologic agents were not identified at the species level. Since then, no outbreaks were reported until late 2016.

The Study

In December 2016, a previously healthy young man was referred to Tsukuba Medical Center Hospital (Tsukuba, Ibaraki Prefecture, Japan) for a fever, rash, malaise, and eosinophilia. He claimed that he had eaten a bear meat dish (Figure 1) at a restaurant in Mito, Ibaraki Prefecture, Japan, with his 4 friends, who all had similar signs and symptoms. Subsequently, a total of 32 patients who had consumed the bear meat were reported to the Ibaraki Prefecture Mito Health Center; 28 patients had been evaluated at hospitals. Ethics approval for this research was obtained from the Institutional Review Board of Mito Kyodo General Hospital, University of Tsukuba, Mito, Japan (No. 16-69). All patients provided informed consent for their data to be included in this study.

Among the 28 patients who underwent evaluation, 21 had signs and symptoms that were compatible with trichinellosis. Each serum sample obtained from the 28 patients was tested 3 times for antibodies to *Trichinella* spp., as previously described (*6*). We performed antibody titer testing with ELISA using excretory–secretory (ES) antigens from *Trichinella spiralis* at the patient's initial presentation and ≥2 weeks after the first serum samples were obtained (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/17-2117-Techapp1.pdf). We defined a confirmed case as illness in a patient with a history of consuming raw bear meat, clinical symptoms compatible with trichinellosis, and serologic evidence of trichinellosis. A probable case was defined as illness in a patient with a history of consuming raw bear meat, clinical symptoms compatible with trichinellosis, and a negative serologic test result (*8*).

In total, 19 (90.4%) patients, all symptomatic, had an antibody titer higher than the cutoff (Table 1); 2 symptomatic patients had an antibody titer lower than the cutoff (titer <200 on convalescent serologic evaluations). All 7 asymptomatic patients had negative serologic test results. Consequently, we identified 21 trichinellosis patients in our study, representing 19 confirmed and 2 probable cases.

We compiled and assessed demographic and clinical data on the 21 patients with confirmed and probable trichinellosis (Table 2). Median age was 35 years (range 23–58 years); 10 (48%) patients were female and 11 (52%) male. Thirteen patients (62%) had consumed ≥3 slices of infected bear meat (≈10 g per slice). The median incubation period was 19 days (range 6–34 days). All patients had a rash (Figure 2), 20 (95%) had a fever, 17 (81%) had myalgia, 10 (48%) had facial edema, and 9 (43%) had peripheral edema. Only 5 (24%) patients had diarrhea (range of onset day 1–16 of illness) during the outbreak. Nine (43%) patients had conjunctivitis, and 2 (10%) had uveitis.

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DOI: https://doi.org/10.3201/eid2408.172117

Figure 1. The bear meat dish implicated in an outbreak of *Trichinella* T9 infection, Japan, December 2016. Bear meat slices are marked with a circle and an arrow.

At the time of initial evaluation, the median eosinophil count was 1.0 × 10^9/L (range 0.1 × 10^9/L to 4.3 × 10^9/L), and the median creatine kinase level was 147 IU/L (range 57–786 IU/L). All patients were treated with albendazole (200 mg or 400 mg, 2×/d for 10–14 days), with or without prednisolone. In 1 case, albendazole was changed to mebendazole because of a mild increase in the patient's aspartate aminotransferase and alanine aminotransferase levels, which was later considered to have occurred because of trichinellosis itself. None of the patients had

Table 1. Serologic test results for 28 patients who consumed bear meat associated with *Trichinella* T9 infection, Japan, December 2016*

Patient no.	Signs and symptoms	Highest blood eosinophil count, cells/L	Initial serologic test titer	No. days postinfection	Convalescent-phase serologic test titer	No. days after first blood collection
1	Yes	7.1 × 10^9	<200	23	6,400	24
2	Yes	4.3 × 10^9	<200	23	12,800	19
3	Yes	2.7 × 10^9	<200	27	1,600	20
4	Yes	10.1 × 10^9	<200	23	800	19
5	Yes	7.8 × 10^9	800	24	3,200	17
6	Yes	8.8 × 10^9	<200	25	1,600	17
7	Yes	3.1 × 10^9	<200	25	1,600	17
8	Yes	1.9 × 10^9	800	25	6,400	18
9	Yes	11.1 × 10^9	<200	25	3,200	18
10	Yes	2.4 × 10^9	<200	24	3,200	15
11	Yes	5.0 × 10^9	400	12	3,200	16
12	Yes	4.2 × 10^9	<200	13	800	16
13	Yes	8.5 × 10^9	200	22	6,400	14
14	Yes	5.3 × 10^9	<200	20	800	15
15	Yes	10.8 × 10^9	200	20	3,200	14
16	Yes	2.9 × 10^9	<200	23	<200	24
17	Yes	4.3 × 10^9	<200	21	<200	16
18	Yes	1.9 × 10^9	<200	20	400	13
19	No	0.1 × 10^9	<200	22	<200	29
20	No	0.2 × 10^9	<200	18	<200	15
21	No	0.1 × 10^9	<200	19	<200	28
22	No	0.1 × 10^9	<200	27	<200	24
23	Yes	2.3 × 10^9	<200	23	6,400	15
24	Yes	2.9 × 10^9	<200	23	400	16
25	No	0.4 × 10^9	<200	25	<200	31
26	No	0.2 × 10^9	<200	21	<200	14
27	No	0.1 × 10^9	<200	11	<200	29
28	Yes	2.0 × 10^9	<200	27	400	13

*ELISA was performed to detect *Trichinella* antigens. The cutoff point (0.148) was 3 times the mean value of A_{414} from the negative serum sample of 100 healthy persons. Of the 28 patients evaluated, 21 had signs and symptoms compatible with trichinellosis. Patients 1–15 also had elevated antibody titers; however, the antibody titers of patients 16 and 17 were not elevated. These cases were defined as probable trichinellosis, as previously described (*8*).

Table 2. Epidemiologic, clinical, and laboratory data for 21 symptomatic patients with probable or confirmed *Trichinella* T9 infection, Japan, December 2016*

Characteristic	Value
Median age, y (range)	35 (23–58)
Sex	
F	10 (48)
M	11 (52)
Consumed ≥3 slices of infected bear meat	13 (62)
Median incubation period, d (range)	19 (6–34)
Signs and symptoms	
Fever	20 (95)
Rash	21 (100)
Myalgia	17 (81)
Fatigue	9 (43)
Facial edema	10 (48)
Peripheral edema	9 (43)
Diarrhea	5 (24)
Conjunctivitis	9 (43)
Uveitis	2 (10)
Median duration from date of eating bear meat to date of blood sampling, d (range)	23 (12–27)
Median leukocyte count, cells/L (range)	7.2×10^9 (3.9×10^9 to 16.9×10^9)
Median eosinophil count, cells/L (range)	1.0×10^9 (0.1×10^9 to 4.3×10^9)
Median aspartate aminotransferase level, IU/L (range)	24 (12–41)
Median alanine aminotransferase level, IU/L (range)	22 (9–73)
Median creatine kinase level, IU/L (range)	147 (57–786)
Median C-reactive protein level, mg/L (range)	9.4 (0.4–67.5)

*Laboratory data were obtained at initial presentation. Values are no. (%) patients except as indicated.

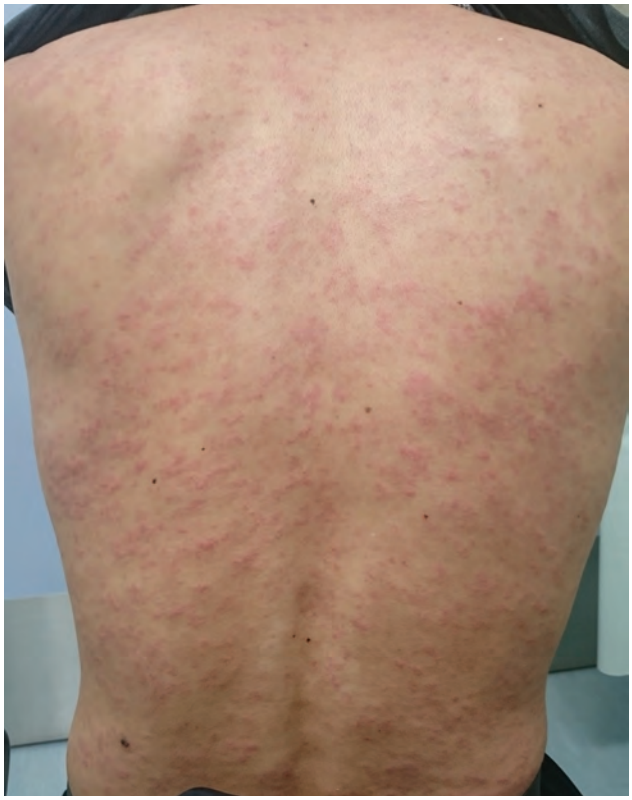

Figure 2. Rash on the back of a patient (patient 10 in Table 1) with confirmed *Trichinella* T9 infection associated with consumption of bear meat, Japan, December 2016. Patient had onset of macular and papular, confluent, and pruritic rash with diffuse blanching on the scalp, face, chest, abdomen, back, and upper and lower extremities. Photo taken 24 days after the patient had consumed the implicated bear meat.

serious complications of trichinellosis or major adverse events during treatment.

The bear meat came from a brown bear (*Ursus arctos*) that had been hunted in Hokkaido Prefecture in November 2016. The meat had been divided into 3 blocks that were preserved in cold storage. Two of these blocks were eaten during this outbreak. The first bear meat block was brought into a restaurant in Mito. In late November, it was seared and served in thin slices with herbs (Figure 1; online Technical Appendix Figure). This bear meat was kept in cold storage and served for 2 days, after which it was preserved in a freezer. The temperature of the cold storage and the freezer were not recorded. Japan Industrial Standard (JIS B 8630) defines the temperature of refrigerated storage as not below 0°C and freezing as ≤–20°C. The bear meat was served after being reheated for a few minutes. The second bear meat block was cooked steak-style to a medium-rare condition; 1 of the patients had eaten meat from this block (online Technical Appendix Figure). The third bear meat block was stored in a freezer without being consumed. We used this meat for the analysis of *Trichinella* spp., which was performed at the National Institute of Infectious Diseases (Tokyo, Japan).

We artificially digested the bear meat with 0.5% pepsin-0.8% HCl solution and then performed a microscopic examination on the sediment. We detected encapsulated larvae with a distinctive esophageal structure (stichosome). The density of the larvae was 84 larvae/g. For the molecular identification of the larvae, we amplified cytochrome *c*-oxidase subunit 1 (*cox1*) and internal transcribed spacer 2 (ITS2) by using PCR with primer pairs described by Kanai

et al. (*9*). A subsequent sequence analysis showed that both sequences (GenBank accession nos. LS361217 for *cox1* and LS361216 for ITS2) were identical to the corresponding sequences of *Trichinella* T9 (GenBank accession nos. KM357420 for *cox1* and AB255886 for ITS2).

Nine species (*T. spiralis, T. britovi, T. nativa, T. nelsoni, T. murrelli, T. zimbabwensis, T. papuae, T. pseudospiralis, T. patagoniensis*) and 3 unclassified genotypes (T6, T8, and T9) are currently recognized in the genus *Trichinella* (*10*). Among them, *T. spiralis* is the most common species in the world (*11*). The taxonomic status of *Trichinella* species in Japan has not yet been fully elucidated. A recent molecular study revealed that the *Trichinella* isolates obtained from animal specimens in Japan included *Trichinella* T9 (*12*) and *T. nativa* (*13*), but *T. spiralis* has not yet been found in Japan (*9*). *Trichinella* T9 has only been reported in Japan. Therefore, it is considered to be native to Japan. *Trichinella* T9 has been detected and confirmed in a brown bear (*13*), raccoons (*14*), raccoon dogs (*13*,*14*), and red foxes (*13*), but no cases of human infection have been reported.

In this outbreak, 2 symptomatic patients and 7 asymptomatic patients had negative serologic test results. A second blood specimen was collected from these patients 33–56 days after the consumption of the bear meat. According to the pertinent literature data (*15*), serum conversion has been observed up to 65 days postinfection. Thus, we need to consider the possibility of a delay in serum conversion for these 9 patients.

Conclusions

We describe an outbreak of trichinellosis that occurred because of the consumption of bear meat infected with *Trichinella* T9. Public awareness should be raised and education should be promoted to prevent further outbreaks of trichinellosis in Japan.

Acknowledgments

We thank Kenji Miyakawa for making the initial clinical diagnosis of trichinellosis and Tomoe Yukawa for diagnosing and managing the patients with uveitis of trichinellosis.

This work was funded in part by a grant to Y.M. from the Japan Agency for Medical Research and Development (grant no. 17fk0108209j0602).

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Variation in Influenza B Virus Epidemiology by Lineage, China

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We used national sentinel surveillance data in China for 2005–2016 to examine the lineage-specific epidemiology of influenza B. Influenza B viruses circulated every year with relatively lower activity than influenza A. B/Yamagata was more frequently detected in adults than in children.

Influenza B virus, first identified in 1940 (*1*), is associated with considerable hospital admissions and deaths worldwide every year (*2*). During the early 1980s, influenza B viruses split into 2 lineages, termed B/Victoria and B/Yamagata (*3*). These 2 lineages showed distinct antigenicity and transmission dynamics (*4*) and have co-circulated during each influenza season since 2001 (*2*). Relatively less attention has been given to influenza B virus epidemiology than to influenza A epidemiology (*2*) because influenza B virus spreads almost exclusively in humans and does not pose a pandemic threat (*5*).

Several recent reports have highlighted potential differences in the epidemiology of B/Victoria and B/Yamagata lineage viruses, including younger average ages of persons with B/Victoria virus infection (*4*,*6*,*7*) and greater transmissibility of B/Victoria viruses (*4*,*6*). Our study aimed to describe epidemiologic patterns of influenza B virus activity in China and to identify and compare the seasonality and age distribution of persons with medically attended influenza B/Victoria and B/Yamagata virus infections.

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DOI: https://doi.org/10.3201/eid2408.180063

The Study

The Chinese Center for Disease Control and Prevention coordinated influenza surveillance in sentinel clinics during October 2005–March 2016. Sentinel hospitals in provinces in southern China conducted year-round surveillance; in northern China (except for Liaoning, Gansu, and Tianjin provinces, where year-round surveillance was conducted), surveillance was suspended from April to September before 2009 because influenza has low activity in summer in these temperate areas of China (*8*). Sentinel surveillance was then expanded from 193 to 554 hospitals conducting year-round surveillance in all provinces since 2009. Sentinel hospitals reported the number of outpatients and the number of outpatients with influenza-like illness symptoms on a daily basis. Respiratory specimens collected from a subset of outpatients with influenza-like illness were tested for influenza viruses. Each sentinel hospital in northern China was required to collect 10–15 samples per week during October–March and 5–15 samples per month during April–September for virus testing, and the hospitals in southern China tested 5–15 samples per week throughout the year. Most laboratories had adopted real-time PCR for lineage identification since 2009; some laboratories still use virus culture followed by hemagglutination inhibition test (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/18-0063-Techapp1.pdf). Individual data on age, sex, and date of specimen collection also were reported for all selected patients for virus testing (online Technical Appendix). Because national influenza sentinel surveillance was part of a routine public health investigation, the study was exempt from institutional review board assessment, and all data were delinked from identifiable personal information.

We used a proxy measure of influenza activity in the communities served by the sentinel locations because it was previously indicated to be a good correlate of the incidence rates of influenza virus infection in the community (*9*). The proxy was calculated as the product of the weekly rates for influenza-like illness consultation and the proportion of sentinel specimens testing positive for each lineage in the same week. The age-specific proportions of sentinel

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specimens testing positive for influenza B virus by lineage were derived as the proportion of sentinel specimens testing positive for each lineage (numerator) among the outpatients recruited for specimen collection (denominator) by exact year of age.

We found that influenza B/Victoria and B/Yamagata lineages circulated every year in mainland China during 2005–2016 and were mostly active during the winter–spring seasons (Figure 1). Influenza B virus activity was generally less intense than influenza A activity and less apparent during the 2005–06, 2010–11, and, particularly, 2012–13 seasons (Figure 1; online Technical Appendix Figure 1).

Influenza B/Victoria activity increased in every season before and during the first wave of infections with influenza A(H1N1)pdm09 virus in China in late 2009, whereas substantial virus detections were only seen in the early 2011–12 and 2015–16 seasons during the postpandemic period. B/Yamagata lineage led to 3 major epidemics during the 2007–08, 2013–14, and 2014–15 seasons (online Technical Appendix Figure 1). These major epidemics were associated with prolonged influenza activity, particularly during summer periods and in provinces and municipalities with lower latitude, which occurred during 2008–2011 for B/Victoria lineage and during the 2007–08 and 2014–15 seasons for B/Yamagata lineage (Figure 1).

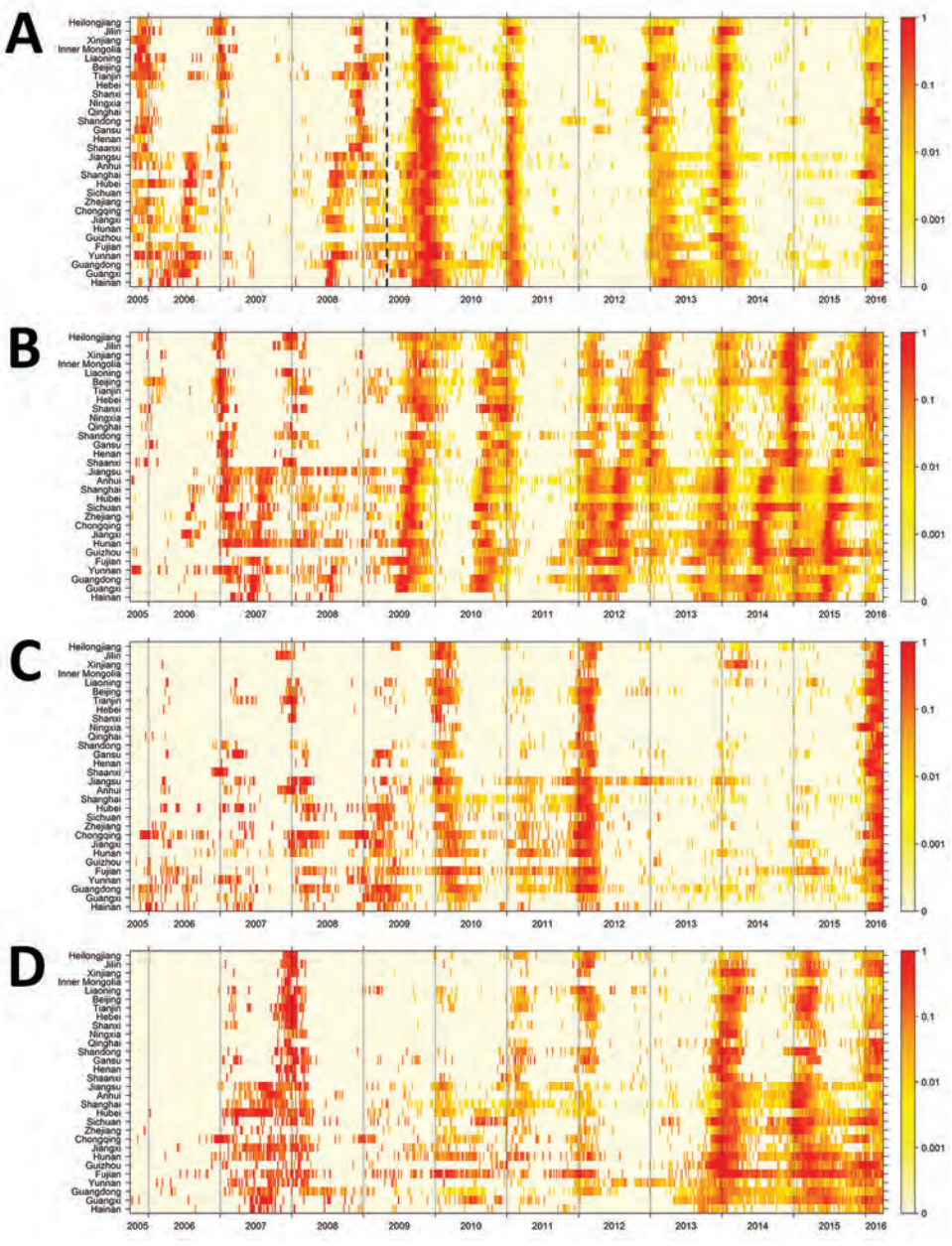

Figure 1. Heatmap of influenza virus activity by lineage in 30 provinces and municipalities (sorted by latitude), China, October 2005–March 2016. A) Influenza A(H1N1); B) influenza A(H3N2); C) influenza B/Victoria lineage; D) influenza B/Yamagata lineage. Map is based on 2,498,735 specimens collected from the sentinel hospitals. Normalized virus activity is shown for each province and municipality as the product of the weekly proportion of influenza-like illness consultations and the weekly proportion of sentinel specimens testing positive for influenza viruses divided by the maximum virus activity in the province or municipality throughout the study period to give a rescaled proxy with values between 0 (no activity) and 1 (highest activity in that province). The dashed line in panel A indicates the start of the H1N1 pandemic in 2009.

Children 5–15 years of age had the highest detection rates among all age groups for both lineages. The rates of detection of B/Victoria lineage viruses decreased with age after peaking at 10 years of age, and the rates of B/Yamagata lineage virus infections generally increased among persons >25 years of age to a second peak in older adults (Figure 2, panel A). The patterns differed somewhat across provinces and municipalities without systematic variation by latitude (online Technical Appendix Figure 2). In comparison, influenza A(H1N1) showed an age pattern similar to that for B/Victoria but with a later peak, at 10–20 years of age; however, influenza A(H3N2) indicated largely comparable virus detections across different age groups (Figure 2, panel B).

Conclusions

Our study showed that influenza B virus generally was relatively less active than influenza A virus (Figure 1; online Technical Appendix Figure 1). Influenza B/Yamagata caused fewer epidemics than B/Victoria during the study period, largely consistent with findings from a study using sentinel surveillance data from multiple countries (*10*). The alternating predominance of the B/Victoria and B/Yamagata lineages, especially after 2009, and the low influenza B virus activity in China during the 2012–13 season might reflect the complex interactions between population immunity and virus evolution of influenza B lineages (*11*).

Our study suggested a potential difference in the age patterns of persons infected with B/Yamagata and

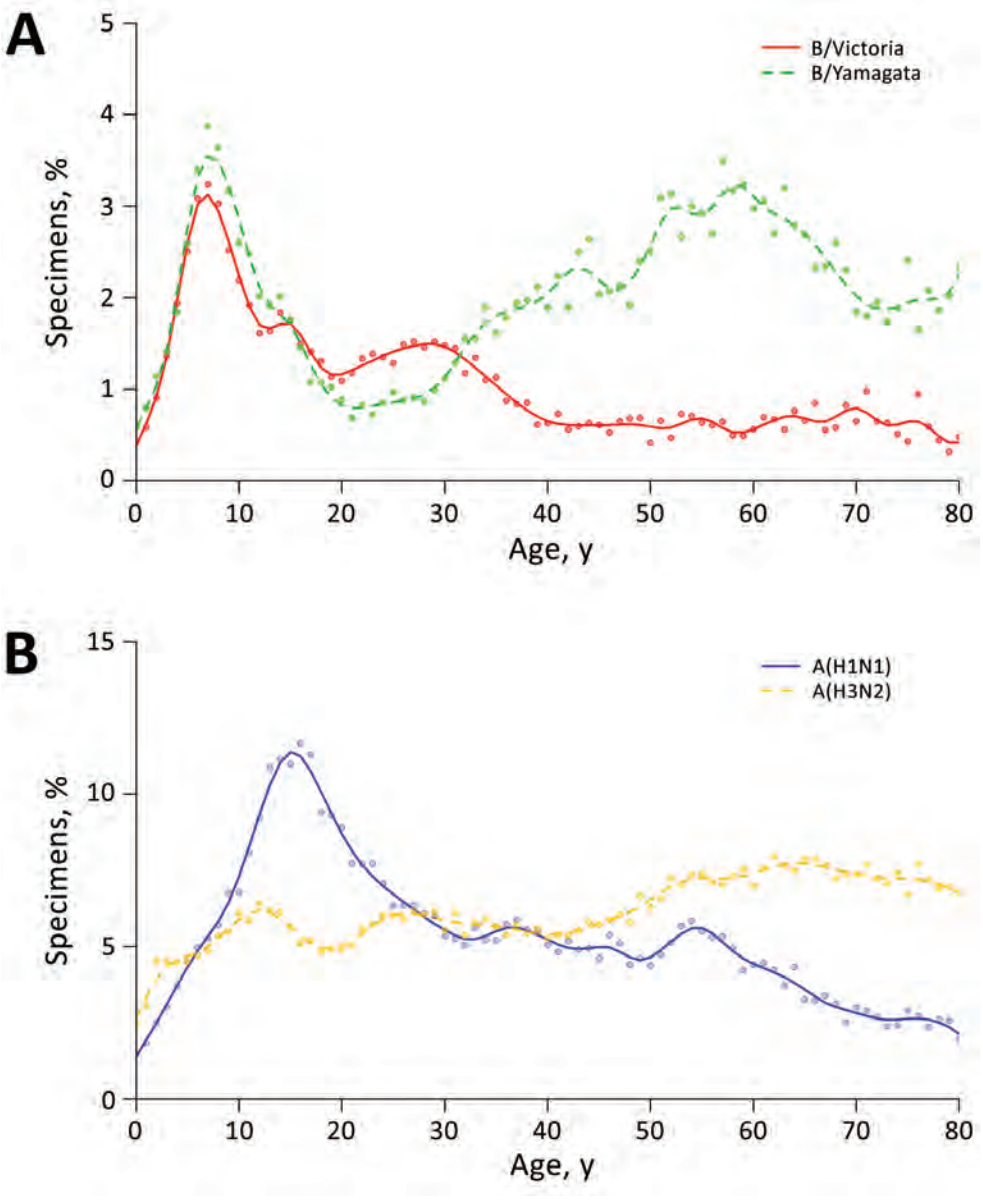

Figure 2. Age-specific proportions of sentinel specimens collected from sentinel surveillance sites testing positive for influenza, China, October 2005–March 2016. A) Influenza B/Victoria and B/Yamagata lineages; B) influenza A(H1N1) and A(H3N2). Findings are based on 2,498,735 specimens collected from the sentinel hospitals. Dots indicate the original data, and lines (solid and dashed) show the estimation from a fitted smoothing function to the pattern by age.

B/Victoria (Figure 2). The elevated proportions of infections with both lineages in children might indicate a lack of exposure to the virus early in life (*4*,*12*). However, the discrepancy in susceptibility to infections with B/Victoria and B/Yamagata in older adults might reflect the genetic difference in viruses of the 2 lineages, although previous exposure to different lineages and vaccination history might have had an effect. Antigenic analysis indicated that circulating B/Yamagata strains in general showed a larger genetic diversity than B/Victoria strains (*4*). This genetic diversity may lead to a substantial number of persons infected with a certain strain of B/Yamagata virus who are susceptible to the other co-circulating strains of the same lineage. The declining frequency of B/Victoria detections with age, however, implied a gradually strengthened immunity in older persons, which could be attributed to accumulated immunity from exposure to virus strains with fewer genetic changes or possibly to the boosted heterologous immunity against B/Victoria viruses induced by exposure to B/Yamagata viruses (*13*).

The study has several limitations. First, expansion of the national sentinel surveillance system in China since 2009 might have affected the observed patterns in virus activity because of inclusion of sentinel clinics providing healthcare services specifically to certain populations, such as patients in respiratory or pediatric outpatient clinics, although we weighted virus activity by age in the analysis. Second, the wider application of PCR in national surveillance laboratories might have led to an artificial increase in virus activity; however, we assumed that this change would not differ between the 2 lineages.

Further work could examine the degree of cross-protection conferred by infections of the opposite lineage, if any (*13*,*14*). Results from such studies would further elucidate the epidemiology of influenza B virus and optimize vaccination strategies in China.

Acknowledgments

We thank the staff members at the hospitals; local health departments; and county-, district-, prefecture-, and provincial-level centers for disease control and prevention for their valuable assistance in coordinating data collection.

This study was funded by the National Science Fund for Distinguished Young Scholars (grant no. 81525023), the US National Institutes of Health (Comprehensive International Program for Research on AIDS grant U19 AI51915), Chinese Center for Disease Control and Prevention's Key Laboratory of Surveillance and Early Warning on Infectious Disease, the Harvard Center for Communicable Disease Dynamics from the National Institute of General Medical Sciences (grant no. U54 GM088558), the Research Grants Council of the Hong Kong Special Administrative Region, China (project no. T11-705/14N), the National Natural Science Foundation of China (grant no. 81402731), and the Natural Science Foundation of Shanghai (grant no. 14ZR1444500).

Y.H. has received investigator-initiated research funding from Sanofi Pasteur, GlaxoSmithKline, bioMérieux Diagnostic Product (Shanghai), and Yichang HEC Changjiang Pharmaceutical Company. B.J.C. has received research funding from Sanofi Pasteur and consults for Crucell NV.

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Invasive Colonic Entamoebiasis in Wild Cane Toads, Australia

Cathy M. Shilton, Jan Šlapeta, Richard Shine, Gregory P. Brown

We detected a disease syndrome in free-ranging Australian cane toads involving atypical behavior and emaciation that is associated with a previously undescribed *Entamoeba* sp. that infiltrates the colonic lining, causing it to slough. The organism may become seasonally pathogenic when toads are under hydric and nutritional stress.

The emergence of new diseases in wildlife substantially threatens global biodiversity in many taxa (*1*), but amphibians face unusually high risk for pathogen-mediated population declines (*2*,*3*). Disease outbreaks among invasive amphibians are of particular concern because the invader may imperil native fauna by transmitting new pathogens (*1*). We documented severe (lethal) colitis of wild cane toads (*Rhinella marina*) in Australia associated with *Entamoeba* spp.

Cane toads were introduced to eastern Australia in 1935 and have now spread 2,000 km westward across the continent. The disease outbreak was observed at the University of Sydney Tropical Ecology Research Facility (TERF), in Australia's Northern Territory. The area experiences a wet–dry tropical climate, with high temperatures year-round but with rainfall limited to a 6-month wet season (November–May). Cane toads reached TERF in 2005, and the disease outbreak occurred 9 years later.

The Study

In August 2014, we noticed dead and moribund toads around the grounds of TERF. In daylight, emaciated toads were found sitting in puddles of water formed under the building's air conditioners. These diurnal observations were unprecedented; toads at this site were normally nocturnal and seen hydrating only in this manner at night. In addition, on several mornings, we observed moribund toads on open areas of lawn, fully exposed to sunlight and apparently too weak to seek refuge. During September and October 2014, we euthanized and necropsied 22 toads found hydrating or otherwise diurnally active near the TERF buildings. For comparative purposes we also necropsied 2 other groups of toads: 7 collected during November 2014 from a lagoon 30 km from TERF and 8 collected during February 2015 from the TERF grounds (Table 1, https://wwwnc.cdc.gov/EID/article/24/8/18-0101-T1.htm).

We detected invasive amebiasis by histologic analysis in all 3 groups, but disease was most prevalent and intense in the dry-season TERF toads (Table 1; online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/18-0101-Techapp1.pdf). The most severe cases were detected in toads in poor body condition with overt illness (online Technical Appendix). Gross pathologic findings ranged from no obvious lesions in mildly affected toads to thickened colonic walls with hyperemic serosal vasculature and hemorrhagic content in severely affected toads (Figure 1, panel A). Histologically appreciable lesions (invasive amebiasis) were commonly limited to the colon, although in severely affected toads, lesions extended through the small intestine and, rarely, into the stomach. The intestinal mucosal epithelium was variably hyperplastic, showing moderate to marked lymphoplasmacytic infiltration, to eroded or deeply ulcerated, showing associated granulocyte and macrophage infiltration. Organisms consistent in morphology with *Entamoeba* spp. were among mucosal epithelial cells, often near the basement membrane and rarely within the lamina propria (Figure 1, panel B; online Technical Appendix) and not present in other organs.

