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Zoonoses

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Artist Unknown. Hercules and the Erymanthian Boar, mid-17th century. Bronze, with red-brown lacquer patina
Height: 17 ½ in / 44.5 cm. Metropolitan Museum of Art, New York, New York; The Jack and Belle Linsky Collection, 1982.

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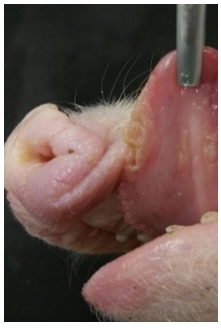

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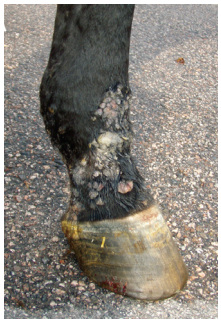

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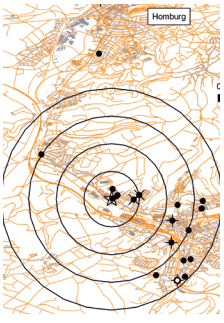

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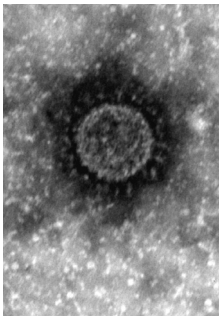

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Turtle-Associated Salmonellosis, United States, 2006–2014

Stacey Bosch, Robert V. Tauxe, Casey Barton Behravesh

During 2006–2014, a total of 15 multistate outbreaks of turtle-associated salmonellosis in humans were reported in the United States. Exposure to small pet turtles has long been recognized as a source of human salmonellosis. The risk to public health has persisted and may be increasing. Turtles are a popular reptilian pet among children, and numerous risky behaviors for the zoonotic transmission of *Salmonella* bacteria to children have been reported in recent outbreaks. Despite a long-standing federal ban against the sale and distribution of turtles <4 in (<10.16 cm) long, these small reptiles can be readily acquired through multiple venues and continue to be the main source of turtle-associated salmonellosis in children. Enhanced efforts are needed to minimize the disease risk associated with small turtle exposure. Prevention will require novel partnerships and a comprehensive One Health approach involving human, animal, and environmental health.

Salmonella spp. cause an estimated 1.2 million human illnesses, 23,000 hospitalizations, and 450 deaths each year in the United States (*1*). Infections are usually acquired through direct or indirect exposure to contaminated food or animals that carry *Salmonella*, including turtles and other reptiles (*1*,*2*). Most of these infections are foodborne, although an estimated 11% of *Salmonella enterica* infections were recently attributed to animal exposure (*2*). Exposure to small turtles (Figure) has been recognized as a source of human salmonellosis in the United States since the 1960s, when small baby turtles first became a popular pet (*3*). By the early 1970s, ≈15 million turtle hatchlings were sold annually in the United States, 4% of all US households owned at least 1 pet turtle at a given time, and 14% of human salmonellosis cases were attributed to exposure to small pet turtles (*4*). In 1975, to prevent turtle-associated salmonellosis among children, the US Food and Drug Administration (FDA) enacted a ban prohibiting the intra- and interstate sale and distribution of turtles with a shell length of <4 in (<10.16 cm) within the United States; after this ban, the small turtle industry turned to the export trade (*5*–*7*). The federal ban was effective, preventing an estimated 100,000 cases of turtle-associated salmonellosis in children each year after its enactment (*8*). By the late 1990s, only 6% of sporadic *Salmonella* spp. infections in the United States were attributed to reptile and amphibian contact (*9*). However, the regulation allows for small turtles to be distributed for bona fide scientific and exhibition purposes and for educational purposes other than use as pets.

The risk of acquiring a *Salmonella* infection from turtles has persisted and may be increasing, as suggested by a recent surge in the number of national salmonellosis outbreaks. The increased number of cases indicates the need for renewed attention to this long-standing public health issue, using a One Health approach involving human, animal, and environmental health.

Healthy turtles carry *Salmonella* spp. as part of their normal intestinal flora and intermittently shed the bacteria in their droppings. Humans become infected through direct contact with a turtle or by contact with its habitat, including contaminated tank water (*10*,*11*). Human salmonellosis typically causes acute gastroenteritis; however, severe invasive illness (e.g., sepsis, septic arthritis, meningitis) and death may occur, especially in persons at high risk (e.g. children <5 years of age, seniors, pregnant women, and immunocompromised persons). Turtle-associated salmonellosis disproportionately affects persons at high risk for severe illness, particularly infants and