We applied environmental DNA sequencing to identify the community of eukaryotes (diversity profile) within the colons of 8 infected and 10 uninfected animals based on histopathologic investigation. From the 18 colon scrapings, we obtained 1,365,109 eukaryotic V1–V3 small subunit (SSU)–rDNA high-quality Illumina MiSeq (Illumina, San Diego, CA, USA) reads clustered into operational taxonomic units (OTU). Three OTUs demonstrated perfect or high-percentage identity with SSU rDNA sequences of the amebae in the genus *Entamoeba*: *E. ranarum* (OTU_16) and 2 new cryptic species (OTU_12 and OTU_119 [Figure 2]). Using SSU-rDNA *Entamoeba* species–specific primers, we confirmed the presence of *E. ranarum* (OTU_16) and *Entamoeba* sp. CT1 (OTU_12) (GenBank accession nos. MG714920–MG714921). The new *Entamoeba* sp. CT1 (OTU_12) was significantly more abundant in toads with histologically diagnosed invasive amebiasis ($t = 2.2$, d.f. = 16, $p = 0.04$; Table 2, https://wwwnc.cdc.gov/EID/

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DOI: https://doi.org/10.3201/eid2408.180101

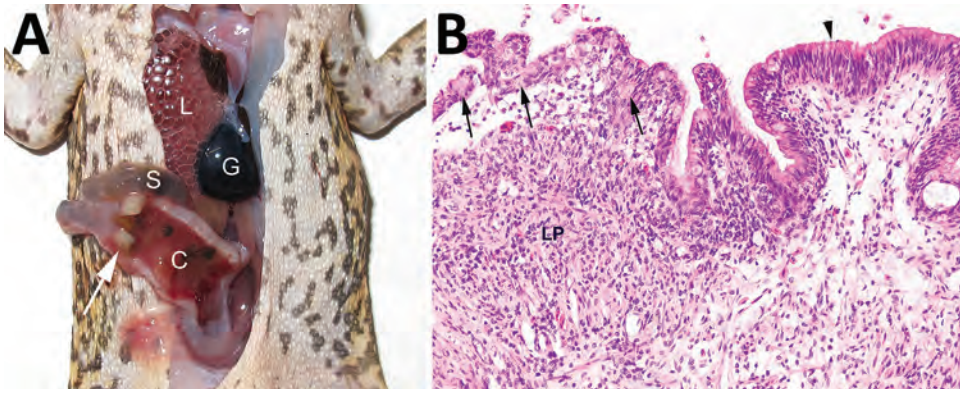

Figure 1. Invasive colonic entamoebiasis in wild cane toads (*Rhinella marina*), tropical Australia, 2014–2015. A) Toad with severe colonic amebiasis. The colon (C) has been opened to show intraluminal hemorrhagic content and blood clots. There is segmental full-thickness necrosis of the colon wall (white arrow). Lung (L), small intestine (S), and gall bladder (G) are annotated for perspective. B) Photomicrograph of colonic amebiasis. The affected segment of mucosal epithelium, which contains several amebae (arrows) is jumbled and sloughing from the underlying lamina propria (LP). Relatively normal colonic epithelium is present at right (arrowhead). There is lymphohistiocytic and granulocytic infiltration of the lamina propria underlying the affected epithelium. Hematoxylin and eosin stain. Original magnification ×200.

article/24/8/18-0101-T2.htm) and significantly more abundant in toads with more severe colonic lesions ($F_{1,16} = 7.0$, $p = 0.017$). OTU_12 was also detected at low levels in clinically healthy toads without histologic evidence of invasive disease from the site 30 km away from TERF (Table 1). *Entamoeba ranarum* (OTU_16) was no more prevalent or abundant in diseased toads than in healthy conspecifics, suggesting that OTU_12 (rather than *E. ranarum*) is the causative agent of the colitis.

Although biologists had monitored toads at the site since 2005, no unusual mortality was observed until 2014. The disease outbreak involved conspicuous behavior, severe clinical disease, and high mortality. Populations of invasive species (including Australian cane toads) often collapse after establishment, but the causes usually are unclear (*4*). An investigation into declines of Australian cane toad populations (*5*) posited an unknown microbial disease as a possible cause. Plausibly, OTU_12 could be that unknown pathogen. It might have remained undetected until now because rapid postmortem decomposition of the colon lining obscures lesions. Euthanizing toads in the final stages of the disease and immediately fixing their tissue enabled us to detect the lesions histologically.

Conclusions

To our knowledge, the only published description of pathology associated with amebic infection in amphibians is a case of renal disease in a single captive cane toad (*6*).

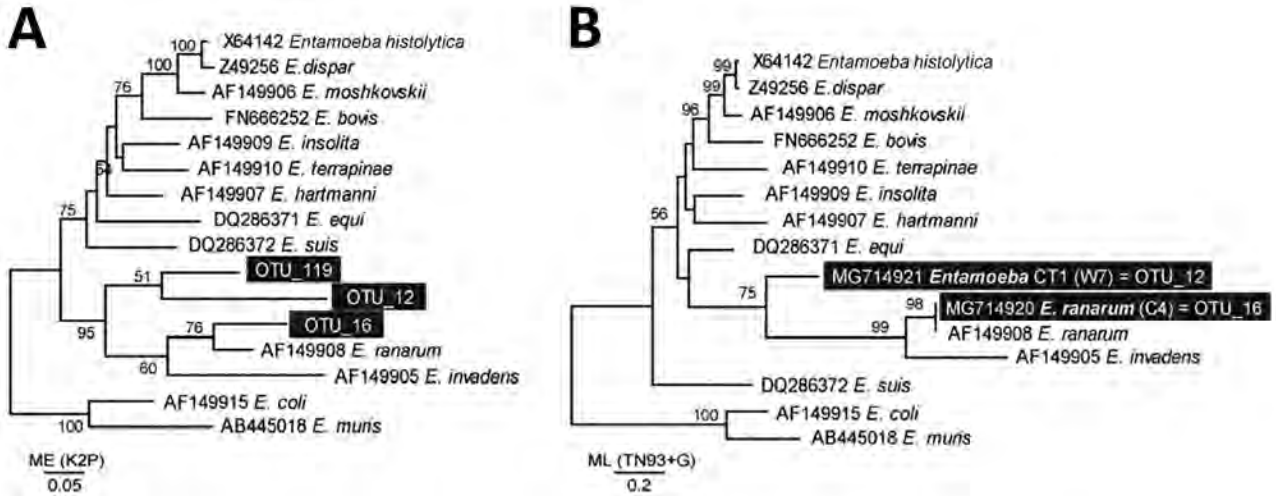

Figure 2. Phylogenetic inference of cane toad (*Rhinella marina*) *Entamoeba* SSU-rDNA sequences. *Entamoeba* SSU-rDNA sequences obtained using environmental next-generation amplicon sequencing (A) and conventional amplification using *Entamoeba*-specific primers (B) were aligned with available representative SSU-rDNA sequences. Each sequence is accompanied by GenBank accession number and *Entamoeba* species name. New sequences are in black boxes. Bootstrap support values (500 replicates) are shown next to the branches. The evolutionary distances were computed using the maximum-likelihood method and are in the units of number of base substitutions per site (scale bars). New sequences are representative of the OTU contigs (A) or are sequences directly from PCR amplicon (B). OTU, operational taxonomic unit; SSU, small subunit.

Although a recent survey of cane toads in Puerto Rico recorded 2 animals with histologic evidence of amebic enteritis (*7*), extensive surveys of intestinal protozoa in Australian toads did not detect amebiasis (*8*). In other wild anurans, amebas (including *Entamoeba* spp.) sometimes are evident cytologically in the intestine (*9*) but have never been linked to disease.

The genus *Entamoeba* infects a range of taxa, often as commensals, and less commonly as pathogens (*10*,*11*). In humans, *E. histolytica* is associated with extensive illness and death (*12*,*13*). However, the presence of *Entamoeba* is inconsistently associated with disease and might depend on interactions between the environment, host, and parasite (*12*,*13*). For example, poor nutritional status facilitates invasive amebiasis in humans (*12*–*14*). Likewise, anorexia predisposes captive herpetofauna to invasive entamoebiasis (*11*). Furthermore, interactions between *Entamoeba* spp. and other organisms in the gut microbiome may affect growth or virulence of the pathogen (*11*,*12*).

Based on this pattern of *Entamoeba* pathogenesis in other species and on knowledge of toad ecology, we speculate the following scenario for the disease outbreak. Toads ingest encysted OTU_12 by foraging on the ground where an infected host has defecated (*12*). Rates of infection increase during the dry season when toads congregate nightly around dwindling water sources (*5*,*15*). Dry-season congregations of toads also decrease food intake as competition for food increases (*15*). Decreased feeding alters the intestinal microbiome and causes *Entamoeba* in the colon to activate genes that enable it to feed on epithelial cells instead of colon contents. Destruction of the colon wall causes fluid imbalance, forcing toads to remain in moist areas to prevent dehydration. As destruction of the colon wall progresses, bacterial infection leads to septicemia, anorexia, and eventual death. Further experimental studies are needed to verify this conjectured chain of causation.

The circumstances underlying the unprecedented mortality event and its implications require further investigation. Of paramount importance is determining the current distribution of OTU_12, its original host, and whether native frog populations are at risk from the disease. Isolating and culturing OTU_12 for reference material and morphologic characterization of cysts and trophozoites would facilitate further study. Determining whether changes in the environment, microbiome, or both cause *Entamoeba* to switch from commensal to pathogenic and the role the disease may play in controlling populations of cane toads also warrant further study.

Acknowledgments

We thank 2 anonymous reviewers for helpful comments.

The Australian Research Council provided funding for this study.

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Detection of Dengue Virus among Children with Suspected Malaria, Accra, Ghana

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We report new molecular evidence of locally acquired dengue virus infections in Ghana. We detected dengue viral RNA among children with suspected malaria by using a multipathogen real-time PCR. Subsequent sequence analysis revealed a close relationship with dengue virus serotype 2, which was implicated in a 2016 outbreak in Burkina Faso.

The accurate diagnosis of nonmalarial febrile illnesses remains a large challenge in many malaria-endemic countries (*1*). The etiologic agents in this context are often not identified because of nonspecific clinical symptoms and diagnostic limitations (*2*); for example, of 457 patients in Nigeria who were presumptively treated for malaria, only 3.9% tested positive (*3*). Because of the decline in malaria transmission over the past decade in many endemic areas, including Ghana, there is a critical need for a comprehensive characterization of the etiology of acute febrile illness (AFI) (*4*). Dengue virus infections cause symptoms that are similar to those of malaria, and considering the increasing reports of dengue outbreaks in countries neighboring Ghana (*5*–*8*), there is an increased need for dengue surveillance.

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DOI: https://doi.org/10.3201/eid2408.180341

The Study

To obtain a description of the pathogens causing febrile illnesses in Ghana, we conducted a hospital-based cross-sectional study among children in 2 geographically distinct areas. Kintampo, in the Brong Ahafo region, is a semiurban area in the forest savanna middle belt and has a population of 42,957. Teshie is a periurban area in the Greater Accra region that has ≈171,875 residents (online Technical Appendix Figure https://wwwnc.gov/EID/article/24/8/18-0341-Techapp1.pdf). We conducted the study during October 2016–July 2017, encompassing parts of the dry season (November–March) and rainy season (April –July). Children 1–15 years of age whose symptoms included fever were examined in the outpatient departments of Kintampo Municipal Hospital or Ledzokuku Krowor Municipal Assembly Hospital in Teshie. The study was approved by the ethics review committees of the Noguchi Memorial Institute for Medical Research, University of Ghana, the Ghana Health Service, and the Kintampo Health Research Centre. We recruited the patients for this study after we obtained written informed consent from their parents or guardians. The inclusion criteria were fever within the preceding 24 hours or measured axillary temperature ≥38°C occurring for <7 days and no severe or known chronic disease. Attending clinicians at the 2 sites screened a total of 10,234 children, and 700 were enrolled for the study on the basis of the inclusion criteria. We collected venous blood (5 mL) from each participant into EDTA-containing tubes (BD Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA) for malaria tests and full blood counts; the remaining blood was stored at –80°C until use. All of the children were treated according to the Ghana Health Service treatment guidelines.

We randomly selected stored blood samples from 166 children diagnosed with AFI and screened the samples by using a customized multipathogen, real-time PCR–based TaqMan probe-array card (TAC; Applied Biosystems, Carlsbad, CA, USA), as described by Liu et al. (*9*). The AFI TAC assay simultaneously tests for 26 pathogens, including 3 protozoa, 7 bacteria, and 16 viruses (Table 1). Each card tests 6 samples and 2 controls (*9*).

As we expected, *Plasmodium* spp. was the predominant pathogen detected in samples from the children (36.8% of samples tested; Table 2). *Salmonella enterica*

Table 1. Pathogens tested for by using the customized AFI TaqMan array card used in study of dengue virus among 166 children with suspected malaria, Accra, Ghana, October 2016–July 2017*

Pathogens
Bartonella spp.
Brucella spp.
Bundibugyo virus
Coxiella burnetii
Crimean-Congo hemorrhagic fever virus
Chikungunya virus
Dengue virus
Ebola virus
Hepatitis E virus
HIV
Lassa virus
Leishmania spp.
Leptospira spp.
Marburg virus
Nipah virus
O'nyong-nyong virus
Plasmodium spp.
Rickettsia spp.
Rift Valley fever virus
Salmonella spp.
Sudan virus
Trypanosoma brucei
West Nile virus
Yersinia pestis
Yellow fever virus
Zika virus

*Applied Biosystems, Carlsbad, CA, USA. AFI, acute febrile illness.

serovar Typhi, *Rickettsia* spp., *Coxiella burnetii*, and HIV-1 were also detected in some samples (Table 2). However, the most notable observation was the detection of dengue virus in samples from 2 children, 3 and 14 years of age, who were admitted to Ledzokuku Krowor Municipal Assembly Hospital; critical cycle threshold (C_t) for these children was 24.40 and 19.35, respectively (Figure 1). Furthermore, 1 of the dengue-positive samples was also positive for *Plasmodium* spp., providing a vivid demonstration of the complex etiology of AFI in malaria-endemic areas. This observation is consistent with a recent report showing that 51% of febrile children in Ghana who were diagnosed with malaria parasitemia were co-infected with ≥1 pathogen (*10*).

No dengue virus infections have previously been reported in Ghana, despite suspected transmission after the

Table 2. Pathogens detected in study of dengue virus among 166 children with suspected malaria by using customized AFI TaqMan array card, Accra, Ghana, October 2016–July 2017*

Pathogen(s)	No. (%) patients
Plasmodium spp. only	61 (36.8)
Dengue virus only	1 (0.6)
Plasmodium spp. + dengue virus	1 (0.6)
Salmonella enterica serovar Typhi	1 (0.6)
Rickettsia spp.	5 (3.0)
Coxiella burnetii + *Plasmodium* spp.	1 (0.6)
HIV	1 (0.6)
Unidentified (negative result)	95 (57.2)

*AFI, acute febrile illness.

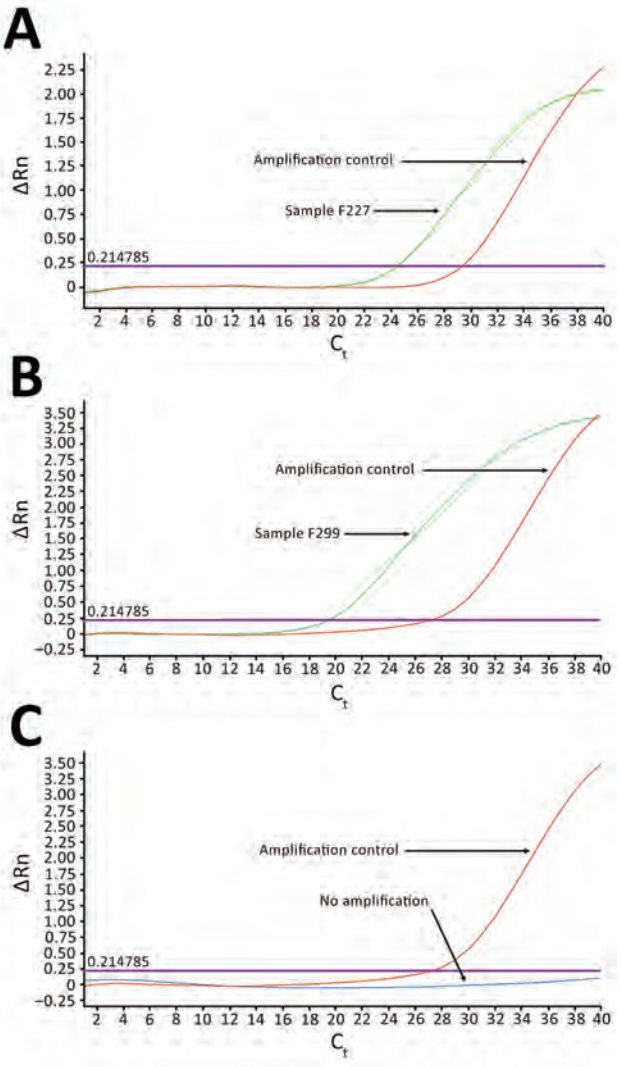

Figure 1. TaqMan array card amplification plots for 2 dengue virus–positive samples and 1 negative sample in study of dengue virus among 166 children with suspected malaria, Accra, Ghana, October 2016–July 2017. Blood samples (2.5 mL of the 5.0 mL collected) obtained from children reporting to the hospital with acute febrile illness (AFI) were screened for 26 pathogens simultaneously by using the real-time PCR TaqMan array card. The cards were in 384-well format, and each well contained 1 µL of reaction mixture (0.75 µL of the extracted total nucleic acid and 0.25 µL of TaqMan Fast Virus 1-step Master Mix; Life Technologies/Applied Biosystems, Foster City, CA, USA). The assays were run on an Applied Biosystems Quant Studio 7 Flex real-time PCR system, according to the manufacturer's recommendations. Two samples tested positive for dengue virus: A) F227, which amplified with critical C_t of 24.40; and B) F299, which amplified with C_t of 19.35. C) All others tested negative, indicated by amplification signals (ΔRn) below threshold levels at quantification cycle cutoff of 35. Each assay included nucleic acid to serve as amplification controls. External controls were spiked into each to monitor extraction and amplification efficiency, and 1 negative control was included for each batch of extraction to monitor laboratory contamination. C_t, cycle threshold.

isolation of dengue virus type 2 (DENV-2) in travelers from Finland who visited Ghana during 2000–2005 (*11*) and our previous study showing serologic evidence of prior dengue exposure among malaria-positive children in Ghana during 2011–2014 (*12*). Therefore, we sought to confirm the results of the TAC screening by using 3 additional molecular methods: 1) the Trioplex TaqMan-based real-time reverse transcription PCR (RT-PCR) developed by the Centers for Disease Control and Prevention (*13*), which differentiates dengue from the arboviruses chikungunya (CHIKV) and Zika; 2) a second TaqMan-based real-time RT-PCR assay developed by Johnson et al. for dengue virus serotype detection (*14*); and 3) a conventional RT-PCR assay by using primers as described by Lanciotti et al. (*15*) (Figure 2). All of these methods confirmed the samples to be dengue positive. C_t values for DENV obtained from the Trioplex assay for the 2 samples were 26.96 and 34.70; results for CHIKV and Zika virus were undetermined (negative). We recorded C_t values of 27.53 and 35.42 on the Johnson et al. real-time RT-PCR assay, which also characterized the 2 samples as DENV-2.

We further sequenced viral RNA by using an ABI 3130XL Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). BLAST search (https://blast.ncbi.nlm.nih.gov/blast.cgi) using default settings of generated nucleotide sequences revealed that the viruses in both positive samples were identical (100% homology) and closely related to the DENV-2 strains isolated in the 2016 outbreak in Burkina Faso (GenBank accession no. KY627763.1). We deposited the nucleotide sequences of the detected virus strain in GenBank under accession nos. MG937762 and MG937763.

Apart from fever, both dengue-positive children reported chills, cough, and vomiting; however, neither reported diarrhea or rash. On a follow-up visit 2 months after enrollment, the children from whom we obtained the dengue-positive samples seemed healthy and afebrile, suggesting that no severe complications of the infection developed. Convalescent-phase blood samples from both patients tested positive for dengue-specific IgG; 1 was additionally positive for dengue IgM (Abcam human anti-dengue virus IgG and IgM ELISA kit; Abcam, Cambridge, UK). The parents reported that these children had not traveled outside the country, indicating that the infection was locally acquired.

Conclusions

Our previous investigations detected dengue antibodies in 21.6% of children in 3 areas of Ghana, including Kintampo and Accra; however, no virus was detected from any of them, suggesting previous exposure rather than acute infections (*12*). In this study, we have now confirmed the presence of dengue virus in the blood of two children, indicating acute infections. In light of other reports from elsewhere in West Africa (*5–7*), this DENV-2 strain may soon become regionally endemic, if it has not already. We have advised the Ghana Health Service accordingly to take measures to intensify surveillance, consistent with prior recommendations (*8*).

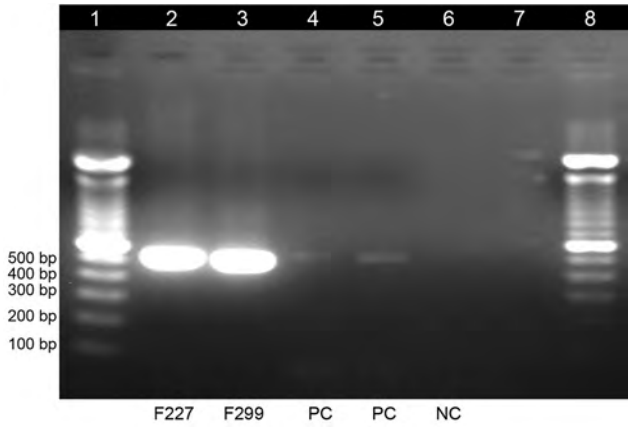

Figure 2. Gel electrophoresis of dengue virus–specific RT-PCR products in study of dengue virus among 166 children with suspected malaria, Accra, Ghana, October 2016–July 2017. We completed a conventional RT-PCR assay by using dengue-specific primers from Lanciotti et al. (*15*) to confirm the results of the TaqMan array card assays. The amplification products (expected size 511 bp) were electrophoresed on 2% agarose gel, stained with ethidium bromide, and viewed under ultraviolet light. Lane 1, molecular weight marker; lanes 2 and 3, test samples; lanes 4 and 5, positive controls; lane 6, negative control; lane 7, empty; lane 8, molecular weight marker. RT-PCR, reverse transcription PCR.

Acknowledgments

We are grateful to the study participants for volunteering for the study and the staff of the Ledzokuku Krowor Municipal Assembly Hospital and the Kintampo Health Research Centre for their support with sample collection. We also thank Wangeci Gatei and Joel Montgomery for facilitating some aspects of the study.

This work was supported by funds from the Government of Ghana through a World Bank African Centres of Excellence grant (ACE02-WACCBIP to G.A.A.), a DELTAS Africa grant (DEL-15-007 and Wellcome Trust 107755/Z/15/Z to G.A.A.), a Royal Society Pfizer Award grant (to G.A.A.), and the Japan Agency for Medical Research and Development.

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Death from Transfusion-Transmitted Anaplasmosis, New York, USA, 2017

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We report a death from transfusion-transmitted anaplasmosis in a 78-year-old man. The patient died of septic shock 2 weeks after a perioperative transfusion with erythrocytes harboring *Anaplasma phagocytophilum*. The patient's blood specimens were positive for *A. phagocytophilum* DNA beginning 7 days after transfusion; serologic testing remained negative until death.

Human granulocytic anaplasmosis (HGA) is caused by the obligate intracellular gram-negative bacterium *Anaplasma phagocytophilum* and transmitted primarily by ticks of the genus *Ixodes* (*1*–*3*). Although HGA is largely asymptomatic, manifestations can range from fever, myalgia, headache, or malaise to life-threatening complications in elderly or immunocompromised patients (*2*,*4*). The prevalence of anaplasmosis is increasing in the United States (*4*). Blood components are not currently screened for *A. phagocytophilum*. Because *A. phagocytophilum* is found predominantly within granulocytes, leukoreduction lowers the risk of transfusion-transmitted anaplasmosis (TTA) by reducing the level of the organism by 300-fold (*5*). To date, 9 cases of TTA attributed to erythrocytes and platelets, including leukoreduced units, have been reported (*5*–*7*).

We report a fatality associated with TTA in an elderly man in New York, New York, USA. The Weill Cornell Medical Center institutional review board (New York, NY, USA) reviewed this case report.

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DOI: https://doi.org/10.3201/eid2408.172048

Case Report

A 78-year-old man with homozygous factor XI deficiency and an extensive medical history, including coronary artery disease, congestive heart failure, diabetes, and chronic kidney disease, was admitted in April 2017 for transurethral resection of the bladder as the result of a urologic malignancy. He had a history of anemia (preoperative hemoglobin 9.9 g/dL; reference range 14–17.5 g/dL); darbepoietin had been prescribed to him as an outpatient, with limited compliance. As an inpatient, he received a diagnosis of iron deficiency; his serum iron level was 13 (reference range 65–175 μg/dL). Minimal blood loss occurred during surgery, but the patient's early postoperative course was complicated by recurrent hematuria and symptomatic anemia. Twelve units of leukoreduced, irradiated erythrocytes were required. The patient also received 14 plasma transfusions to keep his factor XI levels above the hemostatic threshold.

On day 20 of admission, the patient unexpectedly became febrile and developed tachycardia and hypotension. The patient had previously been afebrile and was not taking empiric antimicrobial drugs. Cultures were drawn, and the patient was given broad-spectrum antibacterial drugs (piperacillin/tazobactam, 4.5 g intravenously [IV] every 12 h; and vancomycin, 1.5 g IV every 24 h). Despite antibacterial drug coverage and negative blood, urine, and throat cultures, the patient continued to be febrile and decompensated with dyspnea, hypoxia, and an elevation in troponin. He subsequently developed thrombocytopenia, leukopenia, and transaminitis. On day 4 of fevers, clusters of bacteria within neutrophils, consistent with *A. phagocytophilum*, were seen on peripheral smear (the inclusions were not identified on prior smears, even upon retrospective review) (Figure 1). Doxycycline (100 mg IV every 12 hours) was started immediately, pending *Anaplasma*/*Ehrlichia* serology. The patient went into refractory shock, developed multiple organ failure, and died 24 hours after initiation of doxycycline (Figure 2).

Although the season was early spring, the likelihood of tick-bite transmission was extremely low, as the patient had been homebound for weeks before admission and had no pets (thus eliminating tick exposure via household pets). We pursued an investigation for transfusion-transmitted infection. Nucleic acid–based testing on available patient samples and segments from all transfused erythrocyte units was performed by the New York State Department of Health (NYS DOH). One of 12 donor unit segments tested positive by PCR for *A. phagocytophilum* DNA. This

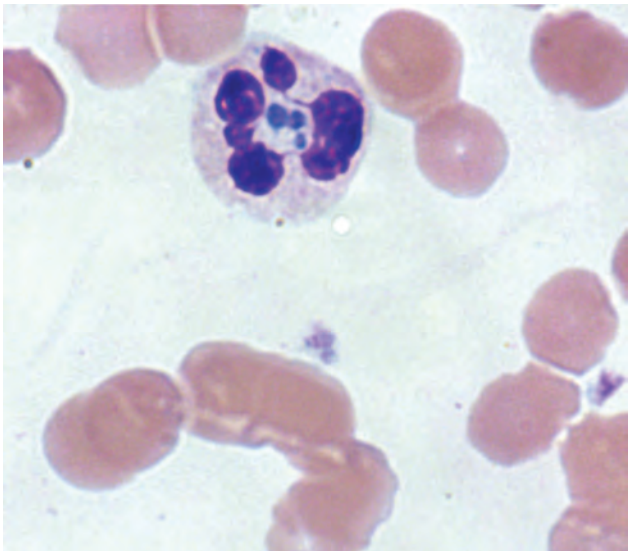

Figure 1. *Anaplasma phagocytophilum* morulae observed on peripheral blood smear from patient in whom anaplasmosis infection developed after a blood transfusion, New York, New York, USA. Intracytoplasmic inclusions (morulae) were first seen 15 days after the patient was transfused with an infected erythrocyte unit, leading to a diagnosis of human granulocytic anaplasmosis later confirmed by PCR (original magnification ×1,000 [oil immersion]).

erythrocyte unit was whole blood derived with 1 co-component from the donation: a plasma unit that had already been transfused with no adverse reactions. The NYS DOH identified *A. phagocytophilum* in each of the patient's blood specimens beginning 7 days after the implicated transfusion (the first patient specimen available for testing) (*8*). However, immunofluorescence assay results for *A. phagocytophilum* antibodies were negative until death (*9*).

At autopsy, fresh tissue from the spleen and bone marrow tested positive for *A. phagocytophilum* by PCR. Results of immunohistochemistry testing of fixed tissue obtained from lung, heart, liver, spleen, and kidney were positive for *A. phagocytophilum* (*9*). This transfusion-related fatality was reported to NYS DOH and the US Food and Drug Administration.

The implicated erythrocyte unit had been stored for 22 days before transfusion; routine testing for *Babesia microti* performed under an investigational new drug application protocol was negative at the time of donation. During investigation of the transfusion-transmitted infection, the donor reported a rash and headache but had no fever around the time of donation. The donor's doctor had empirically treated him for Lyme disease, but exact details of the treatment were not available. The donor did not report a postdonation illness despite a standard reminder during donation to report such information. The donor was deferred from blood donation for 90 days.

This patient's diagnosis was HGA, a tickborne illness previously called human granulocytic ehrlichiosis (*10*). HGA should be included in the differential diagnosis of fever after tick bites in the upper Midwest and northeastern United States and in northern California (*1*,*3*,*4*,*11*). HGA symptoms typically appear 5–21 days after a tick bite and manifest as fever (75%–100%), myalgia (75%), headache (83%), and malaise (97%). Laboratory test abnormalities such as thrombocytopenia (79%), leukopenia (60%), and transaminitis (91%) increase diagnostic specificity (*3*). Although HGA can be asymptomatic in healthy adults, it can have life-threatening consequences in the elderly, patients with immune-compromising conditions (e.g., diabetes or cancer), or patients taking immunosuppressive medications (*2*). Symptoms of severe HGA may include renal failure, respiratory distress syndrome, toxic shock–like syndrome, pneumonia, and disseminated intravascular coagulation or sepsis-like syndrome. Overall, 31% of patients are hospitalized, and 7% require intensive care. The case-fatality rate is 0.6%, with a 16-fold increase in relative risk for death if infection occurs in a patient with an immunosuppressive condition. Anaplasmosis and other tickborne infections should be considered in a differential diagnosis of sepsis, especially in posttransfusion cases, when patients are refractory to routine broad-spectrum antimicrobial drugs. Standard culture

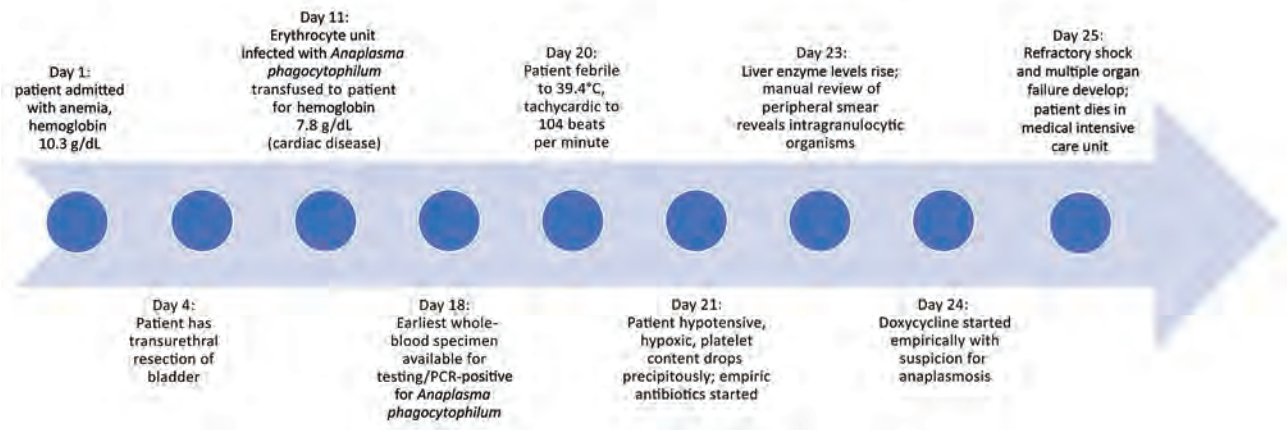

Figure 2. Timeline of patient's hospitalization for anemia followed by *Anaplasma phagocytophilum* infection, New York, New York, USA.

techniques will not detect causative organisms and the infections require directed therapy (e.g., doxycycline) not usually included in standard empiric treatment.

A. phagocytophilum resides intracellularly (primarily in neutrophils), can cause subclinical infection, and can survive refrigeration (*5*,*12*); thus, it meets the criteria for an erythrocyte transfusion-transmitted infection. As of May 2018, a total of 9 cases of TTA have been reported: 7 attributed to erythrocyte units (5 leukoreduced), 1 involving a leukoreduced apheresis platelet unit, and 1 from whole blood–derived platelets. Leukoreduction leads to a 300-fold reduction in *A. phagocytophilum* bacteremia but does not eliminate the risk of infection (*13*). The longest survival of *A. phagocytophilum* previously reported in refrigerated conditions was 18 days (*5*,*7*,*12*); in our case, we report a prolonged survival of *A. phagocytophilum* in a refrigerated erythrocyte unit (22 days).

Conclusions

This case illustrates a challenge in transfusion medicine as a result of limitations of infectious disease screening of the blood supply. No Food and Drug Administration–licensed tests exist for screening donated blood for *A. phagocytophilum.* Serologic or nucleic acid testing of donors is considered unnecessary because transfusion transmission of *A. phagocytophilum* is an extremely rare event. A history of tick bites is generally not obtained from donors because it is neither sensitive nor specific (*14*).

Pathogen reduction would likely have prevented HGA transmission in this case. However, only pathogen-reduced plasma and platelets are currently available. Pathogen-reduced erythrocytes remain under development. The most prudent approach to reduce the risk of transfusion-transmitted anaplasmosis is by the judicious use of blood components and the strict avoidance of unnecessary transfusions in adherence with published guidelines (*15*). This fatality from transfusion-transmitted anaplasmosis is a reminder of the serious residual risks from transfusions, as well as the increasing prevalence of *A. phagocytophilum*.

Acknowledgments

We thank the New York State Department of Health, the New York Blood Center, and the Blood Bank and Central Laboratory staff of New York-Presbyterian Hospital–Weill Cornell Medical Center for their help in this patient's diagnosis and investigation, and Ljljana V. Vasovic for her assistance with this article.

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Capillaria Ova and Diagnosis of *Trichuris trichiura* Infection in Humans by Kato-Katz Smear, Liberia

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We examined human stool samples from Liberia for soil-transmitted helminth ova by Kato-Katz smear and by quantitative PCR. Twenty-five samples were positive for *Trichuris trichiura* by smear but negative by quantitative PCR. Reexamination of samples showed that they contained *Capillaria* eggs that resemble *T. trichiura* in Kato-Katz smears.

Kato-Katz smears are the most commonly used diagnostic tool for detecting and quantifying soil-transmitted helminth (STH) infections in field surveys (*1*). Although this method has some shortcomings, its advantages are field suitability and fast microscopic enumeration of worm eggs. Whereas sensitivity is low for light infections because of the small amount of stool examined (≈41 mg), the specificity of Kato-Katz for diagnosis of *Ascaris lumbricoides* and *Trichuris trichiura* infection is considered to be high (*2*). In contrast, hookworm eggs are difficult to differentiate by morphology, but quantitative PCR (qPCR) enables differentiation among *Necator americanus*, *Ancylostoma duodenale*, and *A. ceylanicum* eggs (*3*–*5*).

Among helminth eggs found in human feces, the barrel-shaped eggs of *T. trichiura* worms are considered to be characteristic, with a length of 50–55 μm, a width of 22–24 μm, and clearly protruding bipolar plugs (*6*). Similar eggs of other members of the *Trichiuridae* family may be differentiated from *T. trichiura* eggs by size and morphology when observed at high magnification, but these eggs have rarely been found in human fecal samples (*7*–*9*). Therefore, the presence of eggs of zoonotic members of the *Trichiuridae* family is generally not considered a confounder for detecting *T. trichiura* by Kato-Katz smear.

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DOI: https://doi.org/10.3201/eid2408.180184

The Study

To assess the effect of mass drug administration using ivermectin and albendazole for the elimination of lymphatic filariasis on STH prevalence and intensity, we collected stool samples over a period of 3 years in 2 different areas in Foya district (Lofa County) in northwestern Liberia and in Harper district (Maryland County) in southeastern Liberia (*10*). We examined a single stool sample per subject by microscopy (magnification ×100) with duplicate Kato-Katz smears (41-mg template). We preserved aliquots of randomly selected specimens on FTA cards (GE Healthcare, Little Chalfont, UK) or in RNAlater (ThermoFisher, Waltham, MA, USA) and shipped them to Washington University School of Medicine (St. Louis, MO, USA) for analysis by qPCR. Two experienced microscopists (L.G., A.T. Momolu) examined the samples by Kato-Katz smear in both study areas. For detection of STH by qPCR, we extracted DNA from ≈100 mg of stool and tested it as described by Pilotte et al. (*5*) with a Quantstudio 6 Flex Thermocycler (Applied Biosystems, Carlsbad, CA, USA) and TaqMan Fast Advanced Mastermix (Applied Biosystems). We used the following primers and probes to detect *Schistosoma mansoni* DNA: forward primer 5′-TGTGGGAGTCTTTGGTTGTT-3′, reverse primer 5′-CAACATGACTGGGAACAGGA-3′, probe 5′-AGGTTCAGGTGG/ZEN/GTGTGTTACGAA-31ABkFQ-3′.

We tested 353 stool samples from Foya district by Kato-Katz smear; 31 (8.8%) were positive for *A. lumbricoides* eggs, 231 (65.4%) for hookworm eggs, 27 (7.6%) for *T. trichiura*–like eggs, and 276 (78.2%) for *S. mansoni* eggs. We tested 225 samples from Harper district by Kato-Katz smear; 163 (72.4%) were positive for *A. lumbricoides* eggs, 65 (28.9%) for hookworm eggs, and 51 (22.7%) for *T. trichiura* eggs (Table 1). There was good agreement between the results of the Kato-Katz and qPCR tests for the specimens from Harper (80.5%–91.6%), but generally qPCR had higher sensitivity. Our results were consistent with results previously reported with samples from other areas (*3*,*11*). Agreement between the 2 diagnostic tests for samples from Foya ranged from 77.3% to 92.9%, but the sensitivity of the qPCR was unexpectedly low, a finding that was especially true for *Ascaris* and *Trichuris* infection (Table 1). Whereas samples positive for *Ascaris* by Kato-Katz but negative by qPCR had low egg counts, samples positive for *Trichuris* by Kato-Katz

Table 1. Comparison of sensitivity of Kato-Katz smear and quantitative PCR results for 778 stool samples tested for soil-transmitted helminths, Foya and Harper districts, Liberia

Site and species	No. positive*	Kato-Katz smear sensitivity, %	qPCR sensitivity, %	McNemar p value
Foya district, n = 353				
Ascaris lumbricoides	34	91.2	17.6	<0.0001
Hookworm†	247	93.5	83.4	<0.0001
Trichuris trichiura	27	100	7.4	<0.0001
Schistosoma mansoni	307	89.9	84.0	0.0573
Harper district, n = 225				
A. lumbricoides	180	90.6	98.9	0.0013
Hookworm†	99	65.7	89.9	0.0005
T. trichiura	86	59.3	94.2	0.0001

*Samples that tested positive by either method.
†Hookworm was *Necator americanus.* No *Ancylostoma duodenale* was detected.

but negative by qPCR had higher counts; 7 samples contained ≥1,000 barrel-shaped eggs/g of stool (Table 2). We repeated DNA extraction and qPCR and also used an alternative qPCR for *T. trichiura* (*3*), but these tests did not improve the agreement between microscopy and qPCR results.

To check further whether Kato-Katz–positive, qPCR-negative stool samples contained *T. trichiura* eggs, we examined direct smears of stool samples preserved in RNAlater by microscopy (magnification ×100 and ×400) (Figure 1). The samples positive by qPCR contained eggs (6 measured) with typical *T. trichiura* morphology; these eggs had a mean (±SD) length of 52 μm (±2.4 μm) and width of 25.5 μm (±1.3 μm). In contrast, qPCR-negative samples contained eggs (31 measured) with a mean (±SD) length of 51.8 μm (±1.5 μm) and width of 32.7 μm (±2.1 μm). The qPCR-negative samples also had less pronounced plugs and a thick, striated shell, features that are consistent with eggs of *Capillaria hepatica* (syn. *Calodium hepaticum*) and some other *Capillaria* species (*Trichuridae*). Eggs of *C. philippinensis* or *C. aerophila* that have been observed in human stool samples previously were either smaller or larger than the *Capillaria* eggs found in Lofa (*12*,*13*). Because polar plugs of these eggs are less prominent than those of *T. trichiura*, and because their shapes are sometimes more oval or round, they can also be confused with *A. lumbricoides* eggs by low-power microscopy, especially if only a few eggs were detected (Figure 1).

Members of the subfamily *Capillaridae* are animal parasites with somewhat divergent life cycles, and most

Table 2. Demographics and Kato-Katz and qPCR results for patients positive for *Trichuris trichiura* infection by microscopy, Liberia*

Demographics				Microscopy, epg				qPCR, cycle threshold			
Year	Patient no.	Age, y/sex	Village	Tt	Al	Hk	Sm	Tt	Al	Na	Sm
2014	P320529	45/F	Yallahun	576	0	360	24	Neg	Neg	31.7	30.5
	P320683	35/F	Kpombu	12	0	0	0	Neg	Neg	Neg	28.4
	P320695	16/M	Kpombu	24	0	0	72	Neg	Neg	Neg	23.8
	P320620	15/M	Foya-Dundu	12	120	0	288	Neg	Neg	32.2	23.5
	P320746	9/F	Bandenin	24	0	0	0	Neg	Neg	Neg	26.51
	P320452	7/F	Felaloe	12	0	0	120	Neg	Neg	Neg	23.9
	P320596	6/F	Foya-Dundu	12	0	0	90	Neg	Neg	Neg	27.6
	P320656	6/F	Kpombu	120	0	0	504	Neg	Neg	Neg	21.3
2016	P331772	36/M	Kpormbu	3,048	0	12	24	Neg	Neg	Neg	Neg
	P331921	35/M	Felaloe	60	0	0	12	Neg	Neg	Neg	Neg
	P331783	34/F	Kpormbu	420	0	0	0	Neg	Neg	Neg	Neg
	P330724	26/M	Keyabendu	4,224	0	0	456	Neg	Neg	Neg	30.4
	P331791	6/F	Kpormbu	12	0	156	12	Neg	Neg	33.1	33.4
	P331962	6/F	Bandenin	12	0	0	168	Neg	Neg	Neg	29.6
	P331983	6/F	Bandenin	36	0	0	5,304	Neg	Neg	Neg	28.1
2017	P341287	61/M	Mendikorma	1,464	0	0	0	Neg	Neg	Neg	33.1
	P341282	56/M	Mendikorma	540	0	216	0	Neg	Neg	28.3	Neg
	P341284	50/M	Mendikorma	60	0	0	132	Neg	Neg	Neg	Neg
	P342148	45/M	Keyabendu	1,368	0	0	192	Neg	Neg	34.5	Neg
	P340246	39/M	Kamatahun	120	0	0	216	Neg	Neg	Neg	30.0
	P340307	19/F	Bambuloe	2,028	0	0	1,188	Neg	Neg	Neg	24.1
	P340133	12/M	Fokolahun	1,020	16,392	0	0	25.3	16.7	Neg	Neg
	P340183	9/F	Kpelloe Ndama	72	0	0	0	Neg	Neg	Neg	36.0
	P341308	9/F	Mendikorma	36	0	108	0	Neg	Neg	Neg	28.5
	P341326	9/M	Mendikorma	456	0	0	0	Neg	Neg	26.5	30.4
	P341327	6/M	Mendikorma	2,076	0	0	0	Neg	Neg	Neg	Neg
	P340147	5/M	Fokolahun	48	0	0	0	30.94	26.93	Neg	Neg

**T. trichiura* infection was confirmed by qPCR in only 2 patients, but 25 had *Capillaria* eggs in their stool. Al, *Ascaris lumbricoides*; epg, eggs per gram of stool; Hk, hookworm; Na, *Necator americanus*; Neg, negative; Sm, *Schistosoma mansoni*; Tt, *T. trichiura*.

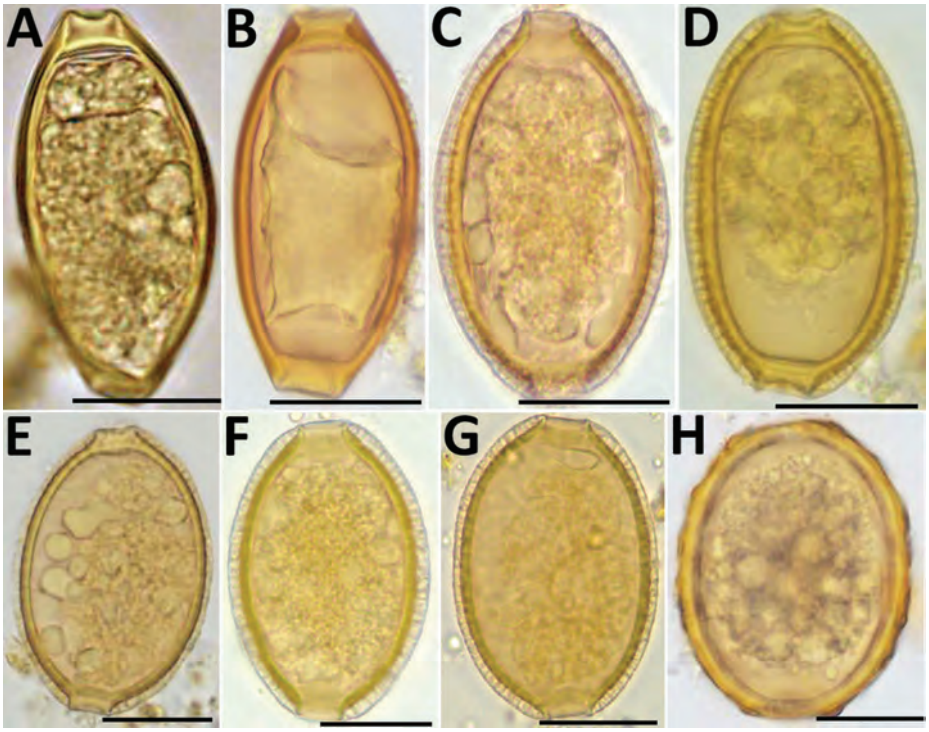

Figure. Helminth eggs found in stool samples from persons in Lofa County, Liberia. A, B) Eggs of *Trichuris trichiura* in samples positive for *T. trichiura* by Kato-Katz smear and by qPCR. C–F) Eggs of *Capillaria* spp. in samples positive for *T. trichiura* by Kato-Katz smear but negative for *T. trichiura* by qPCR. G) Egg of *Capillaria* spp. in sample positive for *Ascari lumbricoides* by Kato-Katz smear but negative for *A. lumbricoides* by qPCR. H) Egg of *A. lumbricoides* in sample positive for *A. lumbricoides* by Kato-Katz smear and qPCR. Scale bars indicate 20 μm. qPCR, quantitative PCR.

do not infect humans. Pseudoinfections with *C. hepatica* occur; eggs found in stool are present because they were consumed in infected animal liver. However, actual infections with *C. hepatica* do not lead to the passing of eggs in stool (*9*). Other species such as *C. philippinensis* cause true infections (and autoinfection) with eggs found in stool; the infection is linked to consumption of raw fish. Human capillariasis has not been reported from Liberia, and only isolated case reports have been published from sub-Saharan Africa (*7*–*9*). We performed DNA sequencing to better characterize the *Capillaria* species found in Foya. Using the primers Kt875351.1 (5′-CCCTAGTTGCGACTTTAAACGA-3′**)** and *Capillaria* 18S1R (5′- TCCACCAACTAAGAACGGCC-3′), we were able to amplify and sequence a 288-bp portion of the 18S rDNA from *T. trichiura* qPCR-negative samples that contained only eggs morphologically identified as *Capillaria* spp. (GenBank accession no. MG859285). The DNA fragment was 100% identical to orthologs of *C. hepatica* (accession no. MF287972.1), *Aonchotheca putorii* (*C. putorii*) (accession no. LC052356.2), and *Pearsonema plica* (*C. plica*) (accession no. MF621034.1), *Capillaria* worm species that have varying life cycles and host species but that are only 95% identical to the ortholog of *T. trichiura*.

The life cycle and the medical importance of the *Capillaria* species found in humans in northwestern Liberia remain to be elucidated. In our study some subjects showed high *Capillaria* egg loads that may indicate a true infection rather than pseudoinfection. However, transient high egg counts have been reported in persons with pseudoinfections (*7*). Whereas consumption of bush meat in Foya is common, consumption of raw or undercooked fish, which is necessary for transmission of *C. philippinesis*, is rare.

Conclusions

This study shows that *Capillaria* eggs similar to those of *C. hepatica* are not uncommon in stool samples collected in Liberia. These eggs can be misidentified by Kato-Katz smear as *T. trichiura* or as *A. lumbricoides*, which can confound results of STH surveys. The misidentification can also lead to an incorrect assumption that antihelminthic treatment was ineffective. Our results also illustrate the value of qPCR for validating Kato-Katz test results and for explaining unexpected findings.

Acknowledgments

We thank Emanuel B. Gray and Aaron T. Momolu for their expert help during field work, as well as Laura Rinaldi and Guiseppe Cringoli for their advice concerning animal *Capillaria* species.

The study was funded in part by grant GH5341 from the Bill & Melinda Gates Foundation.

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Coxiella burnetii Endocarditis and Meningitis, California, USA, 2017

Lao-Tzu Allan-Blitz, Ashyln Sakona, William D. Wallace, Jeffrey D. Klausner

The epidemiology of *Coxiella burnetii* infection in the United States is not well characterized. We report a case-patient with *C. burnetii* endocarditis and meningitis. Infection was diagnosed by detecting high serologic titers for *C. burnetii* and confirmed by sequencing of *C. burnetii* 16S rRNA isolated from resected valvular tissue and PCR of cerebrospinal fluid.

Coxiella burnetii is the bacterium responsible for Q fever. The epidemiology of *C. burnetii* infection in the United States is not well characterized. Chronic infection can result in endocarditis or other complications. We report a case-patient with *C. burnetii* endocarditis and meningitis.

The Case-Patient

A 38-year-old man with no unusual medical history was hospitalized in 2017 for evaluation of a new cardiac murmur detected by his primary care physician. The man lived near the Pacific coast of central California, USA, where he worked from home as a broker selling produce from local farms. He had not visited those farms, but cattle were raised on properties neighboring his home. Three months before hospitalization, he had an episode of bronchitis treated with azithromycin; his symptoms resolved. A few weeks before that illness, he visited a petting zoo with his children where he was exposed to goats and chickens. The patient was not aware of any parturient animals at the zoo. Other animal exposures were limited to his 2 pet dogs. He denied ingestion of unpasteurized dairy products, a history of injection drug use, or recent travel.

Six weeks before hospitalization, a tender nodule developed on the palmar aspect of his left fifth digit. That nodule resolved without intervention, but severe right midfoot pain and swelling developed. The pain and swelling were diagnosed as cellulitis or gout, and the patient was given trimethoprim/sulfamethoxazole and indomethacin.

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DOI: https://doi.org/10.3201/eid2408.180249

Two weeks before admission, fever and severe headache developed, and the man visited the emergency department of another hospital, where a lumbar puncture was performed. Cerebrospinal fluid leukocyte count was 253 cells/μL with 52% lymphocytes and 43% neutrophils, glucose level was 35mg/dL, and protein level was 63 mg/dL. Results for a FilmArray Meningitis/Encephalitis Panel (BioFire Diagnostics, LLC, Salt Lake City, UT, USA) were negative. He was discharged and given a diagnosis of aseptic meningitis believed to be secondary to treatment with indomethacin.

On follow-up with his primary care doctor, the patient reported ongoing fevers, chills, and drenching night sweats for 2 weeks and a 10-pound weight loss in the preceding 2 months. He was admitted to another hospital, where a new cardiac murmur was detected. A transthoracic echocardiogram showed vegetations on the mitral valve. He was then transferred to Ronald Reagan University Medical Center (Los Angeles, CA, USA) for a higher level of care and surgical evaluation.

At admission, he was afebrile and had unremarkable vital signs. A physical examination showed a harsh holosystolic murmur, a decrescendo diastolic murmur, and a splinter hemorrhage. A transesophageal echocardiogram showed a bicuspid aortic valve with thickened, calcific leaflets and severe regurgitation and a mobile vegetation attached to the mitral valve cordae with subvalvular calcifications.

Admission blood cultures and cultures obtained at the previous emergency department visit were negative for bacteria. He was given empiric vancomycin and ceftriaxone and underwent an aortic valve and aortic root replacement and mitral valve repair. At the time of surgery, multiple sets of blood cultures remained negative for bacteria. Intraoperatively, chronic changes in the aortic root near the right coronary cusp were observed and believed to be suggestive of a previous endocarditic process with a healed area of disruption. Calcific lesions involving the mitral subvalvular apparatus were resected and tested by pathologic analysis.

On postoperative day 1, serologic analysis for *C. burnetii* showed complement fixation titers (IgG phase 1, 1:2,077,152; IgG phase 2, 1:8,388,608; IgM phase 1, 1:1,024; and IgM phase 2, 1:2,048). Pathologic evaluation of the valvular specimen showed multiple fragments of

tan/red to tan/brown soft tissue with focal hemorrhage and calcifications. Microscopic evaluation showed valve tissue mononuclear cells. Gram staining showed numerous small clusters of gram-negative coccobacilli, consistent with an intracellular distribution (Figure).

Subsequent 16S rRNA gene sequencing confirmed the presence of *C. burnetii*. Retrospectively, a qualitative PCR on stored cerebrospinal fluid and resected valvular tissue showed a positive result for the 127-bp insertion sequence 1111, consistent with *C. burnetii*. The patient was given doxycycline and hydroxychloroquine for a planned course of 18 months.

He is still receiving therapy and serial complement fixation titers have decreased. After 5 weeks of therapy, his titers were IgG phase 1, 1:262,144; IgG phase 2, 1:1,048,576; IgM phase 1, 1:512; and IgM phase 2, 1:1,024. After 10 weeks of therapy his titers were IgG phase 1, 1:32,768; IgG phase 2, 1:32,768; IgM phase 1, 1:256; and IgM phase 2, 1:128.

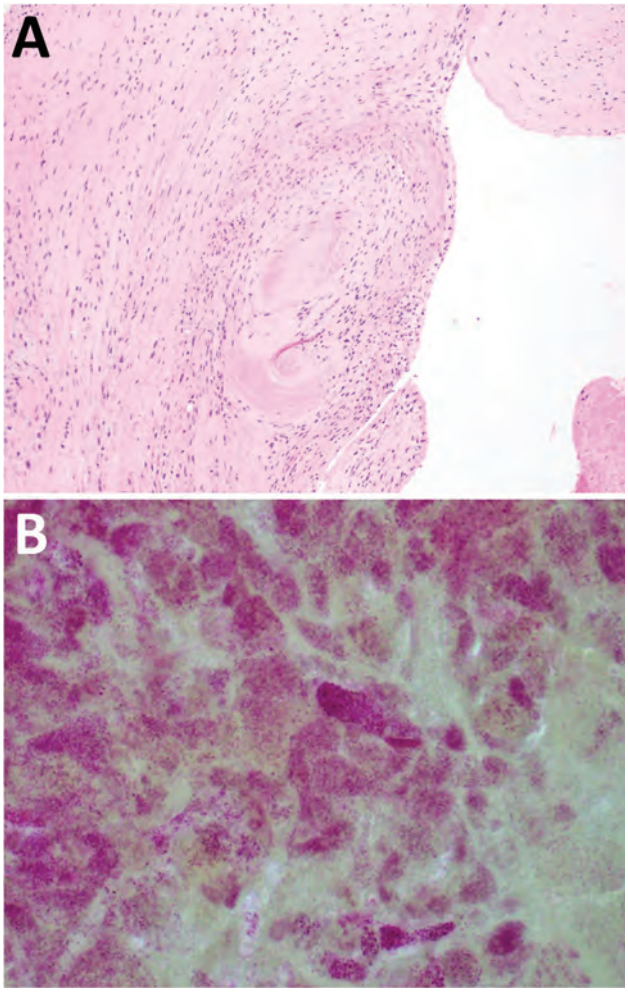

Figure. Results of testing for a 38-year-old man with *Coxiella burnetii* endocarditis and meningitis, California, USA, 2017. A) Cardiac valve tissue showing fibrous scar and chronic inflammation (hematoxylin and eosin stain, original magnification ×100). B) Numerous clusters of gram-negative cocci are consistent with intracellular organisms (Gram stain, original magnification ×1,000).

Conclusions

We report a case-patient with *C. burnetii* endocarditis and meningitis confirmed by 16S rRNA sequencing of resected valvular lesions and a *C. burnetii*–specific PCR of cerebrospinal fluid. Although not confirmed, we suspect that his episode of bronchitis 3 months earlier, which occurred a few weeks after visiting a petting zoo, might have represented atypical pneumonia caused by *C. burnetii*.

The primary reservoirs for *C. burnetii* are goats, sheep, and cattle (*1*). The most common mechanism of infection in humans is inhalation of aerosolized bacteria resistant to environmental stress; however, consumption of poultry or raw or undercooked eggs are other possible routes (*1*). The organism has 2 distinct antigenic phases (phase 1 and phase 2); the immune response to acute infections is predominantly against phase 2, and the response to chronic infection is predominantly against phase 1, although there can be major increases in titers against both phases (*2*).

Acute *C. burnetii* infection is most commonly asymptomatic but can cause symptoms ranging from an influenza-like illness to pneumonia or hepatitis with varying degrees of severity (*3*,*4*). Progression from acute to chronic Q fever occurs in ≈1%–5% of case-patients (*5*,*6*). Chronic Q fever most commonly manifests as endocarditis (60%–70% of cases), which most often occurs in the setting of a valvular lesion (*4*,*7*). This case did not have a known valvular lesion. We believe that the bicuspid valve was likely colonized during the acute phase and resulted in a transition to chronic Q fever endocarditis.

Meningitis is a rare manifestation of chronic infection with *C. burnetii* (*4*). One case series identified a male predominance among persons given a diagnosis of Q fever meningitis and reported a lymphocytic predominance in cerebrospinal leukocyte counts (*8*), as observed for this case-patient. A previous report of concomitant Q fever endocarditis and meningitis attributed central nervous system involvement to embolic phenomenon from a valvular vegetation (*9*). The cause of Q fever meningitis was unclear for the case-patient we report.

Data are limited regarding the prevalence of chronic Q fever in the United States. The Centers for Disease Control and Prevention reported 160 cases of *C. burnetii* infection in the United States during 2014, of which 39 were diagnosed as chronic Q fever (*10*).

Diagnostics for *C. burnetii* are limited because this bacterium is difficult to culture (*11*). In patients with risk factors (e.g., the case-patient we report) and culture-negative endocarditis, *C. burnetii* endocarditis should be considered. Complement fixation is a standard serologic test

for *C. burnetii* at the University of California, Los Angeles. Immunofluorescence assays, which are standard tests at many institutions (*12*), were not available. Diagnostic methods include immunohistochemical analysis of resected heart valves, serologic studies, and qualitative PCRs on freshly resected heart tissue (*13*).

The 16S rRNA gene has regions that are highly conserved across bacteria but with sufficient sequence differences to enable genus, if not species, differentiation (*14*). Thus, use of 16S rRNA sequencing offers a useful diagnostic approach. However, because those regions are highly conserved, 16S rRNA sequencing is not the most sensitive or specific diagnostic approach, but might be most useful when the bacterial cause is unknown.

In summary, *C. burnetii* endocarditis and meningitis should be considered in cases of culture-negative disease for patients with appropriate risk factors. Use of 16S rRNA sequencing might aid in diagnosis of infection.

This study was supported by UCLA Centers for AIDS Research (US National Institutes of Health grant 5P30AI028697) and the Center for HIV Identification, Prevention, and Treatment Services (US National Institutes of Health grant MH58107).

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Probable Locally Acquired *Babesia divergens*–Like Infection in Woman, Michigan, USA

Erica Herc, Bobbi Pritt, Taylor Huizenga, Richard Douce, Matthew Hysell, Duane Newton, Jennifer Sidge, Eve Losman, John Sherbeck, Daniel R. Kaul

We report an asplenic patient who was infected with *Babesia divergens*–like/MO-1. The clinical course was complicated by multiorgan failure that required intubation and dialysis. The patient recovered after an exchange transfusion and antimicrobial drug therapy. Physicians should be alert for additional cases, particularly in asplenic persons.

Babesiosis is an emerging threat in North America. In 2014, this disease was reported in 31 states in the United States (*1*). Protozoan intraerythrocytic parasites of the genus *Babesia* cause infection when transmitted by ticks or blood transfusions. Infections occur most frequently in spring or early summer, coinciding with the host-seeking activity of *Ixodes scapularis* nymphal ticks. Most cases occur in the northeastern upper midwestern United States. Most infections in the United States are caused by *B. microti*, which was first identified in 1966. Other *Babesia* species, including *B. duncani* and *B. divergens*–like/MO-1, have been rarely reported (*2*).

Clinical manifestations of babesiosis can range from asymptomatic to multiorgan failure. Severe illness is frequently seen in elderly, immunocompromised, and asplenic patients (*3*). We report a case of severe babesiosis caused by a *B. divergens*–like/MO-1 organism in an asplenic woman. This case was probably acquired in western Michigan.

The Study

A 60-year-old woman with hereditary spherocytosis status postsplenectomy and a history of pancreatic and colon cancer status post-Whipple procedure was hospitalized in 2017 because she had multiorgan failure. Fatigue, nausea, dyspnea, weakness, and chest pressure without fever had developed 5 days earlier. She was tachycardic and had jaundice but had otherwise reference (normal) vital signs.

Results of testing in the emergency department showed a leukocyte count of 20,800 cells/μL, hemoglobin 8.5 g/dL (reference 10.5 g/dL), creatinine of 5.3 mg/dL, lactate dehydrogenase 7,340 U/L, and haptoglobin <10 mg/dL, and increased levels of liver enzymes (aspartate aminotransferase 128 U/L, alanine aminotransferase 43 U/L, and total bilirubin 9.7 mg/dL). Peripheral blood smear results showed numerous intraerythrocytic parasites consistent with a *Babesia* sp.; parasitemia was 25%–30% (Figure, panel A).

Given her multiorgan failure, the patient was transferred to a tertiary care center for exchange transfusion. At transfer, she was delirious and was admitted to the intensive care unit. She was given quinidine and clindamycin and underwent a 2-volume erythrocyte exchange transfusion. After exchange transfusion, parasitemia decreased to 3.5% (Figure, panel B). The following day she was given clindamycin (600 mg every 8 h), atovaquone (750 mg 2×/d), and azithromycin (250 mg/d) because of prolonged QTc.

Multiplex real-time PCR specific for a 204-bp region of the 18S rDNA gene (*4*) performed at a reference laboratory was positive for *B. divergens*–like/MO-1 and negative for *B. microti* and *B. duncani*. This result was confirmed by additional PCR testing at the Centers for Disease Control and Prevention (Atlanta, GA, USA). Serologic testing results were negative for *Borrelia burgdorferi* and *Anaplasma phagocytophilum* antibodies. No specific serologic analysis was performed for *B. microti* or *B. duncani*.

The patient required mechanical ventilation, pressor support, and renal replacement therapy. Serial peripheral blood smears showed the following consecutive parasitemia values over a 13-day period: 25%–30%, 3.5%, 2%, 1.8%, 0.5%, <0.1%, and 0%. Her hemoglobin and platelet levels returned to reference ranges during this period. Seven days after admission, she was extubated and renal function eventually improved. Antimicrobial drugs were continued after discharge for 4 weeks. At follow-up on day 29 postpresentation after her initial emergency department visit, her clinical status continued to improve, and repeat peripheral blood smears were negative for *Babesia* spp.

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DOI: https://doi.org/10.3201/eid2408.180309

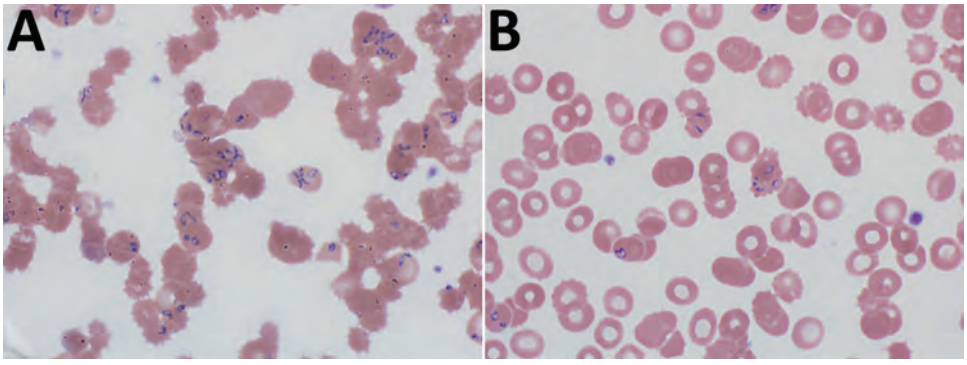

Figure. Peripheral blood films for a 60-year-old woman with probable locally acquired *Babesia divergens*–like infection, Michigan, USA. A) Before erythrocyte exchange transfusion. Parasitemia was 25%–30%. B) After erythrocyte exchange transfusion. Parasitemia was 3.5%. Original magnification ×1,000.

The patient gardened at her home in Berrien County, Michigan, USA, and walked along the Lake Michigan shoreline. She did not report any known tick bites, but the region is known to be endemic for *Ixodes scapularis* ticks. She had traveled to Kansas City, Missouri, USA, 2.5 weeks before symptom onset, but stayed in urban nonpark areas and did not have contact with animals. She did not have blood transfusions during the year before her illness. Given this history, the most likely source of disease acquisition was in Michigan.

Conclusions

We report a case of severe *B. divergens*–like/MO-1 infection in the upper midwestern United States. The patient probably contracted the disease from a tick in southwestern Michigan. She did not have any blood transfusions within the previous year, and the longest reported period between transfusion-transmitted babesiosis and a recipient diagnosis is 384 days (*5*).

Although *B. microti* is the predominant cause of babesiosis in the United States, 5 cases caused by *B. divergens*–like organisms have been reported. Reports include residents of Missouri (1992 and 2010), Kentucky (2001), Washington (2002), and Arkansas (2017) (*4*,*6*–*8*). All case-patients were asplenic and had high levels of parasitemia. Three of these 5 patients died. Neither of the survivors received an exchange transfusion. Of the patients who died, 1 received an exchange transfusion, 1 did not, and the status for the third patient was unknown. *B. divergens*–like/MO-1 parasites have also been identified in cottontail rabbits and *Ixodes* spp. ticks on Nantucket Island, Massachusetts, USA (*9*).

In Europe, *B. divergens* is the most frequent cause of human babesiosis (≈40 reported cases), although the seroprevalence might be higher; 13% of patients with Lyme disease were seropositive in Sweden (*10*,*11*). Further analysis of cases of *B. divergens*–like/MO-1 infection in the United States showed that this infection is distinct from that of *B. divergens* in Europe on the basis of sequence analysis, lack of infectiousness to cattle, and distinct morphologic differences when grown in vitro (*12*). A case of *B. divergens*–like infection was reported in a patient on the Canary Islands in 1994 (*11*). Sequence analysis showed similarities to *B. divergens* but neither the organism nor its vector was present on the islands. Two possible *B. divergens*–like species were also identified in China in 2011 (*11*).

The distribution and number of babesiosis infections in the United States is increasing. In 2014, babesiosis was reported in 31 states, compared with 27 in 2013. The number of reported cases increased from 1,126 in 2011 to nearly 1,744 in 2014. Seroprevalance data from disease-endemic regions ranged from 6% to 16% for *B. microti*, which suggests the reported disease prevalence is underestimated (*2*). In 2016, there were 2 confirmed cases of *B. microti* infection in residents of Michigan, both of whom lived near the Wisconsin border. However, *B. microti* was not detected in the tick vector.

Although microscopic features are similar for all human-infecting *Babesia* species, *B. divergens* and *B. divergens*–like organisms are more likely to have tetrad forms (Maltese cross forms) and accole forms on peripheral blood smears than *B. microti*. Octad forms are rarely seen.

Treatment recommendations for *Babesia* infections are made on the basis of data for *B. microti* because clinical information regarding B. *divergens*–like infections is limited to case reports and treatment recommendations are available elsewhere (*3*). A recent study supports treating persons who are immunosuppressed for ≥6 weeks, including persons with negative blood smears for 2 weeks before discontinuation of therapy (*13*). In case-patients with parasitemia >10% or evidence of end-organ dysfunction, an emergent automated erythrocyte exchange transfusion is indicated. A 2-volume erythrocyte exchange should lead to a 90% reduction of parasite load (*14*,*15*). This procedure not only removes parasite-infected erythrocytes but also removes vasoactive factors, including thromboplastic substances and cytokines, which contribute to development of disseminated intravascular coagulation and renal failure (*14*).

In summary, babesiosis is a potentially fatal disease caused by protozoan parasites of the genus *Babesia*. *B. divergens*–like/MO-1 infections are rare in the United States,

and many patients who are infected had major illness and high mortality rates. Physicians should be alert for additional cases, particularly in asplenic persons. Further epidemiologic investigations of ticks in the upper midwestern United States for *B. divergens*–like organisms are warranted.

Acknowledgment

We thank the Centers for Disease Control and Prevention for confirming the diagnosis of *B. divergens*–like/MO-1 infection in this study.

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Fatal Nongroupable *Neisseria meningitidis* Disease in Vaccinated Patient Receiving Eculizumab

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Patients receiving eculizumab have an increased risk for meningococcal disease, but most reported cases are attributable to encapsulated meningococcal strains. We describe a case in which a nongroupable meningococcal strain, which rarely causes disease in healthy persons, caused fatal disease in an eculizumab recipient despite meningococcal vaccination.

Eculizumab (Soliris; Alexion Pharmaceuticals, New Haven, CT, USA) is currently the only disease-modifying agent approved for patients with paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome. Eculizumab decreases complement-induced hemolysis by preventing cleavage of C5 into C5a and C5b; however, this activity also prevents formation of the membrane attack complex that is essential for meningococcal serum bactericidal activity (*1*).

The Food and Drug Administration–approved eculizumab prescribing information (*2*) includes a warning for increased risk for meningococcal disease and requires a Risk Evaluation and Mitigation Strategy (*3*) to ensure patient and prescriber awareness of the meningococcal disease risk and need for meningococcal vaccination. However, meningococcal disease occurs in eculizumab recipients despite appropriate vaccination (*4*–*6*). Most reported cases have been caused by encapsulated isolates. We describe a fully vaccinated patient with PNH who received 2 doses of eculizumab and died shortly thereafter from overwhelming *Neisseria meningitidis* disease caused by nongroupable meningococci, which rarely cause disease in human hosts.

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DOI: https://doi.org/10.3201/eid2408.180228

Case Report

In March 2016, a 16-year-old previously healthy girl was brought to a children's hospital with abdominal pain, pancytopenia, and laboratory evidence of hemolysis. After extensive work-up, she received a diagnosis of PNH on the basis of peripheral blood flow cytometry. A bone marrow biopsy demonstrated cellularity ≈40%–50% and complete myeloid and erythroid maturation without evidence of dysplasia, aplasia, or an aberrant cell population. In anticipation of possible future eculizumab immunotherapy, she received a booster vaccine targeting *N. meningitidis* serogroups A, C, Y, and W-135 (Menactra; Sanofi Pasteur, Inc., Swiftwater, PA, USA) and a 2-dose series of vaccine targeting *N. meningitidis* serogroup B (MenB-4C) (Bexsero; GlaxoSmithKline, Bellaria Rosia, Sovicille, Italy).

During the first 4 months after diagnosis, the patient exhibited mild pancytopenia and compensated hemolysis but remained transfusion-independent. However, 6 months after PNH diagnosis, she began eculizumab treatment because of worsening symptoms.

Twenty-two hours after her second dose of eculizumab, the patient reported to a local emergency department with generalized body pain, headache, and emesis. She was afebrile, and her physical examination was unremarkable except for tachycardia of 124 beats per minute. Her leukocyte count was 8.9×10^9 cells/L (within reference limits); absolute neutrophil count was 8.46×10^9/L (upper limit 8.00×10^9 cells/L). Her symptoms were attributed to side effects from eculizumab and resolved after treatment with prochlorperazine, ketorolac, diphenhydramine, and intravenous fluids. She was discharged to home after 2 hours of observation.

Approximately 12 hours later, the patient reported weakness and purpura developed. She experienced cardiac arrest in transit to another emergency department, and resuscitative efforts were unsuccessful. Autopsy revealed hemorrhagic necrosis of the adrenal glands and focal hemorrhagic skin purpura, consistent with Waterhouse-Friderichsen syndrome.

By whole-genome sequencing, the meningococcal strain isolated from the meninges was found to be sequence type (ST) 2578 (clonal complex ST-41/44) and nongroupable with a capsule null locus, *cnl*. The inferred amino acid sequences of Factor H binding protein (FHbp) (peptide ID

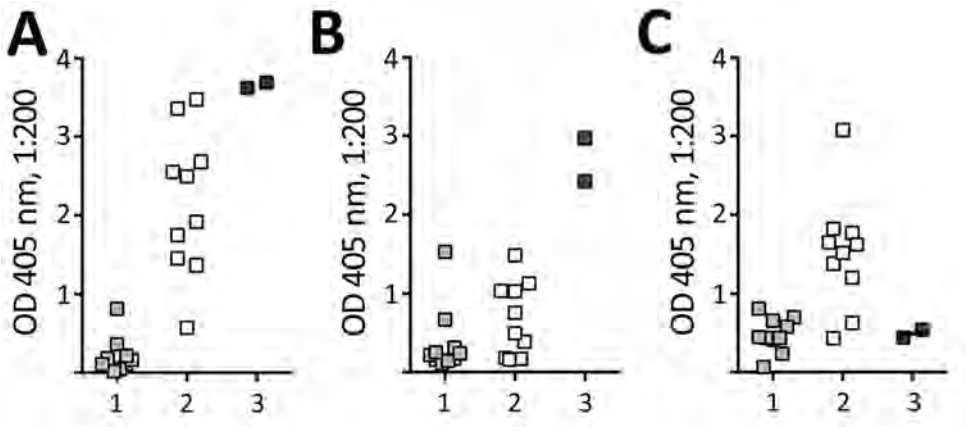

Figure 1. Serum IgG reactivity to 3 recombinant antigens in *Neisseria meningitidis* serogroup B meningococcal vaccine (MenB-4C) (Bexsero; GlaxoSmithKline, Bellaria Rosia, Sovicille, Italy), determined by ELISA. A postmortem serum sample (3) from a 16-year-old girl with paroxysmal nocturnal hemoglobinuria who died of meningococcal disease after treatment with eculizumab. Reactivity was measured in parallel with stored serum from 10 unvaccinated college students (1) and 10 vaccinated college students (2) 7 months after vaccination with MenB-4C (*8*). For the comparison specimens, each data point represents reactivity of an individual person. A) Factor H binding protein. B) *Neisseria* heparin binding antigen. C) NadA, MenB-4C antigens absent. Data points for (3) indicate results of replicate assays. OD, optical density.

100) and *Neisseria* heparin binding antigen (NHba) (peptide ID 2) were 97% and 100% identical to the respective antigens in the MenB-4C vaccine the patient had received, and both antigens were expressed on the surface of live bacteria based on flow cytometry. The remaining MenB-4C antigens were either absent (NadA) or mismatched (strain PorA P1,17,9).

Using 15% IgG-depleted human serum as a complement source, we found the strain was susceptible (titer >40) to bactericidal activity of mouse antiserum to recombinant FHbp ID 1, the subfamily B subvariant in the MenB-4C vaccine but not to mouse antiserum to NadA (not expressed by the strain) or to FHbp ID 22 (a subfamily A subvariant not in the vaccine) (titers <10). Mouse anti-NHba antiserum had a titer of ≤10. The relative resistance to anti-NHba was similar to previous findings that some NHba-expressing serogroup B meningococcal strains resist MenB-4C vaccine–elicited anti-NHba serum bactericidal activity (SBA) in humans (*7*).

A postmortem serum sample had high IgG reactivity against FHbp and NHba, both expressed by the infecting strain, and low reactivity to NadA (Figure 1). The high IgG reactivity specific for the 2 vaccine antigens expressed by the strain, but not for the vaccine antigen absent in the strain, suggests these antibodies may represent an IgG memory response elicited by infection. Given the short duration of symptoms, the likely stimulus for the memory antibody response would be asymptomatic nasopharyngeal colonization before disease onset.

We also measured bactericidal activity against the patient isolate using pooled IgG-depleted serum from 3 healthy unvaccinated adults, performed as previously described (*9*). Whereas 2 invasive serogroup B encapsulated strains survived in the IgG-depleted serum pool (Figure 2, panel A), the nongroupable case-strain was killed (Figure 2, panel B), which could be from complement alone or complement activated by naturally acquired IgM. However, 50 μg/mL of eculizumab (a concentration less than or equal to trough serum levels in treated patients [*10*]) completely blocked killing of the case-isolate (Figure 2, panel B).

Finally, we tested eculizumab's effect on whole blood killing of the unencapsulated case-isolate in an assay that measures a combination of SBA and opsonophagoctyic (OPA) activity (*11*,*12*). Cultures were sterile after 1–3 hours incubation in anticoagulated whole blood from MenB-4C–vaccinated or –unvaccinated adults (Figure 2, panels C, D), but 50 μg/mL eculizumab completely blocked bacterial killing. In contrast, the addition of a mouse monoclonal antibody to C7 (required for SBA but not OPA) did not prevent killing. These findings extend prior studies of whole blood killing of encapsulated serogroup B and C strains, which demonstrated that blocking C5 cleavage inhibited both SBA and OPA (*11*,*12*) killing of these strains, to a nonencapsulated meningococcal strain.

Conclusions

Collectively, these data indicate that eculizumab-treated patients remain profoundly susceptible to meningococcal disease, including from nongroupable meningococcal strains. Neither prior vaccination with MenB-4C, which matched 2 antigens present in the strain, nor the high serum antibody levels to FHbp and NHba in the patient prevented rapidly fatal disease in the presence of eculizumab. Similarly, eculizumab blocked killing of the nongroupable isolate by whole blood from healthy unvaccinated and vaccinated adults.

This case, along with several additional cases of meningococcal disease caused by nongroupable strains, was recently reported in eculizumab recipients in the United States (*13*). In otherwise healthy hosts, virtually all invasive meningococcal disease is caused by encapsulated strains (*14*).

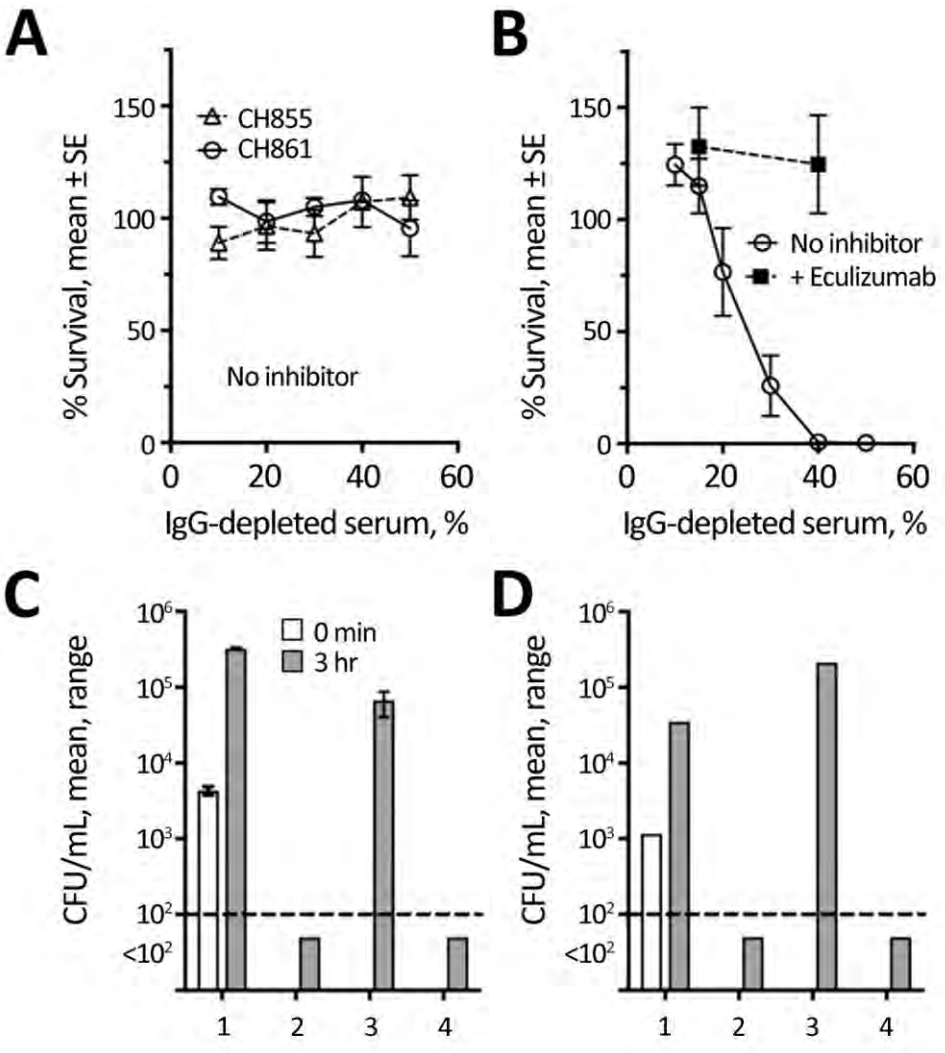

Figure 2. Effect of eculizumab on serum bactericidal activity and killing of *Neisseria meningitidis* by anticoagulated human blood. A) Complement-mediated bactericidal activity of an IgG-depleted human serum pool from 3 unvaccinated adult donors measured against encapsulated serogroup B strains CH855 and CH861 (data from 2–4 replicate assays for each strain). B) Bactericidal activity of pool tested in panel A measured against the nongroupable (NG) case isolate from a 16-year-old girl with paroxysmal nocturnal hemoglobinuria who died of meningococcal disease after treatment with eculizumab (data from 5 replicate assays). The addition of 50 µg/mL of eculizumab blocked bacterial killing of the NG case-isolate (data from 3 replicates). C, D) Killing of the NG case-isolate by anticoagulated human blood from 2 healthy adults, 1 previously vaccinated with 2 doses of *Neisseria meningitidis* serogroup B meningococcal vaccine (MenB-4C) (Bexsero; GlaxoSmithKline, Bellaria Rosia, Sovicille, Italy) with the last dose 14 months earlier (C), the other unvaccinated (D). The addition of 50 µg/mL of eculizumab to the blood from both donors blocked killing of the bacteria by the blood. The addition of a mouse anti-C7 monoclonal antibody, which blocked serum bactericidal activity (data not shown), did not inhibit whole blood killing. Similar results were obtained with blood from a third adult who had been vaccinated with MenB-4C 9 months earlier (data not shown). 1, negative control (plasma-heated at 56°C for 30 min to inactivate complement activity); 2, no inhibitor; 3, eculizumab; 4, anti-C7 (24 whole blood assay).

In contrast, unencapsulated strains are commonly associated with asymptomatic nasopharyngeal carriage. The sequence type of the isolate from this case, ST-2578, is rarely found in the PubMLST *Neisseria* database but previously has been observed primarily among isolates from asymptomatic carriers (*15*). Furthermore, the strain was killed by complement in human serum that had been depleted of IgG and by whole blood from an unvaccinated adult, features of commensal meningococcal strains that lack capsules.

In the United States, there is no official guidance on the use of antimicrobial chemoprophylaxis in eculizumab recipients. However, France and the United Kingdom recommend chemoprophylaxis for the duration of eculizumab therapy (references *16*,*17* in online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/18-0228-Techapp1.pdf), consistent with recommendations in recent case reports (*1*,*13*; reference *18* in online Technical Appendix). Most chemoprophylactic regimens for eculizumab-treated patients use penicillin or erythromycin (*1*; reference *18* in online Technical Appendix). Although the strain isolated from the patient reported was susceptible to penicillin, meningococcal disease has been reported in eculizumab patients receiving penicillin chemoprophylaxis caused by strains with penicillin resistance or intermediate sensitivity (*5;* reference *19* in online Technical Appendix). These breakthrough cases underscore the need for healthcare providers and patients to have a high index of suspicion for meningococcal disease, leading to quick recognition and consideration of early empiric treatment, regardless of the patient's vaccination status or chemoprophylactic regimen.

Acknowledgments

We thank Jarad Schiffer, Gowrisankar Rajam, and Cheryl Ellie for assistance with relevant assays not described here; the Centers for Disease Control and Prevention for characterizing the strain; and Jennifer Picarsic for her contribution to the pathology findings on autopsy.

This work was supported in part by grants from the National Institute of Allergy and Infectious Diseases, National Institutes of Health (R01 AI046464 and R01 AI114701 to D.M.G.). The laboratory work was performed in a facility funded by the Research Facilities Improvement Program grant from the National Center for Research Resources, National Institutes of Health (grant C06 RR016226).

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Distinguishing Zika and Dengue Viruses through Simple Clinical Assessment, Singapore

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Dengue virus and Zika virus coexist in tropical regions in Asia where healthcare resources are limited; differentiating the 2 viruses is challenging. We showed in a case–control discovery cohort, and replicated in a validation cohort, that the diagnostic indices of conjunctivitis, platelet count, and monocyte count reliably distinguished between these viruses.

Zika virus and dengue virus (DENV) are arboviral infections transmitted by the *Aedes* mosquito. Dengue is endemic in Singapore with >10,000 case notifications annually (*1*). Although Zika virus was known through serosurveys to circulate in Southeast Asia (*2*), confirmed infections had been scarce until August 2016, when the first recognized outbreak in Southeast Asia occurred in Singapore, following the epidemic in the Americas (*3*).

Co-circulation of both viruses poses challenges to healthcare providers in distinguishing between the 2 infections. These infections have similar clinical features, including fever, rash, and myalgia. Because most patients enter the primary healthcare setting with nonspecific symptoms, we sought to determine if either infection had distinguishing symptoms, signs, or basic laboratory findings.

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DOI: https://doi.org/10.3201/eid2408.171883

The Study

We conducted a case–control study at the National University Hospital with ethics approval from the hospital's Institutional Review Board. Patients infected with Zika virus and DENV who were seen at the hospital in 2016 constituted the discovery cohort. We confirmed Zika virus infection through testing for viral RNA in serum or urine, as described by Lanciotti et al. (*4*). We confirmed DENV infection through testing for serum DENV nonstructural protein 1 (NS1) antigen (SD BIOLINE Dengue DUO Kit; Standard Diagnostics, Kyonggi-do, South Korea) or by reverse transcription PCR (*5*). The clinical information collected included demographics, symptomatology, examination findings, and laboratory investigations, including complete blood count (with the monocyte count automated) and liver function test.

We compared clinical characteristics of both infections by univariate logistic regression against dichotomous symptomatology and continuous laboratory parameters. We selected predictors that could differentiate Zika virus and DENV infection as input for subsequent multivariate regression models and computed the area under the receiver operating characteristic curve (AUC) to compare model performance. We validated the results in a separate cohort of Zika virus and DENV patients from Tan Tock Seng Hospital, Singapore (*5*). From this validation cohort, we ascertained AUC and accuracy of the derived predictors. There were no pregnant patients in either cohort. We performed all analyses with R statistical software version 3.3.1 (http://www.R-project.org).

We identified 121 patients for the discovery study; 34 had Zika virus and 87 had DENV infection. Fifteen Zika patients (44.1%) were male and 19 (55.9%) female; 57 (65.5%) DENV patients were male and 30 (34.5%) female. Thirty-one Zika patients (91.1%) were PCR positive by urine test and 3 (8.9%) by plasma.

Zika patients sought treatment earlier in their illness than did DENV patients. Whereas viral symptoms including fever and arthralgia were common to both, differences were discernible (Figure 1). Conjunctivitis strongly indicated Zika virus infection (odds ratio [OR] 30.1, 95% CI 9.57–94.44; $p < 0.001$). In contrast, fever (OR 0.05,

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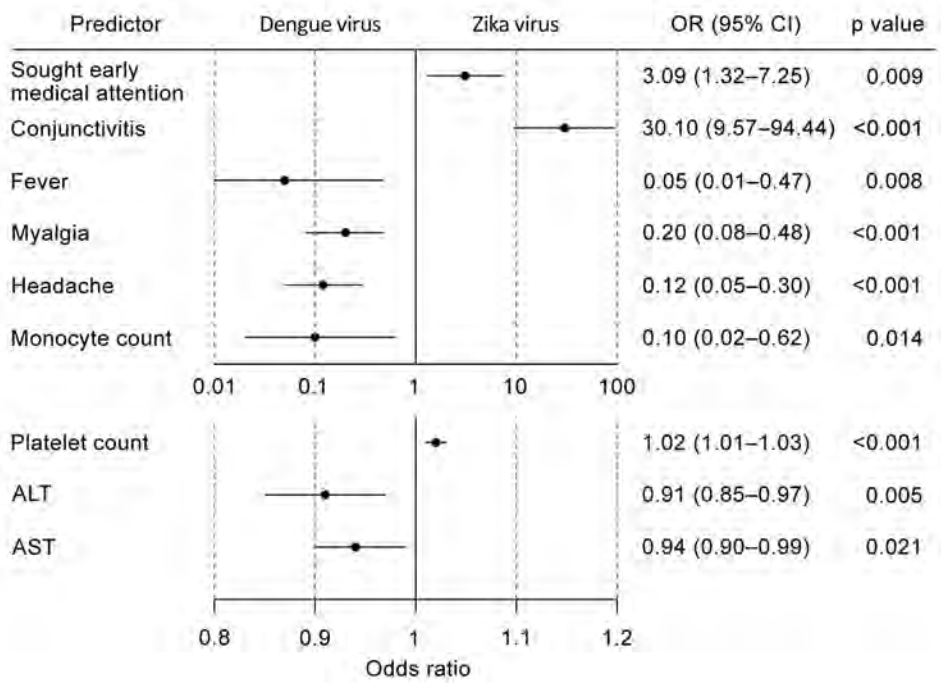

Figure 1. Univariate logistic regression model of clinical characteristics for patients in study of clinical assessments to distinguish Zika and dengue virus infections, Singapore. We analyzed early presentation (seeking treatment within 3 days of symptom onset), conjunctivitis, fever, myalgia, and headache as dichotomous variables, and laboratory findings (monocyte and platelet counts, ALT and AST levels) as continuous variables. For dichotomous variables, odds ratio (OR) >1 is predictive of Zika virus infection and <1 of dengue virus infection; for continuous variables, every unit increase in readout is predictive of Zika virus infection for OR >1 and dengue virus infection for OR <1. Error bars indicate 95% CIs. ALT, alanine aminotransferase; AST, aspartate aminotransferase.

95% CI 0.01–0.47; p = 0.008), myalgia (OR 0.20, 95% CI 0.08–0.48; p<0.001), and headache (OR 0.12, 95% CI 0.05–0.30; p<0.001) were more prominent in patients with DENV infection.

Further, DENV patients tended to have thrombocytopenia (median platelet count 132 × 10^9/μL, range 15–386 × 10^9/μL) and monocytosis (median monocyte count 0.50 × 10^9/μL, range 0.11–1.70 × 10^9/μL), whereas Zika patients tended to have normal platelet (median 225 × 10^9/μL, range 128–326 × 10^9/μL; p<0.001) and monocyte (median 0.35 × 10^9/μL, range 0.13–1.00 × 10^9/μL; p = 0.021) counts. The odds of Zika virus infection increased 2% with every unit (10^9/μL) increase in platelet count (OR 1.02, 95% CI 1.01–1.03; p<0.001) (Figure 1). Lower monocyte counts were associated with Zika virus infection (OR 0.10, 95% CI, 0.02–0.62; p = 0.014).

Patients with DENV had biochemical evidence of liver injury with hepatic alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels >2 times the upper reference limit (ALT, median 51.0, range 12–465 U/L; AST, median 65, range 20–720 U/L). The reference range for ALT is 10–70 U/L, and for AST, 10–50 U/L. In contrast, Zika virus patients did not have pronounced abnormalities in albumin, ALT, AST, or alkaline phosphatase levels.

Our findings point to conjunctivitis, platelet, monocyte, ALT, and AST levels as candidate markers to differentiate Zika virus patients from DENV patients. Conjunctivitis alone had an AUC of 0.79 in identifying Zika virus patients; normal platelet count in addition to conjunctivitis increased the AUC to 0.92, and adding a normal monocyte count further improved the AUC to 0.95 (Figure 2). The use of these 3 indices (conjunctivitis and platelet and monocyte counts) had 88% sensitivity and 93% specificity in distinguishing Zika virus from DENV, with a diagnostic accuracy of 92%. Inclusion of ALT and AST, however, did not further enhance the diagnostic capability.

We applied these 3 indices to a validation cohort consisting of 25 Zika virus and 70 DENV patients (Table

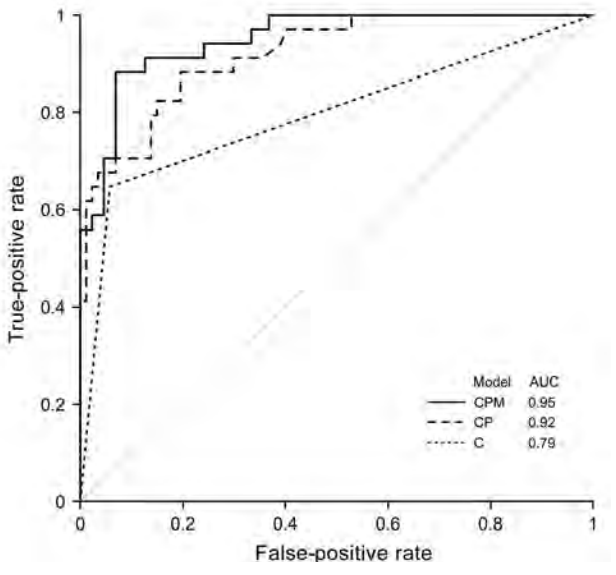

Figure 2. Receiver operating characteristics for different models in study of clinical assessments to distinguish Zika and dengue virus infections, Singapore. AUC is shown for different models: conjunctivitis alone (model C), conjunctivitis with platelet count (model CP), and conjunctivitis with platelet and monocyte counts (model CPM). AUC, area under the curve.

Table 1. Patient profile for validation cohort in study of clinical assessments to distinguish Zika and dengue virus infections, Singapore

Characteristic	Value, N = 95
Age	Median 38, mean 37.9, range 21–67
Sex	M 72, F 23
Day of illness*	Median 5, mean 4.7, range 2–9
Conjunctivitis	Yes 13, no 82
Fever	Yes 93, no 2
Myalgia	Yes 33, no 62
Headache	Yes 33, no 62
Monocyte count, × 10^9/μL	Median 0.32, mean 0.39, range 0.08–1.38
Platelet count, ×10^9/μL	Median 99, mean 115.2, range 13–308
Alanine aminotransferase, U/L	Median 33, mean 55.2, range 12–677
Aspartate aminotransferase, U/L	Median 44, mean 76.5, range 17–715

*Day on which care was sought.

1), resulting in an AUC of 0.90. Applying a cutoff score of 0.34 as determined by Youden's index to maximize sensitivity and specificity of our original model to the new validation dataset, the positive predictive value was 83% and negative predictive value 87%, achieving a similar diagnostic accuracy of 86%.

Zika virus and DENV coexist in many developing nations in equatorial South America and Southeast Asia, where there is limited accessibility to health resources and virus-specific diagnostics are not readily available. Differentiating Zika virus and DENV infections early is important in the prognostication and subsequent monitoring and follow-up of these patients. Although Zika virus infection is self-limiting, concerns about its sequelae in pregnant women and birth defects are well established (*6*). In contrast, severe DENV infection leads to debilitating illness that can cause vascular leakage, dengue shock, and death (*7*).

We applied both definitions from the US Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) for suspected Zika cases (*8*,*9*) in our patient cohort and found them to be unsatisfactory in distinguishing Zika virus from DENV patients (CDC, sensitivity 100%, specificity 2%; WHO, sensitivity 71%, specificity 67%) (Table 2). We therefore sought to develop more accurate indices to identify Zika virus among the backdrop of DENV cases in Singapore.

Our results highlight the utility of conjunctivitis and normal platelet and monocyte counts to distinguish Zika virus infection. We found conjunctivitis to be already a strong predictor of Zika virus infection. The study by Waggoner et al. in Nicaragua had reported conjunctivitis and rash in association with Zika virus infection (*10*). However, rash was not prominent among Zika patients in our study. Headache and myalgia were more common in DENV (*7*) and could help to distinguish DENV from Zika virus in our cohort. Prior studies had not ascertained if incorporation of basic laboratory indices could further enhance diagnostic capability. In our univariate logistic regression model, thrombocytopenia, transaminitis, and monocytosis were notable in DENV infection. Conversely, Zika patients tended to have normal platelet, aminotransaminase, and monocyte levels.

Conclusions

We were able to derive 3 simple clinical predictors on the basis of our findings: in the presence of conjunctivitis and normal platelet and monocyte counts, diagnostic AUC for Zika increased from 0.79 to 0.95, with 92% accuracy (88% sensitivity and 93% specificity). The accuracy of our derived indices exceeds that of WHO's and CDC's definitions for Zika case identification, notwithstanding that performance may differ with disease prevalence or population factors. Distinguishing Zika virus from DENV infection on clinical grounds remains daunting, and it will be ideal to validate these derived indices in a prospective patient cohort. Until then, these simple clinical assessments using conjunctivitis and basic blood count parameters will be helpful in regions of the world where both Zika virus and DENV are endemic.

Table 2. Sensitivity and specificity using CDC and WHO definitions of suspected Zika virus infection in study of clinical assessments to distinguish Zika and dengue virus infections, Singapore*

Case definition		Characteristic					
Source	Criteria	Patient meets criteria	Zika virus positive, n = 34	Zika virus negative, n = 57	Total, n = 91	Sensitivity, %	Specificity, %
CDC	Clinically compatible illness with ≥1 of the following not explained by another etiology: fever, rash, arthralgia, or conjunctivitis†	Yes	34	56	90	100	2
WHO	Fever and/or rash and any of the following: arthralgia, arthritis, nonpurulent conjunctivitis	Yes	24	19	43	71	67
		No	10	38	48		

*CDC, Centers for Disease Control and Prevention; WHO, World Health Organization.
†We excluded 2 additional criteria, complications of pregnancy and neurologic manifestations, because they were not present in our study population.

L.Y.A.C. has been supported by the Clinician Scientist Award (CSA), Individual Research Grant (IRG), Bedside & Bench (B&B) grants, Centre Grant, and the Training Fellowship Award from the National Medical Research Council (NMRC), Singapore. He also receives funding from the Aspiration Grant & Summit Research Program and Bench to Bedside Grant from the National University Health System, Singapore, as well as the Synthetic Biology Research & Development Program of the National Research Foundation, Singapore.

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Leptospirosis as Cause of Febrile Icteric Illness, Burkina Faso

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Patients in Burkina Faso who sought medical attention for febrile jaundice were tested for leptospirosis. We confirmed leptospirosis in 27 (3.46%) of 781 patients: 23 (2.94%) tested positive using serologic assays and 4 (0.51%) using LipL32 PCR. We further presumed leptospirosis in 16 (2.82%) IgM-positive specimens.

Worldwide, approximately 1 million cases of human leptospirosis occur each year, resulting in ≈60,000 deaths (*1*). Although epidemiologic data for Africa are scarce, especially in semiarid and arid regions, some observations suggest that *Leptospira* spp. may be more prevalent than previously thought (*2*). In our study, we tested the hypothesis that leptospirosis is a cause of febrile jaundice in Burkina Faso.

The Study

We conducted the study at Centre Muraz (Bobo Dioulasso, Burkina Faso), a central reference laboratory responsible for the national surveillance of yellow fever. We identified confirmed leptospirosis cases in accordance with World Health Organization criteria (*3*) by symptoms consistent with leptospirosis and a single microscopic agglutination test (MAT) titer ≥1:400, by detection of *Leptospira* DNA by PCR, or both. We identified presumptive cases by symptoms consistent with leptospirosis and the presence of IgM. Specimens testing negative for serologic and PCR were considered negative. We retrospectively tested samples collected during January 2014–July 2015 from adults and children with jaundice and fever ≥38.5°C for the presence of IgM against *Leptospira* spp. using an in-house ELISA (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/17-0436-Techapp1.pdf). We assessed serum that tested positive by ELISA for antibodies to *Leptospira* bacteria in the bacteriology laboratory of Montpellier University Hospital (Montpellier, France), using MAT to confirm the serologic results with a panel of 7 reference serogroups. We also tested for leptospirosis specimens for which a sufficient volume of serum was available by MAT in the French National Reference Center (Paris, France), using a larger panel of 24 serogroups, including the first 7 serogroups (Table 1). We performed real-time PCR for leptospirosis at Centre Muraz using PCR (PUMA LEPTO Kit; Omunis, Clapiers, France) targeting the *lipL32* gene, which is present exclusively in pathogenic *Leptospira* spp. bacteria (*4*).

Of 781 samples, 45 (5.57%) tested positive for leptospira IgM by ELISA (Figure 1). Among those samples, 23 (2.94%) were positive by MAT (≥1:400); consequently, these cases were considered to be confirmed. We considered 6 samples tested negative by MAT and 16 samples with MAT titer ranging from 1:100 to 1:200 (combined, 2.82%) to be presumptive cases. MAT results suggested the existence of multiple serogroups (Table 2), including reacting serogroups Australis, Ballum, Canicola, Grippotyphosa, Icterohaemorrhagiae, Pomona, and Sejroe. In addition, we performed MAT in the Leptospirosis National Reference Laboratory using a larger panel of serogroups applied to 33 ELISA-positive samples. Ten samples tested positive, and we confirmed the presence of all except the Ballum serogroup (data not shown). In 1 sample, we were able to identify Mini as an additional serogroup with a 1:400 titer. In addition to the serologic test, we confirmed leptospirosis cases by *lipL32* PCR in 4/781 (0.51%) samples. All were negative for IgM, but 3 had optical density just above the positive threshold; signal to mean value of the negative controls was between 2 and 3 (data not shown). Hence, screening by serologic assay plus PCR identified a total of 27 (3.46%) cases of confirmed leptospirosis.

Median age for all patients was 20 years (interquartile range [IQR] 12–30 years); 61% were male ($p = 0.65$ by χ^2 test). We observed the highest number of confirmed cases in the age group 10–19 years (data not shown), but the frequencies were not significantly different when cases were analyzed by age group ($p = 0.41$ by χ^2 test). This observation was not unexpected because the population of

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DOI: https://doi.org/10.3201/eid2408.170436

Table 1. *Leptospira* spp. serogroups used for microscopic agglutination test*

Sample no.	Species	Serogroup	Serovar	Strain
1	*L. interrogans*	Australis*	Australis	Ballico
2	*L. interrogans*	Autumnalis	Autumnalis	Akiyami A
3	*L. interrogans*	Bataviae	Bataviae	Van Tienen
4	*L. interrogans*	Canicola*	Canicola	Hond Utrecht IV
5	*L. borgpetersenii*	Ballum*	Castellonis	Castellon 3
6	*L. kirschneri*	Cynopteri	Cynopteri	3522 C
7	*L. kirschneri*	Grippotyphosa*	Grippotyphosa	Moskva V
8	*L. interrogans*	Sejroe	Hardjobovis	Sponselee
9	*L. interrogans*	Hebdomadis	Hebdomadis	Hebdomadis
10	*L. interrogans*	Icterohaemorrhagiae	Copenhageni	Wijnberg
11	*L. noguchii*	Panama	Panama	CZ 214 K
12	*L. biflexa*	Semaranga	Patoc	Patoc 1
13	*L. interrogans*	Pomona*	Pomona	Pomona
14	*L. interrogans*	Pyrogenes	Pyrogenes	Salinem
15	*L. borgpetersenii*	Sejroë*	Sejroë	M 84
16	*L. borgpetersenii*	Tarassovi	Tarassovi	Mitis Johnson
17	*L. interrogans*	Icterohaemorrhagiae*	Icterohaemorrhagiae	Verdun
18	*L. weilii*	Celledoni	ND	2011/01963
19	*L. interrogans*	Djasiman	Djasiman	Djasiman
20	*L. borgpetersenii*	Mini	ND	2008/01925
21	*L. weilii*	Sarmin	Sarmin	Sarmin
22	*L. santarosai*	Shermani	Shermani	1342 K
23	*L. borgpetersenii*	Javanica	Javanica	Poi
24	*L. noguchii*	Louisiana	Louisiana	LUC1945

*Serogroups were tested in the Montpellier CHU laboratory and National Reference Center at Institut Pasteur (Paris, France). ND, not determined.

Burkina Faso is young; almost two thirds of the population is <25 years of age. There was no particular gender distribution for persons with confirmed cases (13 women and 14 men; p = 0.33 by χ^2 test). The repartition of confirmed, presumptive, and negative cases according to rainy season (May–mid-October) versus dry season from mid-October–April was unequal (p = 0.0035 by χ^2 test), with a trend for a higher proportion of confirmed cases among samples received during the rainy season when compared with negative cases (p = 0.065 by χ^2 test; Figure 2).

Our data were in line with a recent publication estimating that some of the West Africa countries, including those in semiarid regions, may have among the highest rates of disability-adjusted life years due to leptospirosis; in Burkina Faso, the rate may be 60–70/100,000 population/year (*5*). Leptospirosis infections have been reported in various parts of West Africa in humans (*6–10*). Studies in Senegal and Mali have shown that cattle, pigs, and sheep are frequently infected (*11*,*12*). Detection of leptospirosis was also recently reported in rodents in Niamey,

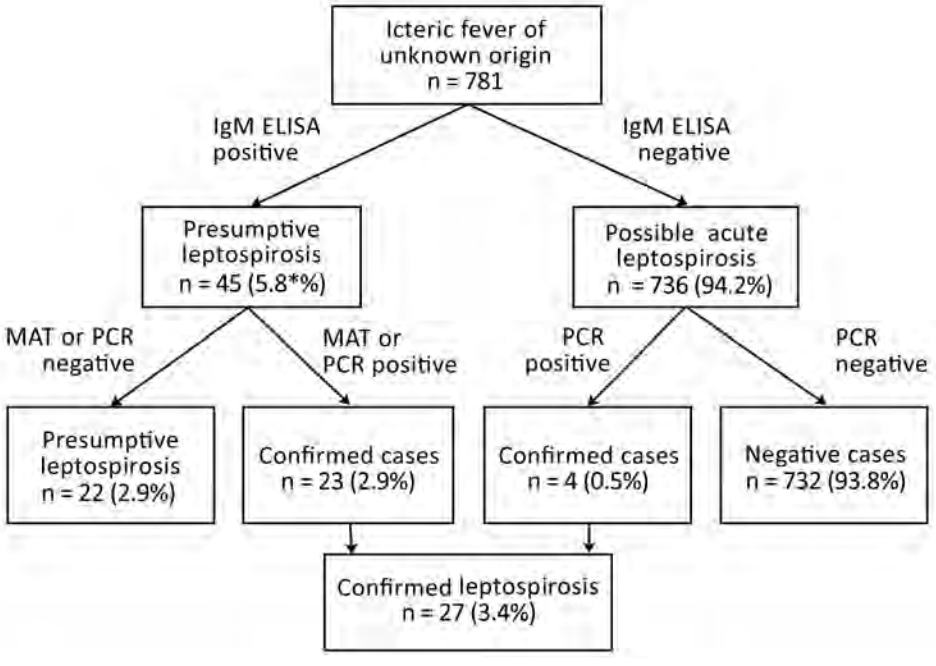

Figure 1. Flowchart used in study of leptospirosis in persons who sought medical attention for febrile jaundice, Burkina Faso. MAT, microscopic agglutination.

Table 2. Confirmed leptospirosis cases by serogroup using microscopic agglutination test, Burkina Faso*

Serogroup	No. positive, N = 23	Titer
Ballum	11 (1†)	1:400–1:1,600
Grippotyphosa	4 (1‡)	1:400–1:1,600
Australis	2	1:400 and,1:800
Canicola	1 1‡	1:400 and 1:800
Sejroe	1†(1§)	1:400
Icterohaemorrhagiae	1	1:800
Pomona	1	1:400
Mini	1§	1:400

*Values in parentheses represent duplicates, which are not included in the total count.
†Titers were equal for serogroups Ballum and Sejroe.
‡Titers were equal for serogroups Grippotyphosa and Canicola.
§Titers were equal for serogroups Mini and Sejroe.

Niger, especially in urban agricultural settings (*13*). In Burkina Faso, agricultural and livestock sectors represent 30% of the gross domestic product and are the backbone of the economy with ≈80% of the working-age population involved in these activities (*14*). Hence, human exposure to *Leptospira* spp. bacteria is probably frequent. Studies conducted in Ghana on patients with febrile illness without an obvious cause of disease found a frequency of 3.2% of confirmed leptospirosis cases and 7.8% of probable cases among icteric patients (*2*). In our study, half of the probable leptospirosis cases characterized by clinical signs consistent with leptospirosis and screened positive for *Leptospira* IgM were confirmed by MAT with a titer ≥1:400; two thirds had a titer ≥1:100 that may also be leptospirosis cases. Collecting and testing a convalescent serum sample might have confirmed the presumptive cases. In addition, the MAT has been shown to be less sensitive than IgM detection using ELISA, especially in acute-phase specimens (*15*). The low rate of molecular test positivity may be explained by the rapid disappearance of *Leptospira* spp. bacteria in the blood at the time the antibody response becomes detectable. Furthermore, transportation from the field to the centralized laboratory and storage at –20°C for ≥1 year before testing had a potentially adverse effect on the detection of low levels of *Leptospira* DNA.

Because our study was retrospective and based on single sample testing, we probably overlooked some cases of leptospirosis. The lack of detail about clinical symptoms and evolution was a limitation of this study. We recruited participants with the presence of jaundice in addition to fever; hence, it is probable that among the cases of confirmed leptospirosis, severe forms were overrepresented.

MAT provided a general insight into existing *Leptospira* serogroups within Burkina Faso, suggesting multiple reservoirs. However, cautious interpretation is invited because of the high degree of cross-reactions among different serogroups, especially in acute-phase serum samples. In addition, 2 instances of seroreactivity against Ballum serogroup observed in the first MAT performed in the Montpellier University Hospital were not confirmed by using an enlarged panel in the National Center for Leptospirosis. This finding may be related to prolonged sample storage and multiple freeze/thaw cycles before testing in the national reference laboratory.

Conclusions

Leptospirosis appears to be an important cause of febrile jaundice in Burkina Faso, suggesting that leptospirosis is probably endemic in this country. Further studies are required to explore animal reservoirs and occupational risk factors associated with human leptospirosis. Awareness of leptospirosis among clinicians, funding for further study, and the possibility of conducting laboratory tests in the field are needed to clarify the extent of the problem in sub-Saharan Africa.

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Dr. Zida works at the Muraz Center as a pharmacist. She is currently preparing a PhD at the University of Montpellier as a member of the U1058 INSERM team.

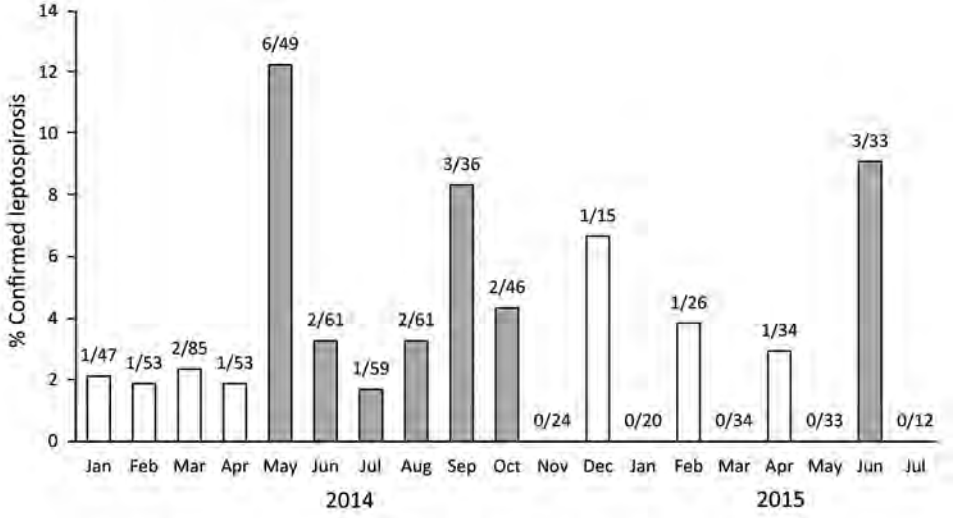

Figure 2. Confirmed cases of leptospirosis among samples received in Centre Muraz within the national network for yellow fever surveillance in Burkina Faso, January 2014–July 2015. White bars indicate months of the dry season, gray bars months of the rainy season. Numbers above bars indicate number of confirmed leptospirosis and the number of specimens tested.

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Direct Detection of *penA* gene Associated with Ceftriaxone-Resistant *Neisseria gonorrhoeae* FC428 Strain by Using PCR

David M. Whiley, Lebogang Mhango, Amy V. Jennison, Graeme Nimmo, Monica M. Lahra

The ceftriaxone-resistant *Neisseria gonorrhoeae* FC428 clone was first observed in Japan in 2015, and in 2017, it was documented in Denmark, Canada, and Australia. Here, we describe a PCR for direct detection of the *penA* gene associated with this strain that can be used to enhance surveillance activities.

Ceftriaxone, either monotherapy or in dual therapy with azithromycin, is the mainstay of treatment of patients diagnosed with *Neisseria gonorrhoeae* infection in most settings (*1*). Therefore, the identification of any strains exhibiting resistance to ceftriaxone is of considerable public health concern. Until 2017, ceftriaxone-resistant strains of *N. gonorrhoeae* had been rare and typically sporadic, including in 2009, H041 in Japan (*2*); in 2010, F89 in France (*3*); in 2011, F89 in Spain (*4*); in 2013, A8806 in Australia (*5*); in 2014, GU140106 in Japan (*6*); and in 2015, FC428 and FC460 in Japan (*7*). However, there is now evidence of sustained international transmission of FC428, reported during 2017 in Canada (*8*) and Demark (*9*) (1 case each) and in Australia (2 cases) (*10*). Rapid and timely detection is pivotal to contain further spread of antimicrobial drug–resistant *N. gonorrhoeae*. Here, we describe a real-time PCR protocol to facilitate enhanced surveillance for the FC428 clone. The study was approved by the University of Queensland Human Research Ethics Committee.

The Study

We designed a real-time PCR to target unique sequences on the *penA* gene of the FC428 *N. gonorrhoeae* clone (*10*). Modifications of *penA*, which encodes penicillin-binding protein 2 (PBP2), are a cause of cephalosporin resistance in *N. gonorrhoeae*. The FC428 clone harbors a mosaic *penA*-allele, designated as PenA-60.001 by results of *N. gonorrhoeae* sequence typing for antimicrobial drug resistance (*10*), and encodes alterations including A311V and T483S that have previously been associated with *N. gonorrhoeae* ceftriaxone resistance in H041 (*2*) and A8806 (*2*,*5*) strains. For this study, we designed 2 primers and 2 allele-specific probes (Table 1) to facilitate specific detection of the *penA* gene of FC428. In brief, the forward and reverse primers were designed to flank the A311V alteration; probe 1 was designed for detection of the A311V alteration; and probe 2 was designed to detect the wild-type A311 sequence. We added probe 2 to act as a blocker probe to limit binding of probe 1 with the wild-type sequence.

We prepared the reaction mix by using the QuantiTect Probe PCR Master Mix Kit (QIAGEN, Doncaster, Victoria, Australia). The reaction consisted of 12.5 μL of the Master Mix, 10 pmol/L of forward and reverse primers (Table 1), 4.0 pmol/L of each probe, and 5.0 μL of specimen nucleic acid, resulting in a total volume of 25 μL. We thermocycled the reaction mix by using the Rotor-Gene 6000 instrument (QIAGEN) and held it at 95°C for 15 min, then cycled (45 cycles) at 95°C for 15 s and 60°C for 60 s. We analyzed data by using the Rotor-Gene allelic discrimination software (QIAGEN).

We initially assessed the analytical performance of the assay by testing cultured isolates of *N. gonorrhoeae* (n = 72) and commensal *Neisseria* and *Moraxella* species (n = 111) (Table 2). We prepared these isolates by using a previously described heat-denaturation method (*11*). The *N. gonorrhoeae* isolates included the 2 FC428 strains recently documented in Australia (*10*), H041 (*2*) and A8806 (*5*); the ceftriaxone-resistant strains; and other local clinical *N. gonorrhoeae* isolates (n = 68). Both FC428 isolates provided strong

Table 1. Primer and probe sequences for PCR to detect *Neisseria gonorrhoeae* FC428 strain*

Designation	Oligonucleotide sequence, 5′ → 3′
Forward primer	CGCAACCGTGCCGTT
Reverse primer	GGGTATTGAATGTGTCTGTTGGA
Probe 1	Fam-TTCA+T+G+A+CA+G+AAC-Iowa Black FQ
Probe 2	Hex-TCA+T+G+G+CA+GA-Iowa Black FQ

*LNA bases are indicated by + preceding the base in the sequence.

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DOI: https://doi.org/10.3201/eid2408.180295

Table 2. *Neisseria* spp. isolates and specimens tested in development of PCR to detect *Neisseria gonorrhoeae* FC428 strain*

Isolates/samples	PCR results for FC428 (C_t)	
	Probe 1	Probe 2
Gonococcal species, n = 144		
Neisseria gonorrhoeae, n = 72		
N. gonorrhoeae FC428, n = 2 (*9*)†	Positive (19.8 and 18.17 cycles)	Negative
N. gonorrhoeae H041, n = 1 (*1*)	Negative	Negative
N. gonorrhoeae A8806, n = 1 (*4*)	Positive (37.8 cycles)	Negative
N. gonorrhoeae, n = 68‡	Negative	Negative
Nongonococcal species, n = 111		
N. cinerea, n = 4	Negative	Negative
N. elongata, n = 1	Negative	Negative
N. flavescens, n = 1	Negative	Negative
N. lactamica, n = 15	Negative	Negative
N. lactamica, n = 1	Positive (42.8 cycles)	Negative
N. meningitidis, n = 55	Negative	Negative
N. meningitidis, n = 1	Negative	Positive (32.6 cycles)
N. mucosa, n = 1	Negative	Negative
N. polysaccharеae, n = 4	Negative	Negative
N. sicca, n = 4	Negative	Negative
N. subflava, n = 14	Negative	Negative
N. weaveri, n = 1	Negative	Negative
Moraxella catarrhalis, n = 7	Negative	Negative
M. osloensis, n = 2	Negative	Negative
N. gonorrhoeae NAAT-positive clinical specimens, n = 358		
Urogenital, n = 172	Negative	Negative
Anal swab, n = 81	Negative	Negative
Throat swab, n = 95	Negative	Negative
Other, n = 10	Negative	Negative

*C_t, cycle threshold; NAAT, nucleic acid amplification test.
†Isolates A7846 and A7536.
‡Other local clinical isolates collected in New South Wales, Australia.

positive signals by using probe 1 with cycle threshold (C_t) values <20 cycles. The A8806 strain provided a late reaction at 37.8 cycles (probe 1), as did 2 commensal *Neisseria* strains: 1 *N. lactamica* isolate at 42.8 cycles for (probe 1), and 1 *N. meningitidis* isolate at 32.6 cycles (probe 2). The Figure shows a sequence alignment of the partial *penA* sequences from these 3 isolates compared with the FC428 PenA-60.001 allele. The A8806 strain shows considerable sequence homology with PenA-60.001 (including 100% match with the A311V Probe 1 sequence), albeit for 2 mutations in the forward primer designed to limit detection of A8806. We do not consider this a limitation of the assay because there has only been 1 reported case of infection with the A8806 strain. Neither the *N. lactamica* nor *N. meningitidis* isolates harbored the A311V alteration.

To compare detection limits, we tested 10-fold dilutions of FC428 DNA by both the FC428 PCR and a previously described in-house *N. gonorrhoeae* PCR, directed at the gonococcal *porA* and *opa* sequences (*12*). The in-house *N. gonorrhoeae* PCR had the lowest detection limit at 0.3 genome copies/reaction, whereas the detection limit of the FC428 PCR was 3.0 genome copies/reaction, indicating the FC428 PCR was 1 log less sensitive than the diagnostic method.

We then applied the assay to a convenience panel of *N. gonorrhoeae*–positive clinical samples (n = 358) submitted to Pathology Queensland Laboratory (Brisbane, Queensland, Australia) during February–September 2017 (Table 2). In brief, these samples comprised remnant nucleic acids from samples that tested positive for *N. gonorrhoeae* by the Cobas 4800 CT/NG test and were confirmed positive by using the in-house *N. gonorrhoeae* PCR (*12*). All samples provided negative results by the FC428 PCR, suggesting that the FC428 strain was not present in Queensland during this period.

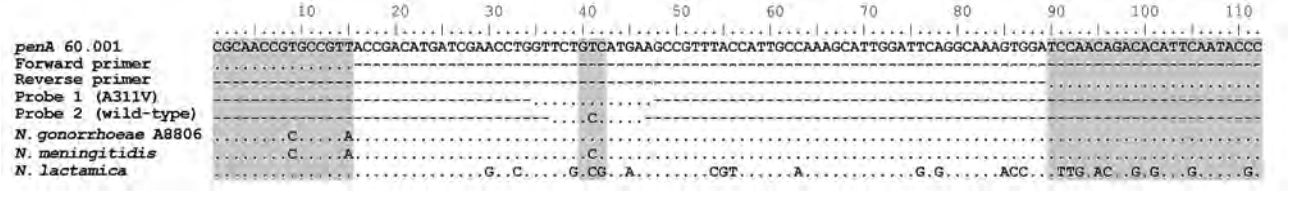

Figure. Sequence alignment showing the expected 112-bp PCR product for the PCR to detect *Neisseria gonorrhoeae* FC428 strain. PenA type 60.001 is provided as the reference sequence. Gray indicates the primer targets and the 311 codon within the probe target sequences. The *penA* sequences from the *N. gonorrhoeae* A8806, *N. meningitidis*, and *N. lactamica* isolates that cross-reacted with the FC428 PCR are also provided. Dots indicate sequence identity.

Conclusions

Overall, our results suggest that the FC428 PCR is suitable for screening for the FC428 *N. gonorrhoeae* clone in clinical specimens for which culture is not available. The method could prove to be a strategic tool to enhance surveillance if FC428 continues to spread. We recommend that positive results be confirmed by, for example, DNA sequencing, particularly if the strain is detected in a pharyngeal sample in which other commensal *Neisseria* species are prevalent.

Acknowledgments

We thank Integrated DNA Technologies for technical support with oligonucleotide design, and the staff at the Molecular Diagnostic Unit of Pathology Queensland for their assistance with the study.

This study was funded by internal funds from the Australian Government Department of Health and Ageing. D.M.W. is a recipient of an NHMRC fellowship and receives research funding from SpeeDx Pty Ltd.

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Identification of Peste des Petits Ruminants Virus, Georgia, 2016

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DOI: https://doi.org/10.3201/eid2408.170334

A phylogenetic analysis of samples taken from reported outbreaks of peste des petits ruminants virus (PPRV) in Georgia revealed a closer relationship to viruses from northern and eastern Africa than to viruses from countries closer to Georgia. This finding has crucial implications for the control of PPRV in the region.

Peste des petits ruminants virus (PPRV) is the cause of a highly infectious transboundary animal disease that affects primarily sheep, goats, and small wild ruminants. Death rates for PPRV in susceptible animals can be as high as 80% (*1*). Because sheep and goats contribute considerably to the household and cash income and nutrition of small farmers in many countries, the control of PPRV is considered an essential element in the fight for global food security and poverty alleviation. For this reason, PPRV is being targeted by international organizations for global eradication by 2030 (*1*).

Currently, 4 genetic lineages of PPRV are circulating globally. The lineages are defined on the basis of sequence comparison of a fragment of either the nucleocapsid (N) or fusion (F) protein genes of the virus. PPRV lineage IV is found predominantly in Asia and the Middle East, whereas all 4 lineages have been reported in Africa (*2*).

During January–March 2016, outbreaks of PPRV in Tushuri sheep were reported in 3 farms located near Tbilisi, the capital of Georgia. Of 3,740 susceptible sheep, 415 (11%) showed symptoms of PPRV infection, which included necrosis of the commissures of the lips; swelling and bleeding of the gums above the dental pad and buccal mucosa, with white cellular debris on all surfaces, including the tongue; bronchopneumonia (in only a few animals); diarrhea (in 50% of lambs); and loss of appetite. Of the diseased animals, 204 (49%) died, 99 (24%) were humanely destroyed, and the rest recovered. The outbreaks were resolved by the end of March 2016 (*3*).

Staff of the National Food Agency in Tbilisi collected nasal swabs and ocular samples, which were tested in the laboratory of the Ministry of Agriculture in Tbilisi using a PPR antigen capture ELISA (IDvet, Grabels, France). Six positive samples were individually adsorbed onto the matrix of a ViveST transport tube (ViveBio Scientific, Alpharetta, GA, USA) and were shipped to the Institute for Veterinary Disease Control, Austrian Agency for Health and Food Safety (Mödling, Austria), for further characterization. A part of the same 6 samples was also shipped to the World Organisation for Animal Health reference laboratory at the Agricultural Research Center for International Development (Montpellier, France) for PPRV testing.

In Austria, we eluted the samples from the ViveST with 1 mL of Dulbecco's modified Eagle medium high-glucose medium and stored them at –80°C. We extracted total RNA from 200 μL aliquots using an RNeasy kit (QIAGEN, Hilden, Germany). We analyzed the extracted RNA samples by reverse transcription PCR (RT-PCR) using the One-Step RT-PCR kit (QIAGEN) to amplify fragments of both the PPRV N and F genes (*4*,*5*). Three of the 6 samples tested were positive by RT-PCR (PPRV/Georgia/G1/2016 [collected January 13, 2016], PPRV/Georgia/G2/2016 [collected February 9, 2016], and PPRV/Georgia/G4/2016 [collected February 9, 2016]). We purified the amplicons and sent them for sequencing using standard Sanger methods at LGC Genomics (Berlin, Germany). The sequences have been deposited in GenBank (accession nos. KY646059–64).

We constructed a phylogenetic tree of N and F gene segments from a representative selection of PPRV sequences available in GenBank, using the maximum-likelihood method available in MEGA6 (https://www.megasoftware.net/) and employing the Kimura-2 parameter model of nucleotide substitution with 1,000 bootstrap replications. The phylogenetic analysis revealed that the PPRVs present in the 3 samples from Georgia were identical and belonged to lineage IV (Figure). Of note, the N gene fragment sequences (Figure, panel A) were more related to those of viruses from Egypt, Eritrea, Ethiopia, and Sudan and the F gene fragment sequences clustered with viruses from Egypt, Ethiopia, and Sudan (Figure, panel B).

Unexpectedly, the N and F gene fragment sequences for viruses isolated from countries close to Georgia (e.g., Turkey, Iran, and Iraq) were less similar to the Georgia viruses than to the ones from Africa. PPRV is a transboundary infectious disease; in many cases, new

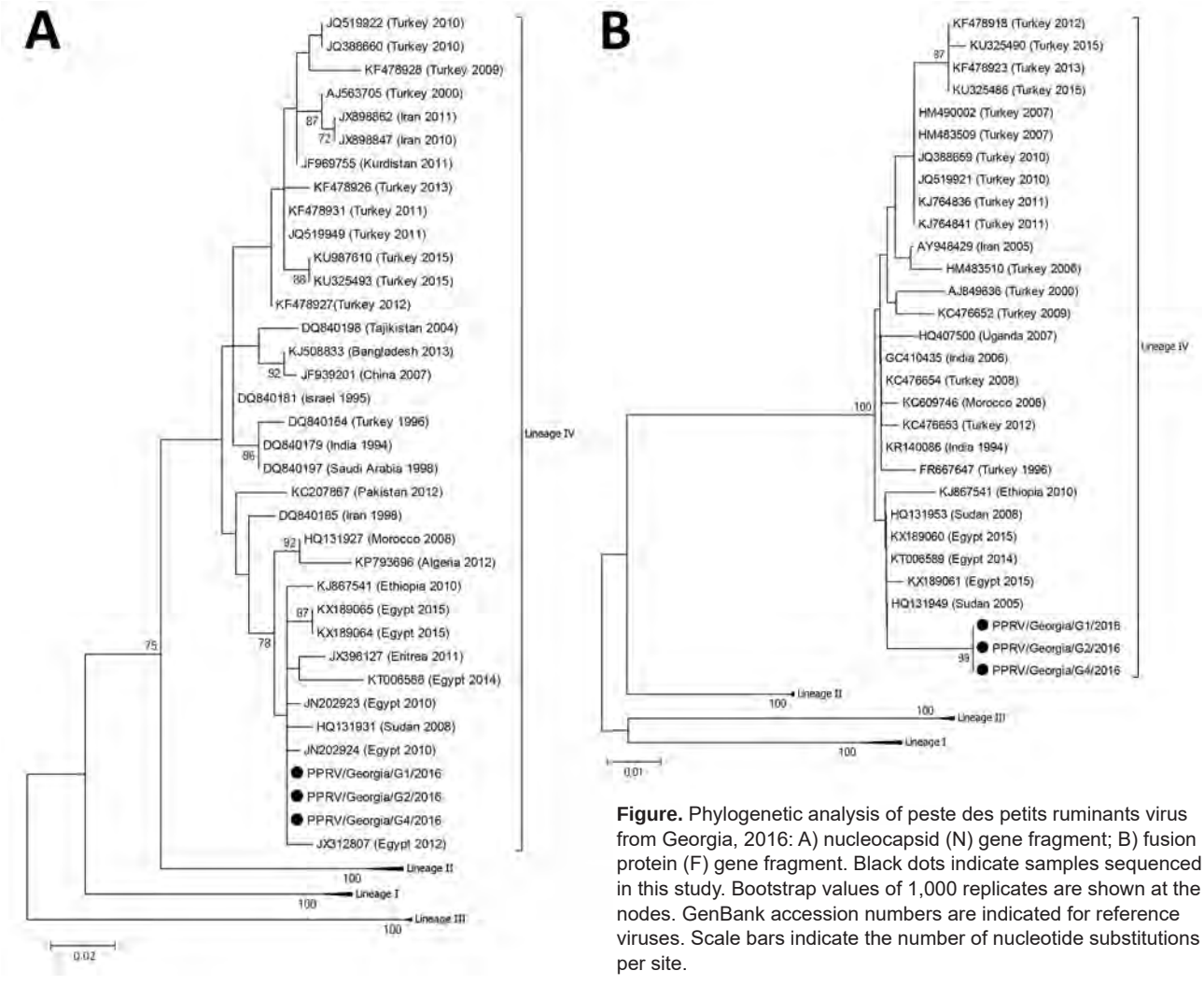

Figure. Phylogenetic analysis of peste des petits ruminants virus from Georgia, 2016: A) nucleocapsid (N) gene fragment; B) fusion protein (F) gene fragment. Black dots indicate samples sequenced in this study. Bootstrap values of 1,000 replicates are shown at the nodes. GenBank accession numbers are indicated for reference viruses. Scale bars indicate the number of nucleotide substitutions per site.

outbreaks are attributable to incursion from neighboring countries (*6*–*8*). Therefore, a similar situation would have been expected for Georgia, which shares borders with Turkey, Armenia, and Azerbaijan in the south and Russia in the north. Several molecular epidemiologic studies of PPRV lineage IV in Turkey have been performed (*9*–*11*), including more than 200 N and F gene sequence submissions in GenBank covering a period of several years (1996–2015) that provide an up-to-date overview of the PPRVs circulating in the country. However, none of these sequences is similar enough to the sequences from Georgia to indicate a common origin (Figure). To date, Azerbaijan, Armenia, and Russia have not reported PPRV in their countries, which makes it difficult to determine the exact origin of the PPRV identified in Georgia. Because there is no obvious connection between Georgia and Egypt, Eritrea, Ethiopia, or Sudan through the trade or import of small ruminants, further work is required to fully clarify the PPRV situation at a regional level.

Acknowledgments

The authors thank the National Food Agency of Georgia for providing the samples and the US Defense Threat Reduction Agency for support.

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Isolation of Complete Equine Encephalitis Virus Genome from Human Swab Specimen, Peru

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DOI: https://doi.org/10.3201/eid2408.171274

While studying respiratory infections in Peru, we identified Venezuelan equine encephalitis virus (VEEV) in a nasopharyngeal swab, indicating that this alphavirus can be present in human respiratory secretions. Because VEEV may be infectious when aerosolized, our finding is relevant for the management of VEEV-infected patients and for VEEV transmission studies.

Venezuelan equine encephalitis virus (VEEV) is one of many alphaviruses transmitted through the bite of infected mosquitoes (*1*,*2*). VEEV primarily infects equine species, causing severe encephalitis and death. VEEV may also infect humans, causing fever and influenza-like symptoms that include headache, chills, myalgia, nausea, and vomiting. In severe cases, human VEEV infection may result in neurologic complications that lead to fatalities. Acute VEEV infection is usually confirmed by PCR, sequencing from blood (*3*), or both, or in the case of encephalitis, from spinal fluid. Nasopharyngeal swabs are rarely tested for alphaviruses like VEEV because they are considered nontraditional sample types for these kinds of pathogens.

The US Naval Medical Research Unit No. 6, in coordination with the local ministry of health, conducts routine surveillance for respiratory and febrile pathogens under Institutional Review Board–approved protocols that comply with all applicable federal regulations governing the protection of human subjects. As part of these efforts, nasopharyngeal swabs and serum samples are tested for a variety of possible etiologies. Frequently, however, a particular sample fails to yield a recognized etiology. This result happens in several scenarios: a patient is infected with an unknown pathogen or with a pathogen for which there is no known diagnostic; a known pathogen has mutated and changed such that a known diagnostic is no longer effective; or a pathogen is present at a concentration too low to diagnose. In these cases, it may be possible to identify the potential etiology using approaches that can identify novel or divergent pathogens, like unbiased next-generation sequencing (NGS).

In 2013, in Iquitos, Peru, we identified a 16-year-old boy who reported a variety of undifferentiated illness symptoms, including fever, chills, general malaise, myalgia, headache, rhinorrhea, sore throat, nausea, vomiting, abdominal pain, retroorbital pain, rash, and

photophobia. A field-administered rapid test for influenza on a nasopharyngeal swab specimen was negative. More thorough laboratory tests, including a highly multiplexed MassTag PCR assay that can detect ≥20 viral and bacterial pathogens (*4*), were also negative (Figure, panel A). The sample entered a pathogen discovery pipeline routinely used to amplify and identify unknown etiologies (*5*). In brief, we cultured 200 μL of the original nasopharyngeal sample in Vero-E6, LLCMK2, and MDCK cells. Cytopathic effect appeared by day 4 in Vero-E6 and LLCKM2 cells and by day 19 in MDCK cells. We used culture supernatant from day 4 LLCMK2 cells as starting material for NGS MiSeq libraries, which we prepared, sequenced, and processed as described (*5*). Sequencing generated 704,444 raw reads, from which 28,555 were de novo assembled into a single 11,412-nt contig (Figure B). When blasted, the contig matched the complete genome of a VEEV-ID strain isolated in Peru from 1994 (GenBank accession no. KC344526.1) with 99% identity.

Given the health implications of detecting live alphaviruses in human respiratory secretions, we sought to confirm the finding by retesting the original nasopharyngeal swab, and by examining available paired serum

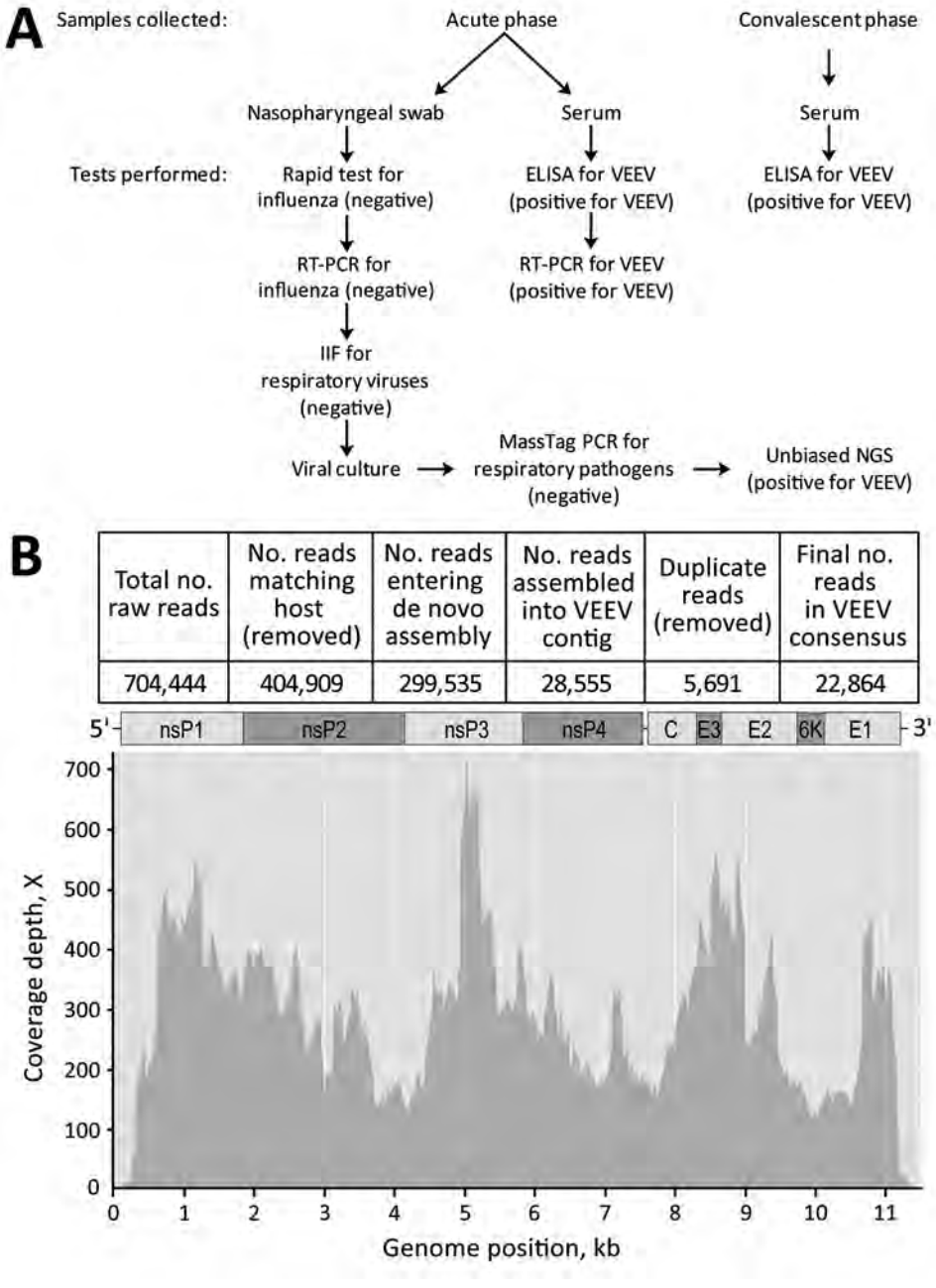

Figure. Testing for respiratory pathogens in a 16-year-old boy in Iquitos, Peru. A) Testing algorithm showing the types of samples collected during both acute- and convalescent-phase periods, the tests performed on each, and the results (in parentheses). B) Depth of coverage plot and schematic representation of the isolated VEEV genome, including all genes (nsP1, nsP2, nsP3, nsP4, C, E3, E2, 6K, and E1), drawn to approximate scale. Genomic information for this isolate has been deposited in GenBank (accession no. MF590066). Flu, influenza virus; IIF, indirect immunofluorescence; NGS, next-generation sequencing; VEEV, Venezuelan equine encephalitis virus.

samples collected during acute and convalescent periods. All confirmatory tests we conducted (*3*) were positive for VEEV: the original swab and the acute serum sample tested positive for VEEV by reverse transcription PCR and confirmatory Sanger sequencing; the acute-phase serum sample enabled isolation of VEEV; and the convalescent-phase serum sample showed a 1:1,600 rise in IgM titer, indicating the patient had seroconverted. Subsequent inspection of the associated metadata also indicated the sample had been collected at a time when VEEV circulated in the region.

Febrile surveillance studies have shown that VEEV is prevalent in many countries throughout Latin America (*3*,*6*). Although respiratory symptoms have been reported in association with VEEV infection (*7*) and there is ≥1 report of VEEV in throat swabs (*8*), human respiratory secretions are seldom tested for alphaviruses. In fact, the presence of VEEV in respiratory secretions, as well as its implications for health and biosafety, are rarely discussed. Although person-to-person transmission of VEEV has not been documented, VEEV can be infectious through aerosolized particles (*9*,*10*), so the potential for an alternate transmission route exists. This possibility should be considered both during individual management of VEEV-infected patients and in studies considering VEEV transmission dynamics or prevention strategies.

Acknowledgments

We thank Dirección Regional de Salud—Loreto for support with this study and Steev Loyola, Gilda Troncos, Alejandra Garcia-Glaessner, and Karla Prieto for technical support.

Funding from the Global Emerging Infections Surveillance and Response System (GEIS) of the Armed Forces Health Surveillance Center (AFHSC) was through work unit number 800000.82000.25GB.B0016, Promis ID#MLeguia-PO166-14, for FY2014.

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Plasmodium ovale wallikeri in Western Lowland Gorillas and Humans, Central African Republic

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DOI: https://doi.org/10.3201/eid2408.180010

Human malaria parasites have rarely been reported from free-ranging great apes. Our study confirms the presence of the human malaria parasite *Plasmodium ovale wallikeri* in western lowland gorillas and humans in Dzanga Sangha Protected Areas, Central African Republic, and discusses implications for malaria epidemiology.

The transmission of infectious diseases between wild animals and humans is a dynamic process in which wildlife populations have been a major source of zoonotic diseases. Six human malaria parasite species have been recognized during the past decade: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. knowlesi*, *P. ovale curtisi*, and *P. ovale wallikeri*. The role of human malaria parasites as zoonotic agents in free-ranging great apes remains unclear. Various *Plasmodium* species have been documented in great apes by using molecular data, including *P. praefalciparum*, *P. adleri*, and *P. blacklocki* in western lowland gorillas (*Gorilla gorilla gorilla*) (*1*). Moreover, parasites closely related to human malaria parasites (e.g., *P. ovale*–like and *P. vivax*–like) have been identified in other free-ranging great apes (*2*). Although contact between free-ranging gorillas and humans has increased steadily, no shared malaria parasites have previously been reported in humans and gorillas. A recent study of western lowland gorillas revealed a unique case of *P. ovale* infection, based on amplification of the mitochondrial cytochrome b (*cytb*) gene from gorilla fecal samples (*1*). To clarify the implications of possible co-circulation of malaria parasites in humans and gorillas, we reevaluated human blood and western lowland gorilla fecal samples from the previous study using state-of-the-art molecular tools.

Dzanga Sangha Protected Areas is located in southwestern Central African Republic, bordering the Democratic Republic of the Congo in the east and Cameroon in the west. We collected 131 gorilla fecal samples during August–October 2012 from 2 habituated western lowland gorilla groups and from several unhabituated gorillas around the Primate Habituation Programme camps of Mongambe and Bai Hakou. We collected human blood samples in 2012 as part of a health monitoring program for National Park and Primate Habituation Project employees. We received 95 blood samples from asymptomatic participants.

We performed PCR within the nuclear 18S small subunit ribosomal RNA gene, as previously described (*3*,*4*). This gene is the most commonly used for diagnosis of *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* (primers rOVA1WC/rOVA2WC), *P. ovale curtisi* (rOVA1/rOVA2), *P. ovale wallikeri* (rOVA1v/rOVA2v), and *P. knowlesi* in humans. We amplified parts of the mt *cytb* gene to obtain ≈939-bp fragments (*1*). We also analyzed a shorter fragment of ≈480 bp with hemosporidian-specific primers (*5*). We used a hemosporidian-specific nested PCR performed within the mitochondrial cytochrome c oxidase subunit 1 gene (primers: cox1a/cox1b and cox1c/cox1d) to amplify a 964-bp fragment (*6*). For further analysis of *P. ovale*, we used the primers Porbp2TMfwd/Porbp2TMrev, binding a 120-bp fragment of the nuclear *P. ovale* reticulocyte binding protein 2 gene (*7*).

Overall, 23 out of 95 (24.2%; 95% CI 16.7%–33.7%) asymptomatic human samples had positive results in *Plasmodium*-specific PCRs. Species-specific analysis revealed *P. falciparum* monoinfections in 19 cases (20%; 95% CI 13.2%–29.1%) and *P. ovale wallikeri* in 2 cases (2.1%; 95% CI 0.6%–7.3%). We found a mixed infection of *P. falciparum* and *P. malariae* (2.1%; 95% CI 0.6%–7.3%) in 2 samples. In the previous study, 42 of 131 fecal samples from western lowland gorillas tested positive for *Plasmodium* parasites, namely *P. adleri* (n = 21), *P. praefalciparum* (n = 9), and *P. blacklocki* (n = 7), but also *P. ovale*–like (n = 1) and *P. vivax*–like (n = 3) (*1*). In this study, reevaluating the gorilla fecal DNA extracts for human malaria parasites confirmed 1 sample from a habituated individual as positive for *P. ovale wallikeri*. Identical sequences were obtained in mt *cytb* (GenBank accession no. KJ930413) and mt *cox*1 (GenBank accession nos. MG251662, MG251663) with the gorilla *P. ovale wallikeri* isolate and 1 of the human isolates (Figure). The analysis of the nuclear small subunit rRNA and the *porbp*2 gene gave positive results only for the human isolate (GenBank accession nos. MG251661, MG255222).

[1]These authors contributed equally to this article.

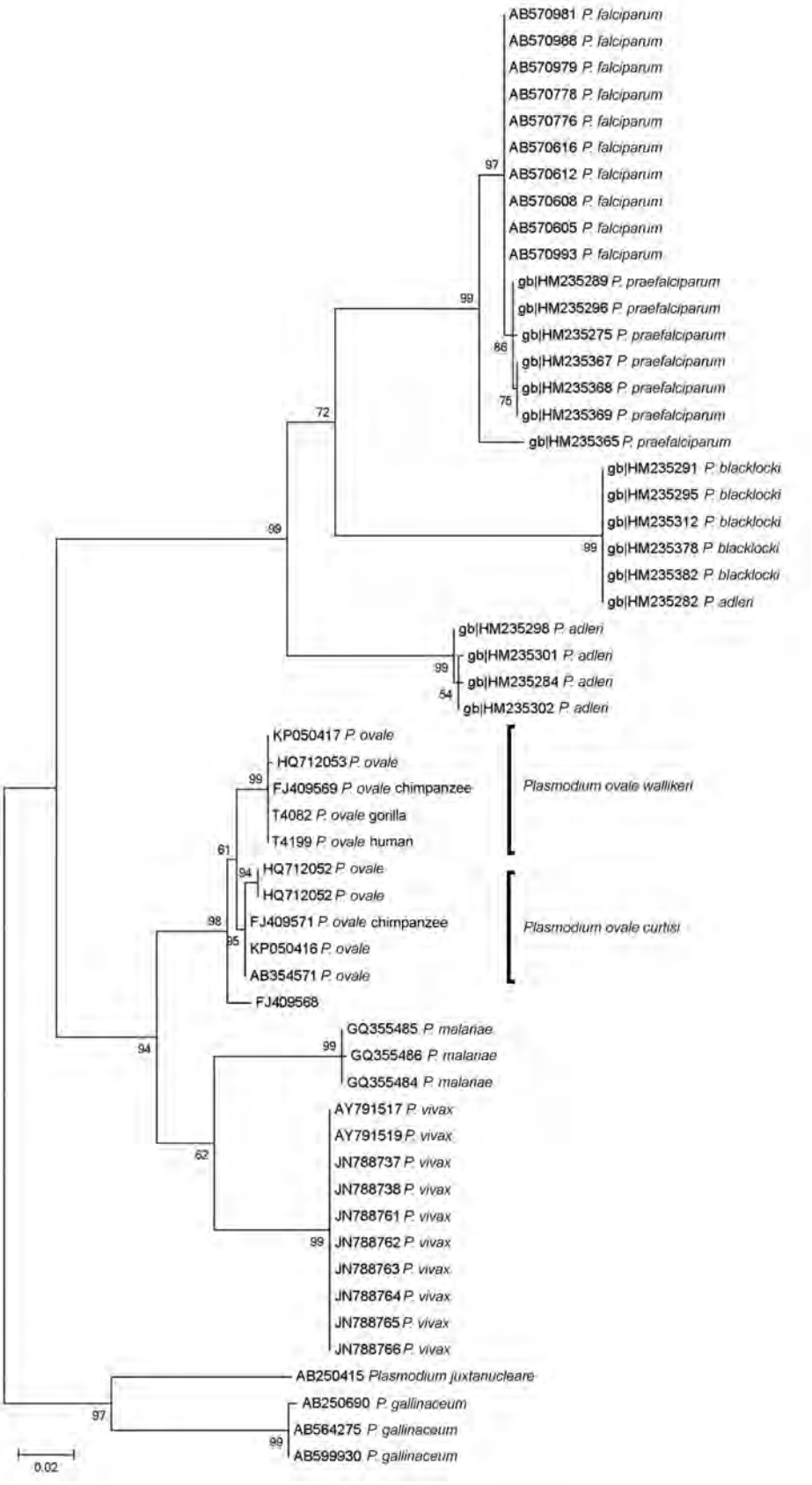

Figure. Maximum-likelihood phylogenetic trees of cytochrome C oxidase subunit 1 (*cox*1) gene (656-bp) sequences from African great apes and human *Plasmodium* spp. reference strains. GenBank accession numbers are indicated. Scale bar represents nucleotide substitutions per site.

In this study, we found the human malaria parasite *P. ovale wallikeri* in both asymptomatic humans and western lowland gorillas in Dzanga Sangha Protected Areas. Molecular analysis revealed that the genotype of the *P. ovale wallikeri* DNA found in a gorilla was genetically identical to that of a human isolate within the mt *cytb* and mt *cox*1 genes, indicating potential human–ape transmission. Analysis of nuclear genes failed in gorilla feces; thus, it remains unclear which parasite stages can be detected in feces (*8*). Although the mt *cytb* and mt *cox*1 genes are not the best-suited genes for genotyping human malaria parasites because of their homogeneity, these genes allow clear species discrimination from *P. ovale*–like parasites found in, for example, chimpanzees in Côte d'Ivoire (*9*) and the Democratic Republic of the Congo (*10*), which have never been reported in humans. This finding in a western lowland gorilla corroborates a finding of *P. ovale* in a chimpanzee from Cameroon (*6*) with a sequence identical to *P. ovale wallikeri*. However, further studies are required to evaluate the role of great apes as reservoirs of human malaria parasites and vice versa, and the implications of these findings for malaria epidemiology.

Acknowledgments

We thank Barbara Eigner for her excellent technical support and Colin Sutherland for the critical assessment of the manuscript. We also thank the government of the Central African Republic and the World Wildlife Fund for granting permission to conduct our research in the Central African Republic; the Ministre de l'Education Nationale, de l'Alphabetisation, de l'Enseignement Superieur, et de la Recherche for providing research permits; and the Primate Habituation Project for providing logistical support in the field.

This study was supported by the European Social Fund, the state budget of the Czech Republic (project OPVK CZ.1.07/2.3.00/20.0300), and a grant from the Czech Academy of Sciences (IGA 1230-21-IG 121 231).

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Dapsone Resistance in Leprosy Patients Originally from American Samoa, United States, 2010–2012

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DOI: https://doi.org/10.3201/eid2408.180033

Skin biopsies from US leprosy patients were tested for mutations associated with drug resistance. Dapsone resistance was found in 4 of 6 biopsies from American Samoa patients. No resistance was observed in patients from other origins. The high rate of dapsone resistance in patients from American Samoa warrants further investigation.

Standard treatment for leprosy is multidrug therapy with dapsone, rifampin, and clofazimine (*1*). Resistance to dapsone and rifampin has been observed in many leprosy-endemic regions of the world (*2*–*5*). The Global Sentinel Surveillance for Drug Resistance in Leprosy program was established by the World Health Organization to monitor global leprosy drug resistance among cases of relapse (*6*). With this program, 9 cases of dapsone resistance and 1 case of rifampin resistance were found among 72 leprosy relapse patients from 8 participating countries in 2010 (*7*). However, some leprosy-endemic countries and countries with low incidence, such as the United States (≈200 cases/y), are not involved in this program, even though most US patients with leprosy migrated from endemic areas where drug resistance has been identified (*8*).

Previously, we performed a survey of drug resistance among US leprosy patients referred to the National Hansen's Disease Programs (Baton Rouge, Louisiana, USA) during 2010–2012 (*8*). Of 39 patients with origins in the Pacific Islands (n = 18), Central or South America (n = 5), Asia (n = 2), and the United States (n = 14), 1 patient had dapsone-resistant *Mycobacterium leprae* and 1 had dapsone- and rifampin-resistant *M. leprae* (*9*). Both of these cases originated in American Samoa.

We expanded this previous survey by evaluating an additional 11 US leprosy patients from American Samoa and US patients from other geographic origins for susceptibility to dapsone and rifampin. All specimens had been referred to the National Hansen's Disease Programs for histopathologic diagnosis and were tested by using previously published molecular drug susceptibility protocols (*6*). We were able to obtain susceptibility results for only 4 of the 11 US patients from American Samoa. Mutations in *folP*1, a gene associated with dapsone resistance (*4*), were detected in 2 of these patients; no drug resistance was observed in US patients from other origins (Table). Combining these results with our previously published data, 4 of 6 US patients from American Samoa had dapsone-resistant *M. leprae*, and 1 of these was also resistant to rifampin. All 4 dapsone-resistant isolates had distinct mutations (P55L, P55R, T53I, and T53A) in the drug resistance–determining region of the *M. leprae folP*1 gene. The data strongly suggest that these *M. leprae* isolates are not clonal in origin (i.e., did not originate from a single dapsone-resistant clone). In addition, all patients with resistance seemed to have primary resistance to dapsone because biopsies were taken before known treatment with antileprosy drugs.

The registry of the National Hansen's Disease Programs indicates that 23 patients from American Samoa were given leprosy diagnoses in the United States during 2002–2014. Because of insufficient DNA or specimen unavailability, drug susceptibility of only 6 patients could be determined. Findings indicate that at least 4 (17%) of these 23 patients were infected with dapsone-resistant *M. leprae*. Biopsy results of 20 US leprosy patients known to be originally from other locations given diagnoses during this time interval did not demonstrate dapsone or rifampin resistance. Eleven (55%) of these patients had Pacific Island origins.

Dapsone resistance in American Samoa could have developed before implementation of multidrug therapy in this population, when dapsone was used as a monotherapy for leprosy. Several patients from American Samoa indicated that frequent visits occur between friends and relatives in American Samoa and Western Samoa; however, no information is currently available regarding drug resistance in Western Samoa.

Dapsone resistance might not necessarily have clinical significance when patients take multidrug therapy as recommended by the World Health Organization. However, in patients with a high bacteria load, resistance to dapsone essentially results in dual therapy with rifampin and clofazimine, placing the burden on just 2 drugs in the multidrug therapy regimen. Moreover, in this regimen, rifampin is taken only once monthly, so the patient is receiving only 1 effective drug (clofazimine) daily. In addition, patient noncompliance might result in the selection of multidrug-resistant *M. leprae*. In another study, resistance to dapsone and rifampin was found in 1 of 4 dapsone-resistant cases, with the 1 case occurring in a relapse patient (*9*). Therefore, *M. leprae* drug resistance (including the identification and evaluation of new markers for dapsone resistance) should be further studied in

Table. Drug resistance in leprosy patients with and without origins in American Samoa, USA, 2012–2015*

Sample no.	Clinical classification†	Drug susceptibility‡		DRDR sequence		Location§
		Dapsone	Rifampin	*folP*1	*rpoB*	
US patients of American Samoa origin						
NHDP1	BL	No	Yes	T53I	Wild type	Louisiana, USA
NHDP2	LL	Yes	Yes	Wild type	Wild type	Hawaii, USA
NHDP3	LL	No	Yes	P55R	Wild type	Washington, USA
NHDP4	LL	Yes	Yes	Wild type	Wild type	Massachusetts, USA
NHDP5 (*9*)	BL	No	No	T53A	S456L	Hawaii, USA
NHDP6 (*8*)	LL	No	Yes	P55L	Wild type	California, USA
US patients not of American Samoa origin						
NHDP7	BL	Yes	Yes	Wild type	Wild type	Africa
NHDP8	LL	Yes	Yes	Wild type	Wild type	Burma
NHDP9	LL	Yes	Yes	Wild type	Wild type	Micronesia
NHDP10	LL	Yes	Yes	Wild type	Wild type	Micronesia
NHDP11	LL-BL	Yes	Yes	Wild type	Wild type	Micronesia
NHDP12	BL	Yes	Yes	Wild type	Wild type	Micronesia
NHDP13	LL	Yes	Yes	Wild type	Wild type	Pacific Islands
NHDP14	LL	Yes	Yes	Wild type	Wild type	Pacific Islands
NHDP15	LL	Yes	Yes	Wild type	Wild type	Marshall Islands
NHDP16	BL	Yes	Yes	Wild type	Wild type	Marshall Islands
NHDP17	BL	Yes	Yes	Wild type	Wild type	Marshall Islands
NHDP18	BL	Yes	Yes	Wild type	Wild type	The Philippines
NHDP19	LL	Yes	Yes	Wild type	Wild type	The Philippines
NHDP20	BL	Yes	Yes	Wild type	Wild type	Alaska, USA
NHDP21	LL	Yes	Yes	Wild type	Wild type	Alaska, USA
NHDP22	LL	Yes	Yes	Wild type	Wild type	Florida, USA
NHDP23	LL	Yes	Yes	Wild type	Wild type	Florida, USA
NHDP24	LL	Yes	Yes	Wild type	Wild type	Florida, USA
NHDP25	LL-BL	Yes	Yes	Wild type	Wild type	Louisiana, USA
NHDP26	LL	Yes	Yes	Wild type	Wild type	Louisiana, USA

*BL, borderline leprosy; DRDR, drug resistance–determining region; LL, lepromatous leprosy; NHDP, National Hansen's Disease Programs.
†Classification according to the Ridley-Jopling scale.
‡Molecular drug susceptibility testing done according to the World Health Organization guidelines (*6*). A susceptible *Mycobacterium leprae* isolate had no mutations in the DRDR of *folP*1 or *rpoB* (i.e., the isolate was wild type). A drug-resistant *M. leprae* isolate had a mutation in the DRDR of the *folP*1 or *rpoB* gene (*4*,*5*).
§Location for patients of American Samoa origin refers to the US state where the leprosy diagnosis was made. Location for patients not of American Samoa origin refers to their location of origin.

the American Samoa population (*5*). This research will most likely require correlation of epidemiologic, clinical, and molecular drug susceptibility data from a large number of leprosy patients in this leprosy-endemic region (*10*).

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Autochthonous Hepatitis E during Pregnancy, France

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DOI: https://doi.org/10.3201/eid2408.180105

Acute hepatitis E virus infections occurred during the third trimester in 2 pregnant women in France who sought treatment with nonspecific symptoms or asymptomatic elevation of liver enzymes. Infection cleared quickly in both women. We detected no hepatitis E RNA in 1 newborn's feces at 3 weeks of age.

In low-income countries, estimates of 20 million hepatitis E virus (HEV) infections and 70,000 deaths have been reported for 2005. Increased mortality rate in pregnant women is well described but unexplained; pregnant women with symptomatic HEV infection have a 10-fold higher probability of death, especially in countries of the Indian subcontinent and Africa in which genotypes 1 and 2 are prevalent (*1*). In addition, up to 50% mother-to-child transmission rates have been reported, resulting in both premature births and prenatal deaths (*2*). In high-income countries with high seroprevalence rates, infection is usually asymptomatic. Most symptomatic cases are caused by HEV genotype 3 (*3*); illness ranges from mild to fulminant acute hepatitis, as well as chronic hepatitis in immunocompromised patients. No increased severity has been observed in the few cases reported during pregnancy (*4*–*8*).

We report 2 infections acquired during pregnancy in immunocompetent women in France, neither of whom reported having traveled abroad or eaten raw or undercooked pork. In accordance with hospital policy, patients were informed at hospital admission of the potential use of their anonymized medical data for research purposes.

The first case-patient was a 43-year-old woman, hospitalized at 39 weeks 2 days' gestation, who had nausea, headache, and increased serum alanine aminotransferase (ALT) (Figure). Hepatitis led to a cervical ripening and labor induction on the next day. At 39 weeks 5 days' gestation, she delivered a 3,040-g healthy baby. We diagnosed maternal HEV infection at admission by detectable IgM and HEV RNA (5.75 log IU/mL), with a 3c genotype. Maternal cytolysis decreased significantly after 2 days in the hospital (from 600 to 300 U/L); physical examination remained normal in the weeks after delivery, so no further biologic follow-up was performed. At birth, the baby had normal liver enzymes; HEV RNA in stools was negative twice at birth and at weeks 2 and 3.

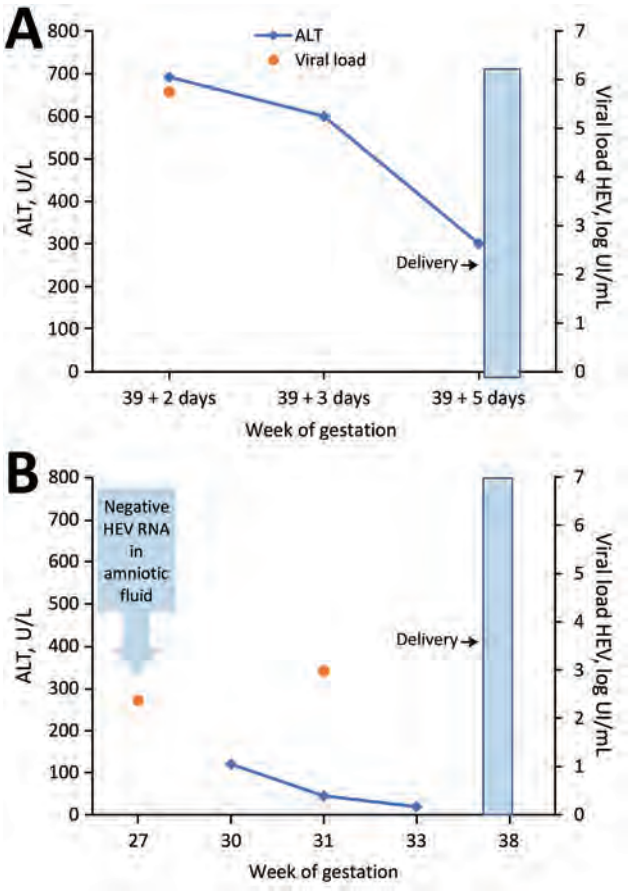

Figure. Relationship of HEV RNA and alanine aminotransferase to gestation and delivery time for 2 pregnant women, case-patient 1 (A) and case-patient 2 (B), France. Upper limit of normal for ALT values is 40 U/L. HEV RNA was quantified by a commercial real-time reverse transcription PCR assay targeting open reading frame 2/3. ALT, alanine aminotransferase; HEV, hepatitis E virus (Ceeram, La Chapelle sur Erdre, France).

The second case-patient was a 38-year-old woman with intrauterine growth restriction found at 24 weeks' gestation. Increased ALT at 30 weeks' gestation led to HEV diagnosis with detectable HEV IgM and viremia (2.98 log IU/mL). In retrospective testing, we detected HEV RNA in blood (2.37 log IU/mL) but not in the amniotic fluid at the time of amniocentesis (27 weeks' gestation). HEV genotyping was unsuccessful. The patient's liver abnormalities

resolved by 33 weeks' gestation. We performed no fetal or neonatal monitoring. The woman delivered a low-weight (2,350-g) but otherwise healthy baby at 38 weeks' gestation. The maternal physical examination remained normal in the weeks after delivery; we performed no further hepatitis follow-up.

Both patients had nonspecific symptoms or asymptomatic ALT elevation despite acute HEV infections in the third trimester of pregnancy. The rapid decline in ALT suggested a rapid clearance of infection in both. The lack of HEV RNA in the feces of 1 newborn indicated that infection was not transmitted; the lack of detectable RNA in the amniotic fluid suggested the same in the second case. We cannot rule out fetal HEV infection in patient 2 after 27 weeks' gestation, even though such an infection is unlikely because both children were born healthy. These mild courses of illness are similar to previous reported outcomes involving HEV genotype 3 (*4*,*5*,*7*,*8*) but dissimilar to the findings of a retrospective case series from Israel (*6*). That study reported significant illness in 8 autochthonous cases: indeed, all but 1 case required hospitalization (from 1 week to 2 months in duration), and fulminant hepatitis was described in 2 postpartum cases. HEV genotyping was not available in this study. Poor outcomes, including spontaneous abortions, stillbirths, and premature delivery, have been related to impaired maternal immune and hormonal responses but have been limited to genotype 1. Genotype 1 replication has been shown in human placenta (*9*), which suggests a major role of the viral genotype in pathogenesis. This replication may account for the high rates of mother-to-child transmission and poor fetal outcomes in highly HEV-endemic areas. Recently, HEV infection in a rabbit model also demonstrated vertical transmission (*10*). However, rabbit HEV is a distant member of genotype 3 that causes only partial cross-species infections in nonhuman primates; thus, pathogenesis could be different for this specific HEV genotype 3 variant. We observed no mother-to-child transmission in the 2 cases we describe.

HEV infection among pregnant women in industrialized countries is rarely reported, perhaps because it is rare in this setting, or infection may be underdiagnosed because symptoms are mild or nonspecific. Indeed, no acute infection or seroconversions were observed among pregnant women in a prospective study in France (*11*). The cases we report further suggest that no maternal, fetal, or neonatal complications occur following HEV genotype 3 infection.

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Misdiagnosis of Babesiosis as Malaria, Equatorial Guinea, 2014

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DOI: https://doi.org/10.3201/eid2408.180180

We report a case of babesiosis, caused by *Babesia microti*, in a missionary who worked in Equatorial Guinea but also visited rural Spain. The initial diagnosis, based on clinical features and microscopy, was malaria. The patient's recovery was delayed until she received appropriate treatment for babesiosis.

Babesia parasites are naturally transmitted by ixodid ticks; the parasites invade erythrocytes, causing babesiosis in animals and humans. The disease can be clinically silent or can progress to a fulminant malaria-like disease. Of the 4 characterized *Babesia* species involved, *B. microti* is the one that mostly infects humans and is found worldwide; most cases occur in the United States (*1*). Babesiosis in humans in Africa has rarely been reported (*2*–*4*), but the similarity to malaria, in symptoms and appearance in blood smears, may confuse diagnosis and result in underreporting (*5*,*6*).

In March 2014, a 43-year-old woman with fever, chills, fatigue, and general malaise was admitted to the General Hospital of Douala, Douala, Cameroon. Giemsa-stained blood smears showed intraerythrocytic parasites, leading to a diagnosis of malaria. The patient, who had previously had malaria, was given dyhydroartemisin plus primaquine, improved slightly, and was discharged. A few days later, she was admitted to the Hospital La Paz in Bata, Equatorial Guinea, with similar symptoms. Over an 8-month period, she received 6 consecutive diagnoses of malaria; treatment with quinine, artemether, atovaquone/proguanil, or artemether/lumefantrine led to no clear improvement. Because all antimalarial therapies had failed, the patient's case was reevaluated.

Chest radiographs and abdominal ultrasonograms were unremarkable. The patient had an intact spleen of normal size and had not received any blood products. Laboratory findings were unremarkable except for a high proportion of neutrophils (86%) (Table). New Giemsa-stained thin blood smears were examined and, in addition to ring forms, rare tetrads (Maltese crosses, which do not occur in *Plasmodium* infections) were observed. A diagnosis of babesiosis with a parasitemia of >0.5% was determined, and the patient then received oral azithromycin (500 mg/d) and atovaquone/proguanil (250 mg/100 mg every 8 h). Untreated blood was sent to the National Center for Microbiology and Hospital La Paz, both in Spain, where *Babesia* spp., but not *Plasmodium* spp., were detected by PCR (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/8/18-0280-Techapp1.pdf**)**. The partial amplified product was cloned by using TOPO TA vector (ThermoFisher Scientific, Inc., Carlsbad, CA, USA) and sequenced. The short nucleotide sequence of a 157-bp fragment of the *B. microti* 18S RNA gene (online Technical Appendix) showed 100% identity with the *B. microti* Munich type (GenBank accession nos. AB366158, AY789075, AB071177, KT271759, and KX758442). However, precise identification of the strain of parasite involved would have required larger fragments of the 18S rRNA gene. Attempts to amplify the β-tubulin gene were not successful.

One week after diagnosis and commencement of specific treatment, the patient traveled to Spain and was admitted to the Unit for Tropical Diseases at the tertiary Hospital La Paz in Madrid, where diagnostic tests for *Babesia* spp., *Plasmodium* spp., and other pathogens were conducted. An indirect immunofluorescence assay (IFA) (Fuller Laboratories, Fullerton, CA, USA) obtained *B. microti* titers of 128. Not surprisingly, anti–*Plasmodium falciparum* antibodies were also detected (titer 640) by IFA by a *Falciparum*-Spot IF kit (bioMérieux S.A., Lyon, France). Diagnostic test results were negative for *Schistosoma* spp., *Strongyloides stercoralis*, *Trypanosoma brucei*, *Leishmania*,

Table. Initial laboratory test results from blood samples of a patient with suspected babesiosis, Equatorial Guinea, 2014

Test	Result (reference range)
Hematology	
Leukocytes, × 10^3 μL	10.29 (3.0–10.0)
Hemoglobin, g/dL	13.2 (12.0–16.2)
Mean corpuscular volume, fL	85.9 (79–93)
Hematocrit, %	40.3 (33–44)
Platelet count, × 10^3 μL	205 (120–400)
Neutrophils, %	86.7 (49–70)
Lymphocytes, %	8.6 (20–50)
Monocytes, %	4.3 (4–8)
Eosinophils, %	0.4 (0–6)
Biochemistry	
Aspartate aminotransferase, U/L	15 (15–46L)
Alanine aminotransferase, U/L	20 (13–69)
Gamma-glutamyl transferase, U/L	14 (12–56)
Lactate dehydrogenase, U/L	450 (300–618)
Alkaline phosphatase, U/L	58 (30–130)
Total protein, g/dL	6.9 (6.3–10)
Albumin, g/dL	4.3 (3.5–5.5)
Bilirubin, mg/dL	0.7 (0.3–1.3)
Indirect bilirubin, mg/dL	0.7 (0.2–0.9)

[1]These authors contributed equally to this article.

Borrelia, *Anaplasma*, filariae, *Treponema pallidum*, hepatitis viruses (A, B, and C), HIV, and dengue virus. Treatment for babesiosis was continued for another 14 days, after which PCR results were negative and the patient's general condition had clearly improved. One year later, PCRs indicated that she was still free of *Babesia* parasites.

We do not have solid evidence of the source of this patient's babesiosis. Every year since 2001, she spent a week in rural areas in Spain, where at least 1 case of human babesiosis caused by *B. microti* (also "Munich" type) has been recorded (*7*). However, initial symptoms occurred while the patient was in Equatorial Guinea, having arrived there several months earlier from Valencia, Spain, where the *B. microti* vectors in Europe, *Ixodes ricinus* ticks, are not known to occur. However, no data are available on the presence of *Ixode*s ticks or of vertebrate reservoirs infected with *B. microti* in Equatorial Guinea. This lack of information, together with the fact that the patient traveled to different locations inside and outside Africa, makes it difficult to determine whether the infection was acquired in Equatorial Guinea. In such regions, where infrastructure and resources are limited, molecular and serologic diagnostic methods are usually lacking, and diagnoses of febrile diseases are based on symptoms, physical findings at examination, and microscopy. These limitations, and the similarities between malaria and babesiosis, are sufficient to explain why this patient's babesiosis was initially misdiagnosed as malaria. Because of this misdiagnosis, the patient was treated for malaria 6 times over 8 months. An accurate diagnosis and appropriate treatment for babesiosis was necessary to end this sequence of mistakes. Increased awareness of the possibility of babesiosis, together with appropriate diagnosis, may result in the discovery of more cases of babesiosis in malaria-endemic areas.

Acknowledgments

We thank the Parasitology Department of the Centro Nacional de Microbiología of Spain, which provided the results of IFA testing for *P. falciparum*.

This work was funded by the Surveillance Program of the Centro Nacional de Microbiología, the Center for Collaborative Research (RETIC-RICET), and grants from Ministerio de Economia y Competitividad of Spain (grant nos. AGL2010-21774 and AGL2014-56193-R awarded to E.M. and L.M.G.).

Written informed consent for publication of this article was obtained from the patient at the Hospital Universitario La Paz-Carlos III, Madrid, Spain.

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Paenibacillus assamensis in Joint Fluid of Man with Suspected Tularemia, China

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DOI: https://doi.org/10.3201/eid2408.180260

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Paenibacillus assamensis is a bacterium usually found in warm springs. We detected *P. assamensis* in a man with suspected tularemia. The strain isolated from the man's knee joint fluid was identified as *P. assamensis* after analysis of a homologous sequence of the 16S rRNA gene.

The genus *Paenibacillus* comprises >89 species. Some of these are pathogens in honeybees or other invertebrates; others are occasional opportunistic pathogens in humans (*1*,*2*). Bacteria belonging to the genus *Paenibacillus* have been isolated from various environments, such as soil, water, rhizospheres, vegetable matter, forage, and insect larvae, as well as from clinical samples (*3*). Tularemia, caused by the gram-negative intracellular pathogen *Francisella tularensis*, is highly virulent in humans and animals. An isolate from the joint fluid of a man in China in whom suspected tularemia was diagnosed recently was identified as *Paenibacillus assamensis*, a species usually found in warm springs.

A 44-year-old farmer was admitted to the surgical department of the Hospital of Traditional Chinese Medicine of Qianxinan Prefecture (Xingyi, China) on June 28, 2016. He had swelling and aching in his left knee that had appeared without an obvious cause and lasted for ≈6 months (Figure, panel A). He denied being bitten by a mosquito or other insect. Moreover, he had no contact with any animal before onset. In January 2016, he was admitted to the local county hospital for fever, cough, and pectoralgia. An antituberculosis regimen was started 3 days later. After he took the prescribed medicine for 15 days, his left knee began to swell and ache, accompanied by limitation of movement. However, no improvement was evident after drug withdrawal.

His body temperature fluctuated from 36.0°C to 36.8°C. A semisolid mass of 9.7 × 2.1 cm^2 was detected on ultrasound examination 0.4 cm from the left popliteal fossa to the subcutaneous surface. No acid-resistant bacilli were detected in the articular puncture fluid, which was inoculated using blood plate media. Gray migratory colonies of gram-negative bacteria appeared after 2 days. The presumptive identification of the isolate using the gram-negative card on the VITEK 2 Compact System (bioMérieux, St. Louis, MO, USA) was *F. tularensis*. A Cystine heart agar enriched with chocolatized erythrocyte medium was inoculated with the isolate, and gray opaque colonies ≈1 mm in diameter emerged after 2 days (Figure, panel B). The latex-agglutination test showed that the fresh strain and the patient's serum were negative for *F. tularensis*–specific antigens and antibodies.

We amplified the 16S rRNA gene of the bacterial genomic DNA using 2 universal bacterial primers, 27f and 1492r (*4*). Next, a 1,379-nt continuous stretch (GenBank accession no. MG847149) was obtained by 2-way sequencing of high-quality amplicons, which we used as a query to search for homologous sequences in the GenBank database. The query coverage was 100%, and the highest consistent sequence was that of *P. assamensis* strain GPTSA 11 16S rRNA gene. We further amplified the 16S rRNA gene of the patient's DNA using another pair of primers, 8–27f and 1500r (*5*). The highest consistent sequence obtained this time was 1,429 nt (GenBank accession number MG847150), still in the 16S rRNA gene of *P. assamensis* strain GPTSA 11. The analysis of the sequences indicated that the 2 amplicons contained the consensus signature sequence stretches PAEN 515F (*6*) and PAEN 862F (*7*), which are mostly found among different species within the genus *Paenibacillus*.

The local hospital detected *F. tularensis* in the patient with suspected tularemia on the basis of PyrA-positive results using the VITEK 2 Compact System. No recent studies have reported the association between PyrA and *F. tularensis*. The biochemical tests also showed that the strain could

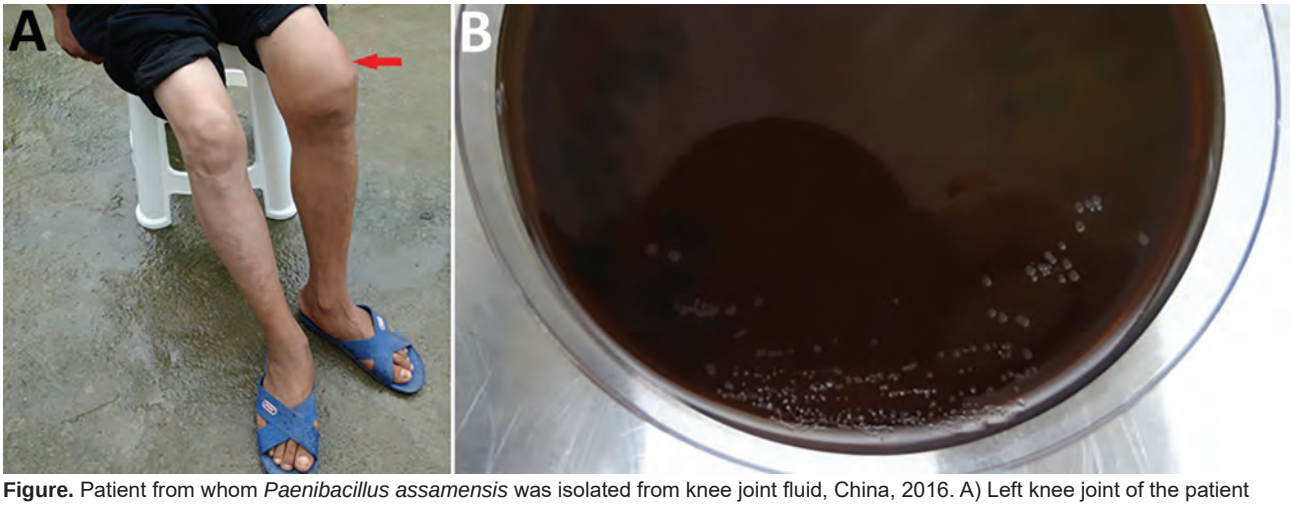

Figure. Patient from whom *Paenibacillus assamensis* was isolated from knee joint fluid, China, 2016. A) Left knee joint of the patient was obviously swollen (arrow). B) *P. assamensis* isolated from the patient's articular fluid appeared on chocolatized erythrocytes. A color version of this figure is available online (https://wwwnc.cdc.gov/EID/article/24/8/18-0260-F1.htm).

not ferment glucose and maltose. However, *F. tularensis* type A and type B are capable of fermenting glucose and maltose (*8*). Moreover, *F. tularensis*–specific antigen and antibody tests did not confirm that this strain was *F. tularensis* (*9*).

Both amplicons of the patient's 16S rRNA gene contained PAEN 515F and PAEN 862F signature sequences. After searching the homologous sequence of the 2 amplicons, the 16S rRNA gene sequence of *P. assamensis* GPTSA 11 displayed higher homology. Therefore, we concluded that the bacterium isolated from the patient's joint fluid was not *F. tularensis* but *P. assamensis*.

In 2005, a new species of *Paenibacillus* named *P. assamensis* GPTSA 11 was isolated from a warm spring in Assam, India (*10*). Since then, *P. assamensis* had not been isolated from other environments or patients. Our findings emphasize the need to consider new approaches for preventing infection in the environments where *P. assamensis* exists.

This patient remained at home to recuperate because of his obscure symptoms and financial constraints, but his symptoms did not abate until the follow-up in July 2017. He was advised to return to the hospital for treatment with drugs targeting *P. assamensis*. Isolation of *P. assamensis* from the living and working environments of patients, including soil and water, can successfully reveal the source of infection.

Acknowledgments

We thank Yingqiong Wang, Xiang Li, Bei Feng, Heng Wang, Jinlong Ran, Xiucheng Wu, and Tao Wu for their support.

This work was funded by the National Natural Science Foundation of China (no. 81473032).

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Plasmodium falciparum Plasmepsin 2 Duplications, West Africa

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DOI: https://doi.org/10.3201/eid2408.180370

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Dihydroartemisinin/piperaquine (DHA/PPQ) is increasingly deployed as an antimalaria drug in Africa. We report the detection in Mali of *Plasmodium falciparum* infections carrying plasmepsin 2 duplications (associated with piperaquine resistance) in 7/65 recurrent infections within 2 months after DHA/PPQ treatment. These findings raise concerns about the long-term efficacy of DHA/PPQ treatment in Africa.

Artemisinin combination therapy has been the cornerstone of malaria control in sub-Saharan Africa for the past 10 years and is typically represented by artemether/lumefantrine and artesunate/amodiaquine. Because of the notorious capacities of *Plasmodium falciparum* to develop drug resistance, many antimalarial programs have recently included dihydroartemisinin/piperaquine (DHA/PPQ) as a second-line antimalarial drug. This decision is sensible, considering the recent reports of substantially decreased artemether/lumefantrine cure rates in some regions, signaling a potential focus of lumefantrine resistance (*1*).

DHA/PPQ has shown near-perfect efficacy levels in clinical trials conducted in Africa; the combination also has been proposed as a tool for intermittent preventive approaches (*2*). Unfortunately, full *P. falciparum* resistance to DHA/PPQ treatment has been reported recently in Cambodia (*3*,*4*). These events were directly associated with increased copy number variations (CNVs) in the plasmepsin system, including the *pfpm2* gene (PF3D7_1408000) coding for the food vacuole enzyme plasmepsin II, which is speculated to be a major piperaquine target.

CNV is generally considered as emerging at relatively rapid mutation rates (a rate several orders of magnitude higher compared with that of single-nucleotide polymorphisms [*5*]) and is able to generate substantial diversity (*6*). Therefore, preexisting *pfpm2* duplications in Cambodia might have been rapidly selected by DHA/PPQ, aided by a less effective protective action of the artemisinin derivative (*7*). Such a scenario suggests that this mutation may already be present in Africa.

To investigate this possibility, we analyzed a subset of archived *P. falciparum* DNA samples from clinical infections, derived from a set of large, multicenter comparative artemisinin combination therapy efficacy trials conducted in West Africa by the West African Network for Antimalarial Drugs (*8*). These trials, performed during October 2011–February 2016 in Mali, Burkina Faso, and Guinea, had a randomized double-blind design with a 2-year follow-up for monitoring repeated treatment. Here we focus on the DHA/PPQ trial conducted at the village of Bougoula-Hameau in Mali, located ≈350 km south of the capital city of Bamako, near the border with Burkina Faso. The weekly control follow-up for each episode at Bougoula-Hameau was 63 days, and the DHA/PPQ arm involved a total of 224 patients who were ≥6 months of age.

We conducted a pilot study analyzing the 96 recurrent infections associated with the shortest interepisode periods, assuming that this subgroup, among whom initiation of recurrent infection ranged from 23 to 65 days posttreatment (Figure), would be the most likely to include *pfpm2* duplications. The study was reviewed and approved by the Ethics Committee of the Faculty of Medicine, Pharmacy, and Odonto-Stomatology, University of Sciences, Techniques and Technology of Bamako.

We determined copy number by using a SYBR-green–based quantitative PCR (ThermoFisher Scientific, Waltham, MA, USA) in a protocol modified from the one previously described by Witkowski et al (*4*). We used the *P. falciparum* β-tubulin gene as the internal nonduplicated standard and the 3D7 clone as a parallel 1 copy control. We ran the quantitative PCR thermal cycle at 98°C for 3 min, followed by 45 cycles at 98°C for 15 s, 63°C for 20 s, and 72°C for 20 s on a C1000 Thermal Cycler (Bio-Rad, Marnes-la-Coquette, France) with the CFX96 Real-Time System (Bio-Rad) detection system. We executed all procedures in triplicate.

The analysis was conclusive in 65 of the 96 samples. We confirm the presence of 7 infections carrying 2 copies of *pfpm2*, representing ≈10% of the successfully analyzed infections. We did not identify any trend of earlier recurrence associated with this group of infections (Figure), a preliminarily observation that needs to be further explored in a larger sample set.

Our results clearly show that piperaquine resistance–associated *pfpm2* duplications are probably already frequent in Africa, which is of concern given the long half-life of piperaquine (>20 days). In high-transmission areas, this long period of decreasing drug exposure is likely to progressively select less sensitive, potentially *pfpm2* CNV–carrying parasites. Parallel studies conducted in these areas have not detected substantial altered parasite clearance dynamics or K13 mutations associated with artemisinin-derivative therapy (*9*,*10*), indicating that these *pfpm2* duplications are emerging despite the efficacy of dihydroartemisinin. Further studies are urgently needed to clarify the clinical implications of piperaquine resistance and to monitor occurrence in other areas of high malaria transmission in Africa.

Acknowledgments

We are grateful to Hamadoun Diakite, Sekou Toure, Amadou H. Togo, Sekou Koumare, and the entire WANECAM team of Bougoula-Hameau. We thank the study patients and their parents and legal guardians, the respective communities involved, and the healthcare authorities of Sikasso.

This work was supported by a Swedish Research Council Grant (no. VR-2014–3134). The WANECAM study is funded by the

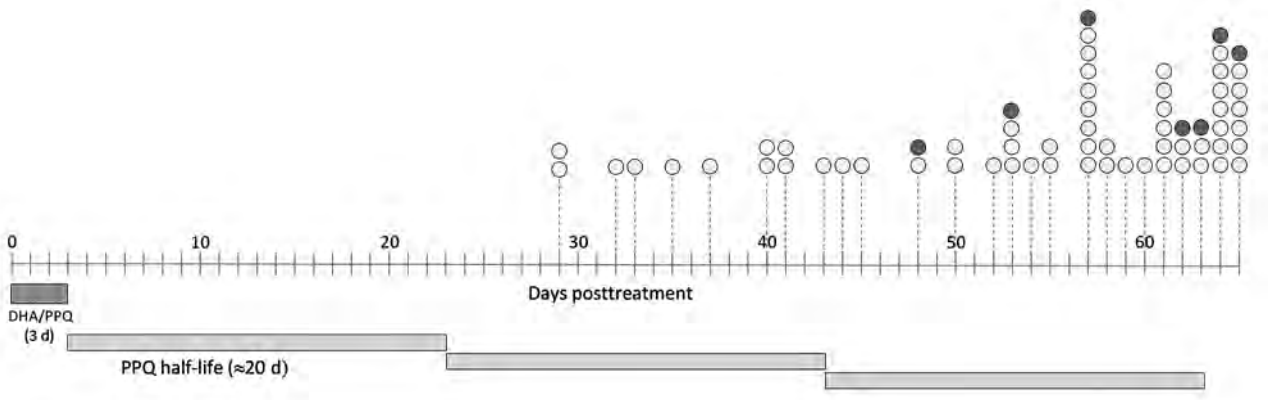

Figure. Timeline distribution of *Plasmodium falciparum pfpm2* copy number status during post–DHA/PPQ treatment followup for artemisinin combination therapy efficacy trials conducted by the West African Network for Antimalarial Drugs, Mali, Burkina Faso, and Guinea, October 2011–February 2016. Dark gray bar highlights the period (3 d) of treatment; lighter, longer gray bars represent PPQ average half-life (≈20 d). Circles represent recurrent infections; white circles indicate 1 *pfpm2* copy, and gray circles indicate 2 *pfpm2* copies. DHA/PPQ, dihydroartemisinin/piperaquine; PPQ, piperaquine.

European and Developing Countries Clinical Trial Partnership and by the Medicines for Malaria Venture (Geneva, Switzerland) and is co-funded by the United Kingdom Medical Research Councils, the Swedish International Development Cooperation Agency, the German Ministry for Education and Research, the University Claude Bernard (Lyon, France), the University of Science, Techniques, and Technologies of Bamako (Bamako, Mali), the Centre National de Recherche et de Formation sur le Paludisme (Burkina Faso), the Institut de Recherche en Sciences de la Santé (Bobo-Dioulasso, Burkina Faso), and the Centre National de Formation et de Recherche en Santé Rurale (Guinea).

J.I. was supported by EuroInkaNet/Erasmus Mundus Program. Fundação para a Ciência e Tecnologia supports M.S. (grant no. SFRH/BD/129769/2017), M.I.V. (grant no. SFRH/BPD/76614/2011), and P.E.F. (grant no. IF/00143/2015).

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Progressive Multifocal Leukoencephalopathy after Treatment with Nivolumab

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DOI: https://doi.org/10.3201/eid2408.180460

Progressive multifocal leukoencephalopathy (PML) is increasingly being reported in patients undergoing immunotherapy. We report a case of progressive multifocal leukoencephalopathy after treatment with nivolumab, a PD-1 blocker that is used to restore impaired T-cell responses in patients with cancer and infections. Data for 4 other cases were obtained from pharmacovigilance databases.

Progressive multifocal leukoencephalopathy (PML) is a life-threatening disease of the central nervous system caused by JC polyoma virus (JCV). Although incidence of this disease reportedly has increased markedly in association with HIV/AIDS, a new complication caused by immune-mediated therapies, particularly monoclonal antibodies, has emerged (*1*).

Nivolumab, a cancer immunotherapy, is a checkpoint inhibitor that functions by blocking the programmed cell death 1 (PD-1)/programmed death ligand 1 pathway and restoring immunity against tumor cells (*2*). We report a case of PML that occurred after 12 months of treatment with nivolumab in a patient with refractory stage IV Hodgkin lymphoma.

A 54-year-old HIV-negative man was hospitalized in November 2017 for new-onset acute back pain that was not responsive to high-dose morphine. In 2013, he had been given a diagnosis of stage IV Hodgkin lymphoma, for which he underwent several chemotherapy sessions, including ABVD (adriamycin, bleomycin, vinblastine, dacarbazine); DHAP (dexamethasone, high-dose cytarabin, cisplatin); ICE (ifosfamide, carboplatin, etoposide); ChIVPP (chlorambucil, vinblastine, procarbazine, prednisolone); etoposide, gemcitabine, liposomal doxorubicin plus methylprednisolone; and brentuximab plus bendamustin. In 2016, a positron emission tomography scan showed new locations of lymphoma (lymph nodes, spine, and lungs).

He was then given nivolumab (3 mg/kg every 2 wk) during October 2016–November 2017. He was also taking hydrocortisone (30 mg/d) for hypocorticism; 110 mg/d starting in March 2017). At admission, corticosteroid therapy was increased to 40 mg/d of methylprednisolone to treat hyperalgesic spine locations.

Three weeks later, progressive hemiparesis developed on the left side of his body. Magnetic resonance imaging of the brain showed multiple nonenhancing lesions that were suggestive of PML (Figure). Analysis of cerebrospinal fluid showed identical concentrations of leukocytes and erythrocytes (1 cell/mm^3). PCR analyses of showed a JCV concentration of 2,230 copies/mL in cerebrospinal fluid and 9,720 copies/mL in blood, confirming the diagnosis of PML.

Nivolumab was discontinued, and corticosteroid dose was decreased. CD4 lymphocyte subpopulation counts were 227 cells/mm^3 (12%) and CD8 lymphocyte subpopulation counts 1,547 cells/mm^3 (83%). The patient was still alive 5 months later but showed no neurologic improvement and had persistent JCV viremia (2,506 copies/mL).

PD-1 is a transmembrane receptor expressed on dendritic, NK, CD4, CD8 and T-cells and involved in downregulation of the immune system by promotion of activated T-cell apoptosis and diminution of regulatory T-cell apoptosis to stop unrestrained cytotoxic functions (*3*,*4*). Blockade of pathways involving PD-1 and its ligand is a promising treatment for cancers (melanoma, nonsmall cell lung cancer, metastatic renal cell carcinoma, head and neck carcinomas, Hodgkin lymphoma, and urothelial carcinoma) (*4*), although the role of PD1/programmed death ligand 1 in immune suppression and the mechanism of action of antibodies remain to be better defined.

Checkpoint inhibitors can cause unique toxicities, known as immune-related adverse events (IRAEs), including rashes, colitis, hepatitis, nephritis, pneumonitis, and thyroid disturbance, but these rarely comprise neurologic manifestations (<1%) such as aseptic meningitis and immune encephalitis (*3*,*5*,*6*). Neurologic IRAEs occur rapidly after treatment with checkpoint inhibitors and disappear after treatment interruption and introduction of steroids (*6*). Therefore, we excluded a diagnosis of neurologic IRAEs for this patient. Infections related to treatment with checkpoint inhibitors are infrequent (*4*).

Checkpoint inhibitors have been studied as treatment for chronic infectious diseases by restoring T-cell depletion (*2*,*3*,*7*), including PML, in which PD-1 on CD4 and CD8 cells has been reported (*1*,*8*). In a series of 740 patients given checkpoint inhibitors, serious infections developed in 7.4%, particularly in patients taking ipilimumab and nivolumab, steroids, or tumor necrosis factor antibodies for treatment of IRAEs, but few opportunistic infections and no PML were reported (*9*).

We found 4 unpublished cases of PML related to nivolumab in pharmacovigilance databases, but few data are available for these cases. One case of PML in the

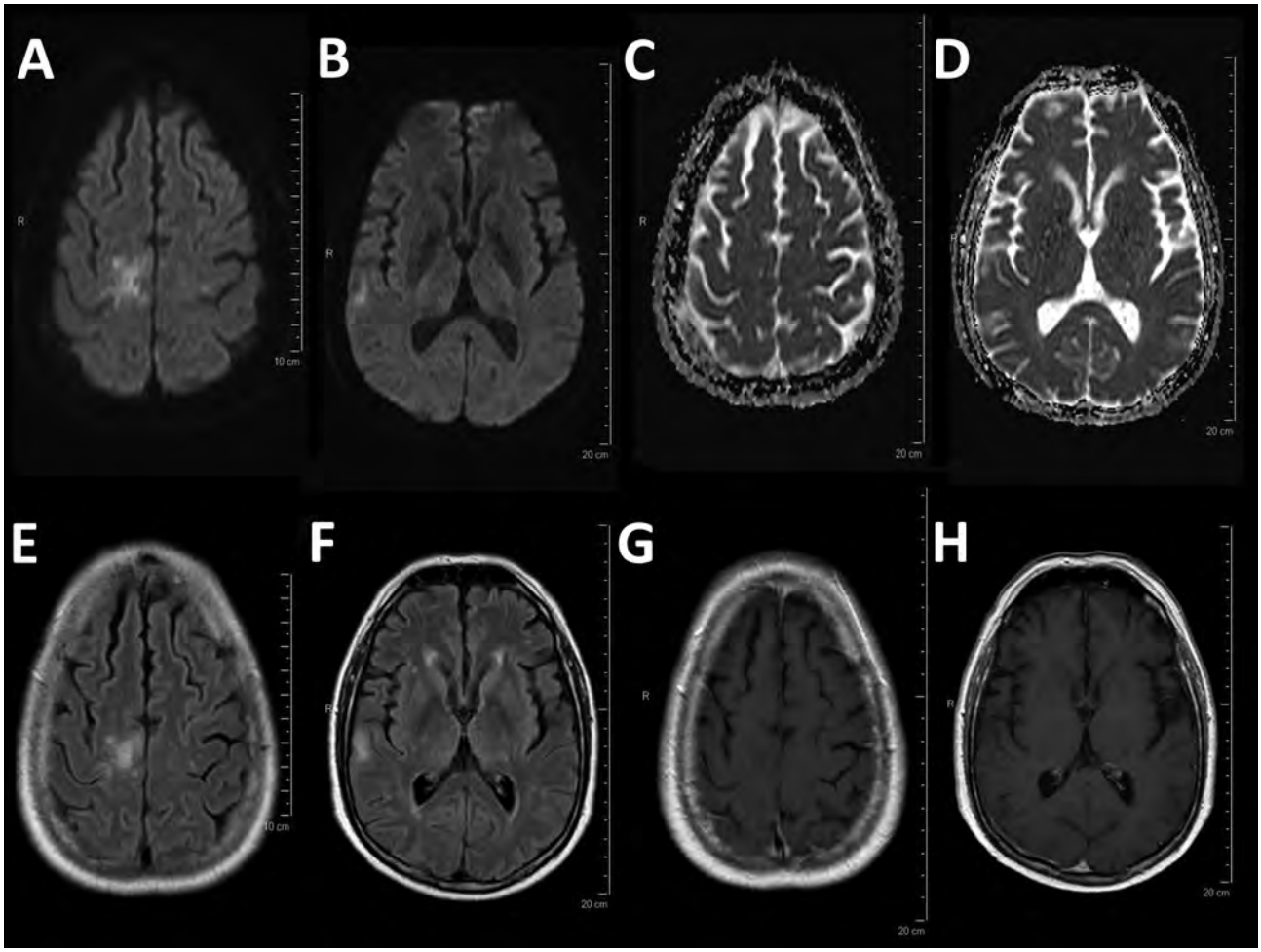

Figure. Magnetic resonance imaging for a 54-year-old man with progressive multifocal leukoencephalopathy after treatment with nivolumab, showing typical multifocal lesions: diffusion weighted imaging hyperintensity (A, B) with no decrease in the apparent diffusion coefficient (C, D), corresponding patchy corticosubcortical hyperintensities on fluid-attenuated inversion recovery image (E, F) without enhancement on T1-weighted imaging after administration of gadolinium (G, H).

European database EudraVigilance did not contain any detailed information. We found 3 cases in the World Health Organization pharmacovigilance database: a 70-year-old man with metastatic melanoma who was treated with ipilimumab for 3 months and nivolumab for 9 months; a 61-year-old woman with lung cancer who was given nivolumab (300 mg) 21 days before PML developed; and a 61-year-old woman who was treated with nivolumab (300 mg every 15 d for 5 mo).

Another reported mechanism of development of infections with use of checkpoint inhibitors is restoration of T-cell function that mimics immune restoration syndrome (IRIS), which can cause reactivation of latent tuberculosis (*4*) and acute progression of aspergillosis. IRIS has been reported in patients with PML, but for our patient, use of steroids and onset of PML long after treatment with nivolumab was started did not support a diagnosis of IRIS.

This patient was highly immunocompromised because of lymphoma and use of steroids. Thus, it is difficult to determine whether nivolumab contributed to development of PML or whether PML was caused by combinations of different factors. PML cases have been reported even long after cure of the initial condition, but the incidence of PML among patients with hematologic malignancies is rare (0.07%) (*10*). However, because of the complexity and unknown mechanisms of checkpoint inhibitors and reported cases of PML, physicians should be vigilant when using this treatment.

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Isolation of *Candida auris* from Ear of Otherwise Healthy Patient, Austria, 2018

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DOI: https://doi.org/10.3201/eid2408.180495

The emerging pathogen *Candida auris* is isolated mostly from hospitalized patients and often shows multidrug resistance. We report on the isolation of this yeast in Austria from an outpatient's auditory canal. The isolate showed good susceptibility against antifungals except for echinocandins; the patient was treated successfully with topical administration of nystatin.

The yeast *Candida auris* was first isolated from the external auditory canal of a person in Japan in 2009 (*1*). Reports from all continents except Australia (*2*) exist, but an outbreak in Europe was reported only recently, from a hospital in London (*3*). Major outbreaks of *C. auris* from countries in Europe have been reported from the United Kingdom and Spain, and sporadic isolations have been reported in Germany, France, Belgium, and Norway (*2*,*4*). We report on an isolation of *C. auris* in Austria, cultivated from an infection of the external auditory canal (otitis externa).

In January 2018, an otherwise healthy 22-year-old man came to an established otorhinolaryngology practice with therapy-refractory otitis externa in both ears that had persisted for almost 4 years despite antimicrobial drug treatment. The patient is an Austrian citizen with Turkish ancestry. He used to travel to Turkey frequently; his last visit was in 2017, and other trips abroad were not reported. A smear test was taken and sent to a microbiological laboratory for bacterial examination. A yeast grew after 48 hours at 37°C; the yeast was transferred to chromogenic mycological media, and an intense pink-colored yeast was cultivated on Brilliance Candida Agar (Oxoid, Basingstoke, UK). The isolate showed a pale rose color on BBL CHROMagar Candida Medium (Becton Dickinson, Heidelberg, Germany), and on Candida ID medium (bioMérieux, Marcy l'Étoile, France) (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/8/18-0495-Techapp1.pdf). On Sabouraud glucose agar and on malt extract agar (both from Becton Dickinson), the colonies were white. The maximum temperature at

which the isolate grew on the chromogenic media and on malt extract agar was 42°C.

We further examined the isolate using the VITEK 2 system (bioMérieux). This system (version 08.01) identified the yeast as *C. auris* but with only 90% probability. We transferred the isolate to another laboratory and to the Austria national reference laboratory for antifungal susceptibility testing and molecular identification. Repeated examination with 2 more VITEK 2 systems with different versions (07.01, 99% probability *C. haemulonii*; 08.01, 98% probability *C. duobushaemulonii*) and the VITEK MS system (no identification) failed to identify the yeast correctly. Two different MALDI Biotyper Systems (Bruker Daltonics, Bremen, Germany) failed to identify the isolate with the in vitro diagnostic library. However, using the research use only library, we identified the isolate as *C. auris* with scores of 1.72 (formic acid extraction with additional washing step) to 1.96 (formic acid extraction) of a log(score) value between 0.00 and 3.00, which translates to a low confidence identification.

For molecular analysis, we extracted DNA from the isolate and performed PCR with the primer pairs ITS5 and ITS4 for the internal transcribed spacer region of the rRNA gene and NL-1 and NL-4 for the D1/D2 region of the large subunit of the rRNA gene (*5*,*6*). We then purified the amplicons and sequenced them with the same primers as for PCR. We submitted the sequence data of both sequences to GenBank under accession nos. MH071441 (ITS) and MH071440 (D1/D2). We deposited the strain for public use in the CBS-KNAW yeast collection of the Westerdijk Fungal Biodiversity Institute in Utrecht, the Netherlands, where it was assigned strain no. CBS 15366.

For antifungal susceptibility tests, we used 3 systems: the European Committee on Antimicrobial Susceptibility Testing (EUCAST) microdilution method (http://www.eucast.org/ast_of_fungi/), Micronaut (Merlin Diagnostica, Bornheim, Germany), and Etest (bioMérieux). Despite reports from other studies (*2*,*7*), our strain showed good antifungal susceptibilities for the tested antimycotics except for the echinocandins; their MICs were higher than those for the EUCAST breakpoints for *C. albicans*, for example. However, no breakpoints have been determined for *C. auris*. In the case of anidulafungin, Etest revealed a lower MIC than did the other tests. The susceptibility results (in MIC, μg/mL, by test) in detail are as follows: amphotericin B, 0.5–1.0 (EUCAST, Micronaut); anidulafungin, 0.012–0.125 (EUCAST, Micronaut, Etest); caspofungin, 0.032–0.125 (EUCAST, Micronaut); micafungin, 0.064–0.125 (EUCAST, Micronaut, Etest); 5-flucytosine, ≤0.064 (EUCAST, Micronaut); fluconazole, 0.25–0.5 (EUCAST, Micronaut, Etest); itraconazole, ≤0.03 (Micronaut, Etest); posaconazole, ≤0.008–0.032 (EUCAST, Micronaut, Etest); voriconazole, ≤0.008–0.016 (EUCAST, Micronaut, Etest); and isavuconazole, 0.002 (Etest).

We treated the patient twice weekly for 3 weeks with an oral suspension of nystatin; the patient did not return for the next 4 weeks. Thereafter, we examined the patient's ears by otoscopy and found that the external ear canal was only slightly reddened and there were no signs of fungal growth.

In conclusion, *C. auris* is isolated not only from hospital settings and from severely ill patients but also from otherwise healthy persons. Despite new developments of databases and libraries for mass spectroscopy and biochemical-based instruments, molecular identification (e.g., ITS or D1/D2 domain of rDNA) is still the most, or in many cases the only, tool to identify *C. auris* reliably.

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Phylogeny of Yellow Fever Virus, Uganda, 2016

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DOI: https://doi.org/10.3201/eid2408.180588

In April 2016, a yellow fever outbreak was detected in Uganda. Removal of contaminating ribosomal RNA in a clinical sample improved the sensitivity of next-generation sequencing. Molecular analyses determined the Uganda yellow fever outbreak was distinct from the concurrent yellow fever outbreak in Angola, improving our understanding of yellow fever epidemiology.

Yellow fever virus (YFV) remains a public health threat; outbreaks occur frequently in regions of Africa and South America to which it is endemic. Genetic analyses have identified 5 YFV genotypes circulating in Africa in distinct geographic regions (*1*,*2*). This information can be used to identify the origin of outbreaks.

In December 2015, a yellow fever outbreak was identified in Luanda, Angola (*3*). A rapid increase in the number of cases was observed in January 2016, and the outbreak subsequently spread to other areas of Angola and neighboring Democratic Republic of the Congo (*4*). In April 2016, yellow fever was identified in the southwestern district, Masaka, of Uganda (*4*). By June 2016, the Ministry of Health of Uganda had reported 68 suspected yellow fever cases, of which 3 probable and 7 confirmed cases were in the Masaka, Rukungiri, and Kalangala districts (*5*). The Uganda Virus Research Institute (Entebbe, Uganda) collaborated with the Centers for Disease Control and Prevention (CDC, Fort Collins, CO, USA) to confirm the presence of YFV RNA in human clinical samples and determine the molecular epidemiology of virus causing the Uganda outbreak.

Serum specimens from the Uganda 2016 outbreak were determined to be YFV RNA-positive by real-time reverse transcription PCR at the Uganda Virus Research Institute, and CDC confirmed the results using a previously published method (*6*). One serum sample was selected as the most viable candidate for next-generation sequencing because of its relative concentration of viral RNA, as determined by real-time reverse transcription PCR (cycle threshold <30). The sample was prepared for sequencing on the Ion Torrent Personal Genomics Machine system (Life Technologies, Carlsbad, CA, USA), as previously described (*7*). Initial sequencing did not result in any sequence reads aligning with a YFV reference template (SeqMan NGen; DNASTAR, Madison, WI, USA), suggesting that the YFV RNA in the sample was of low quality and/or quantity.

To enhance sequence coverage, we subjected RNA extracted from the selected serum sample to a targeted RNase-H (Epicentre, Madison, WI, USA) digestion to remove the contaminating carrier and ribosomal RNA, as previously described (*8*). Then we prepared a standard cDNA library and conducted Ion Torrent sequencing. Fastq files were again aligned to a YFV reference template in SeqMan NGen (DNASTAR). Targeted RNase-H treatment of the RNA sample resulted in 37,637 sequencing reads aligning to the reference template or 1.2% of all sequencing reads corresponding to 38% coverage of the complete YFV genome. Contigs representing partial sequences of 7 coding regions (capsid, membrane, envelope, nonstructural [NS] 1, NS2B, NS3, and NS5) of the 10 YFV genes were identified. The longest contigs and deepest coverage were identified in partial coding regions of the envelope (693 nt; GenBank accession no. MG757496), NS3 (963 nt; GenBank accession no. MG757497), and NS5 (450 nt; GenBank accession no. MG757498), which were subjected

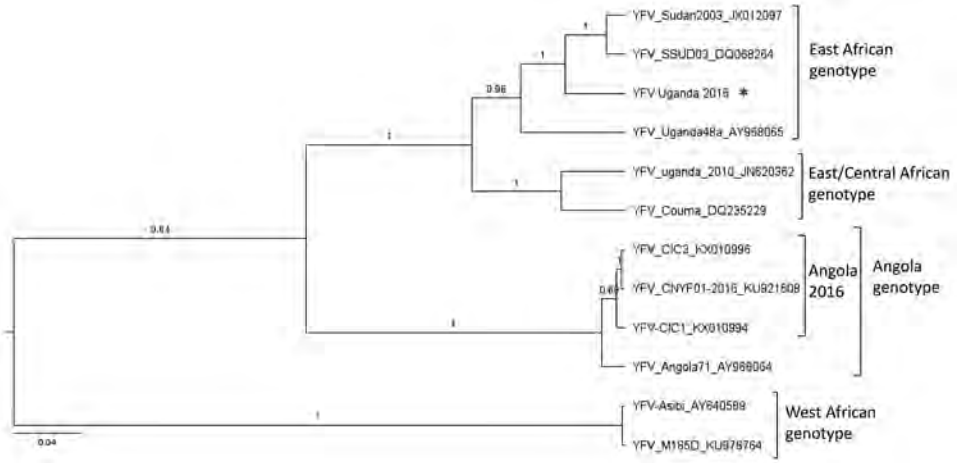

Figure. Bayesian maximum clade credibility tree of the Uganda 2016 YFV. Phylogenetic inference of the Uganda 2016 YFV strain (asterisk) representing partial coding regions of the membrane and envelope genes compared with reference YFV genotypes. Posterior probabilities are shown for each branch. Reference YFV strains are labeled with strain designation and GenBank accession numbers. YFV, yellow fever virus. Scale bar indicates nucleotide substitutions per site.

to BLAST analyses (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The YFV Uganda 2016 strain envelope sequence was aligned with reference YFV genomes by using MAFFT through the EMBL-EBI server (http://www.ebi.ac.uk), and phylogenies were generated with BEAST 1.8.4 (*9*), as previously described (*7*).

BLAST analyses determined that the highest percentage identity (95%) is shared between the Uganda 2016 strain and strains from South Sudan 2003 in the envelope region (the only region for which data from the Sudan strain are available) versus 83% with Angola 2016 strains from the same region. Furthermore, the Uganda 2016 sequences corresponding to the NS genes NS3 and NS5 have the highest percentage identities (94% and 95%, respectively) with a Uganda 1948 strain relative to 85% and 84% with the Angola 2016 strains in the same regions. Together these BLAST analyses indicate that the Uganda 2016 YFV is most similar to strains in the East African genotype. Phylogenetic analyses confirm the BLAST analyses and place the Uganda 2016 YFV in a well-supported clade along with these East African genotype strains, whereas the Angola 2016 strains group with an Angola 1971 YFV (Figure), indicating that the Uganda outbreak in 2016 was not seeded by the Angola outbreak.

These findings reiterate the endemicity of YFV throughout the tropical regions of Africa because at least 2 concurrent yellow fever outbreaks of independent origins were identified in 2016. Our findings also highlight the importance of assessing the molecular epidemiology of the virus in outbreak investigations. These data improve our understanding of YFV epidemiology in Africa and support the previous studies of Mutebi and colleagues (*2*). In addition, removal of contaminating ribosomal RNA proved to be an effective method for unbiased enrichment of viral RNA in degraded samples to enhance sequencing sensitivity.

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LETTERS

Visceral Leishmaniasis in Traveler to Guyana Caused by *Leishmania siamensis*, London, UK

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DOI: https://doi.org/10.3201/eid2408.172147

To the Editor: In a case report of visceral leishmaniasis in a traveler returning from Guyana, Polley et al. identified *Leishmania siamensis* as the causative agent (*1*). However, we believe that the parasite responsible for this infection has been misidentified. Classification of parasites formerly identified as *L. siamensis* has recently been revisited (*2*) after description of a new species

(*L. martiniquensis*) from the West Indies (*3*). All previously described *L. siamensis* strains, except 1, are now reported as *L. martiniquensis*. Their rDNA internal transcribed spacer 1 sequences are still deposited in GenBank under the name *L. siamensis*. The exception, reported from Thailand (GenBank accession no. JX195640), is the only known *L. siamensis* sample to date.

New analysis of *Leishmania* (*Mundinia*) sequences available in GenBank and of *L. infantum* showed no variability in *L. martiniquensis*, including the sequence (GenBank accession no. LT577674) reported by Polley et al. (*1*), and sequence divergence when compared with *L. siamensis* (32.4%), a *Leishmania* sp. from Ghana (32.3%) (*4*), *L. enrietti* (30.6%), and *L. infantum* (43.6%). *L. martiniquensis* has been reported worldwide (Florida, West Indies, central Europe, and Southeast Asia). However, *L. siamensis* has been reported only once (in Thailand).

If one considers possible quiescence of the parasite, and that the patient was from Guyana, migrated to the United Kingdom in 1967, and had a relevant travel history, including visits to France (2003), Ghana (2005), Caribbean Grenada (2012), and Guyana (2012 and 2013), the geographic origin of this infection is unknown. Moreover, the mode of transmission of *L. martiniquensis* is not yet clearly defined. In contrast to the report of Polley et al. (*1*), although the genus *Sergentomyia* could play a role in some foci of leishmaniasis, it has never been recorded in the Americas (*5*).

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Visceral Leishmaniasis in Traveler to Guyana Caused by *Leishmania siamensis*, London, UK

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DOI: https://doi.org/10.3201/eid2408.180192

To the Editor: Polley et al. reported a case of *Leishmania siamensis* infection outside Thailand (*1*). In Thailand, 2 *Leishmania* species, *L. siamensis* (MON-324, World Health Organization code MHOM/TH/2010/TR) and *L. martiniquensis* (MON-229, World Health Organization codes MHOM/TH/2011/PG and MHOM/MQ/92/MAR1), are sporadically reported in immunocompetent and immunocompromised patients and cause cutaneous and visceral leishmaniasis (*2*). Cases of asymptomatic visceral leishmaniasis caused by both species were also detected in HIV-infected patients in Thailand (*3*).

Before 2017, *L. siamensis* was described as having 2 lineages: PG and TR. Additional information from zymodeme and genetic analysis indicated that these 2 lineages are different species (i.e., lineage PG is *L. martiniquensis* and lineage TR is *L. siamensis*) (*2*). A review of leishmaniasis cases in Thailand during 1999–2016 (*2*) summarized the biological characteristics of *L. martiniquensis* and *L. siamensis* and clarified *Leishmania* species reported in humans (Thailand and Myanmar), animals (Thailand, Germany, Switzerland, and the United States), and sand flies (Thailand).

Polley et al. (*1*) reported phylogenetic analysis of internal transcribed spacer 1 sequences of 8 isolates of *L. siamensis* (GenBank accession nos. EF200012, JX195637, GQ281279, GQ226034, JQ866907, JQ617283, JQ001751, and GQ293226) against reference sequences of other *Leishmania* species. Their results confirmed that these sequences clustered with *L. siamensis* sequences as a monophyletic group, supported by bootstrap values of 100%.

However, 7 of these sequences (GenBank accession nos. EF200012, JX195637, GQ281279, GQ226034, JQ866907, JQ001751, and JQ617283) are *L. martiniquensis* sequences (MON-229), as reported in our article (*2*). Thus, we have revised and updated our sequences submitted to GenBank regarding the species of *L. martiniquensis* (MON-229) and *L. siamensis* (MON-324) for future analysis.

The patient had a history of traveling to Caribbean Grenada, which is in the same geographic area where *L. martiniquensis* was first reported (*4*). Thus, we believe

that the correct diagnosis for the 65-year-old woman in the study by Polley et al. (*1*) was visceral leishmaniasis caused by infection with *L. martiniquensis*.

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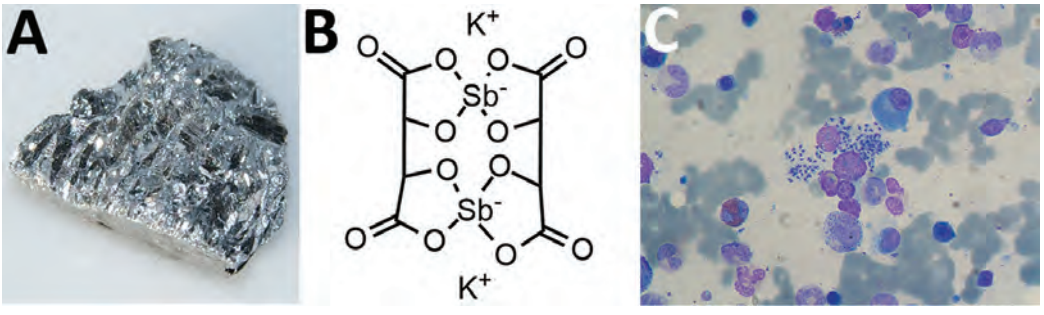
etymologia

Antimony *[an'tĭ-mo"ne]*

Mark D. Walker

One hundred years ago, John Brian Christopherson (1868–1955) discovered that antimony potassium tartrate was an effective treatment against schistosomiasis. Antimony had been previously used against visceral leishmaniasis, *Trypanosoma brucei gambiense*, and yaws. The ancient Egyptians used antimony paste as mascara. In the Middle Ages, it was used as a laxative, which, after swallowing and retrieval, could be reused. Alchemists used it to harden lead.



A) Antimony, unknown author, http://images-of-elements.com/, CC BY 3.0, https://commons.wikimedia.org/w/index.php?curid=9084452;
B) Antimony potassium tartrate trihydrate, Chargelot, own work, CC BY-SA 4.0, https://commons.wikimedia.org/w/index.php?curid=47342907;
C) Bone marrow aspiration: Leishmaniasis (*Leishmania* sp.) in liver transplant recipient, Paulo Henrique Orlandi Mourao, 2009, https://en.wikipedia.org/wiki/Leishmaniasis#/media/File:Leishmania_2009-04-14_smear.JPG;

Its name might have been derived from the Egyptian word for the metal *sdm*, from which the Greek *stimmi*, then the Latin *stibium*, then the French *antimoine* were derived. A more interesting, but unlikely, origin is that the French *antimoine* translates as monk's killer, referring to its toxicity to religious alchemists. Antimony potassium tartrate remained the treatment of choice for schistosomiasis until the development of praziquantel in the 1980s.

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DOI: https://doi.org/10.3201/eid2408.ET2408

Ben Taylor (aka Mometo) (1970–), *The Host* (2014) (detail)**.** Oil and textile on canvas.39.4 in × 39.4 in/100 cm × 100 cm). Image used by permission of the artist. Dartmoor, United Kingdom.

A Worm's Eye View

Byron Breedlove and Richard S. Bradbury

Seeing a several-centimeters-long worm traversing the conjunctiva of an eye is often the moment when many people realize they are infected with *Loa Loa*, commonly called the African eyeworm, a parasitic nematode that migrates throughout the subcutaneous and connective tissues of infected persons. Infection with this worm is called loiasis and is typically diagnosed either by the worm's appearance in the eye or by a history of localized Calabar swellings, named for the coastal Nigerian town where that symptom was initially observed among infected persons. Endemic to a large region of the western and central African rainforests, the *Loa loa* microfilariae are passed to humans primarily from bites by flies from two species of the genus *Chrysops*, *C. silacea* and *C. dimidiate*. The more than 29 million people who live in affected areas of Central and West Africa are potentially at risk of loiasis.

Researcher Chris Desjardins notes that "while *Loa loa* does less damage than a few of its filarial cousins, sometimes causing pain or swelling under the skin, it is

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DOI: https://doi.org/10.3201/eid2408.AC2408

still 'horrifying' since the worm often goes unnoticed until it enters the eye." Count contemporary artist Ben Taylor among those who discovered he was a host for *Loa loa* when he spotted one of the worms in his eye.

Taylor, who was born in Australia and grew up in Africa and Scotland, was an industrial model maker before becoming a computer-generated imagery artist. He eventually left the corporate world to create sculptural works and paintings, the former influenced by his time spent as a model maker, the latter by his work as a computer-generated imagery artist (B. Taylor, pers. comm., 2018 June 29). Taylor also has, in his words, "spent a lifetime living and travelling in far flung lands."

In 2013, Taylor visited the jungles of Gabon for several weeks. After his return to England, he started, but could not complete, an elaborate abstract painting that involved meticulously scraping the wet paint to reveal the underlying dry layers, "forming long undulating lines that, with hindsight, had a distinctly wormy look and feel" (B. Taylor, pers. comm., 2018 June 29). As Taylor notes on his website, "I was not able to fully bond with the piece at that time, it was a struggle to complete and I was not satisfied with the end result. I had no idea what compelled me to paint it, or what it was trying to say, and it rested in this unsatisfactory state for many months."

At that time, Taylor was plagued by health problems that were proving challenging to diagnose or treat, including what he described as a blinding transient pain in his eye, which would recur and disappear. About 4 months after Taylor had set aside this painting, he witnessed something wriggling in his eye and promptly sought medical care, which resulted in an eye surgeon extracting a *Loa loa* worm from Taylor's eye. Taylor notes that this event "was the start of new adventures as a medical novelty exhibit" during his week-long stay at the London Hospital for Tropical Diseases for "an intensive bout of treatment."

Taylor discovered that he had returned from this travels not just with *Loa loa* but with two additional parasitic diseases, hookworm infection and strongyloidiasis. Finally having a diagnosis for his ongoing health issues was tinged with an awareness of "how serious the treatment was likely to be" (pers. comm., 2018 June 29).

While he was recovering from this trio of parasitic infections and related treatments, Taylor rediscovered the unfinished painting stashed upside down in his studio: "I immediately saw that what I had painted was an eye made out of worms. Out came the oil paints, and a few days later, the painting was finished." The artist dubbed his finished work "The Host," perhaps his way of winking at the adversity he experienced in providing a home for this parasite.

The translucent white ribbon that coils across the conjunctiva and pupil dominates the image on the canvas and seems to rise above its surface. Though it is natural to gaze at the dark blue center of the eye, the viewer's attention keeps returning to the disturbing pale segmented shapes. A constellation of wormlike patterns that swirl toward the center of the eye is rendered with detailed density. The completed work depicts what the artist describes as his "descent into sickness and desperation as no diagnosis was forthcoming for my illness."

Loiasis is believed to affect an estimated 12 million people in regions where it is endemic. In many of those places, the public health and medical infrastructure and expertise essential for its successful diagnosis and treatment are lacking. The compelling artwork on this month's cover may help boost awareness concerning the public health burden posed by this overlooked tropical parasitic infection.

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https://www.asm.org/

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October 3–7, 2018
ID Week
San Francisco, CA, USA
http://www.idweek.org/

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Hong Kong, China
www.keystonesymposia.org/18S2

October 28–30
International Society for Vaccines 2018 Annual Congress
Atlanta, GA, USA
www.ISVCongress.org

October 28–November 1
ASTMH
American Society of Tropical Medicine and Hygiene
New Orleans, LA, USA
http://www.astmh.org/

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Article Title

Unilateral Phrenic Nerve Palsy in Infants with Congenital Zika Syndrome

CME Questions

1. Your patient is a 3-day-old boy with evidence of congenital Zika syndrome (CZS) and respiratory distress. On the basis of the case series by Rajapakse and colleagues, which one of the following statements about clinical features and course in 4 infants with CZS and diaphragmatic paralysis is correct?

A. Three of the 4 infants had severe microcephaly
B. Diaphragmatic paralysis was on the right in 2 infants and on the left in the other 2, confirmed by ultrasound or fluoroscopy
C. All 4 patients were term female infants born to primiparous mothers with symptoms suggestive of Zika virus infection during the first trimester of pregnancy
D. Two infants died from progressive respiratory failure, 1 died from intraventricular hemorrhage, and 1 was still living at 6-month follow-up

2. According to the case series by Rajapakse and colleagues, which one of the following statements about the significance of diaphragmatic paralysis among infants with CZS is correct?

A. Congenital Zika virus infection appears to be an infectious cause of congenital unilateral diaphragmatic paralysis
B. The study proves that a demyelinating neuropathy caused diaphragmatic paralysis in these infants
C. The respiratory insufficiency and subsequent failure in these infants is typical in CZS
D. Bilateral diaphragmatic involvement was ruled out in all 4 infants

3. On the basis of the case series by Rajapakse and colleagues, which one of the following statements about the significance of arthrogryposis among infants with CZS is correct?

A. Two of the 4 infants had talipes equinovarus
B. All 4 infants had involvement of only 2 joints
C. Arthrogryposis in CZS is thought to be only of upper motor neuron origin with MRIs showing less frequent periventricular calcifications than in infants with CZS without arthrogryposis.
D. Infants with CZS and arthrogryposis may be a more severely affected subgroup because of earlier fetal infection and more severe interruption in neuronal migration and/or cortical organization

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Article Title

Epidemiology of *Diphyllobothrium nihonkaiense* Diphyllobothriasis, Japan, 2001–2016

CME Questions

1. You are seeing a 30-year-old woman who complains of having diarrhea and abdominal pain for 2 weeks. She has attended an "all you can eat" sushi and sashimi night at a Japanese restaurant for years, but she does not believe that has anything to do with her present symptoms. You wonder whether she has diphyllobothriosis. What should you consider regarding the parasitology of this infection?

A. Diphyllobothriosis is closely associated with the consumption of raw Pacific salmon
B. The only mammals to be infected with *Diphyllobothrium nihonkaiense* are humans
C. *D. nihonkaiense* can grow up to 4 cm in length
D. *D. nihonkaiense* has only salmon as an intermediate host

2. Which one of the following statements regarding the epidemiology of diphyllobothriosis is most accurate?

A. It was most commonly diagnosed in September through November
B. Only a minority of cases were caused by *D. nihonkaiensis*
C. The timing of cases implicates mature chum salmon as a principal intermediate host
D. Most cases were reported in large metropolitan areas

3. What should you consider regarding the clinical presentation of diphyllobothriosis as you evaluate this patient?

A. Most patients were female
B. Few patients noted the expulsion of strobili while defecating
C. The most common presentation was no clinical symptoms
D. Weight loss occurred in nearly one quarter of patients

4. Which one of the following treatments was used in all cases of diphyllobothriosis in the current study?

A. Praziquantel
B. Mebendazole
C. Metronidazole
D. Erythromycin

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Article Title

Ancylostoma ceylanicum Hookworm in Myanmar Refugees, Thailand, 2012–2015

CME Questions

1. You are advising a Thailand public health department regarding anticipated needs for hookworm infection among Myanmar refugees. On the basis of the cohort study by O'Connell and colleagues, which one of the following statements about the epidemiology of hookworm infection among Myanmar refugees living in 3 camps along the Thailand–Myanmar border is correct?

A. Baseline hookworm infection of any type detected by qualitative PCR ranged from 12.9% to 19.8%, depending on the camp of residence
B. *Ancylostoma duodenale* was more prevalent than *Ancylostoma ceylanicum*
C. Cases mainly appeared to be imported, rather than acquired in the camps
D. *A. ceylanicum* is likely a more important human pathogen than previously recognized, with epidemiology and treatment response different than for *Necator americanus*

2. According to the cohort study by O'Connell and colleagues, which one of the following statements about risk factors for hookworm infection among Myanmar refugees living in 3 camps along the Thailand–Myanmar border is correct?

A. *A. ceylanicum* was more common than *N. americanus* in younger subjects and in those with higher blood eosinophil counts
B. Male sex was associated with a lower risk for infection with either hookworm
C. Co-infection was not a risk factor for infection with either hookworm
D. *Strongyloides stercoralis* and *Giardia duodenalis* coinfection were risk factors for *N. americanus* infection

3. On the basis of the cohort study by O'Connell and colleagues, which one of the following statements about the response to treatment of hookworm infection assessed through molecular analyses among Myanmar refugees living in 3 camps along the Thailand–Myanmar border is correct?

A. Cure rates after a single course of albendazole were lower for *A. ceylanicum* than for *N. americanus*
B. *N. americanus* cure rates were associated with ß-tubulin single-nucleotide polymorphisms at codon 200 or 167
C. Deworming strategies may enable the zoonotic hookworm *A. ceylanicum* to fill a niche left by the decrease in anthropophilic hookworms
D. Reinfection rates over the course of 3 months were approximately 30%

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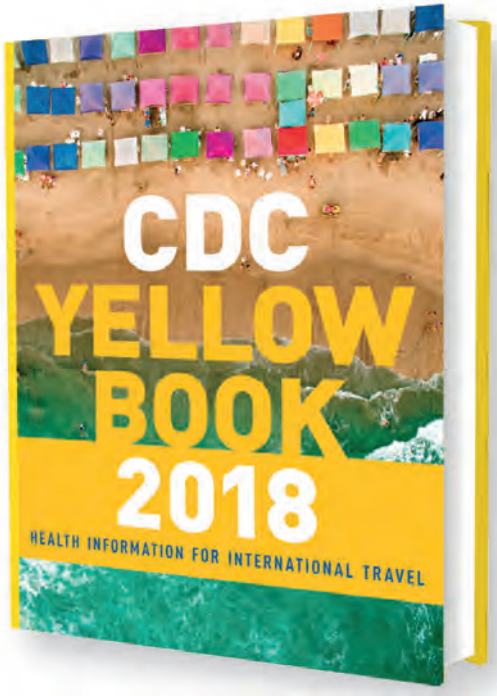

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Types of Articles

Perspectives. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words in the main body of the text or include more than 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section comprises case series papers and concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

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Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

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ISSN 1080-6040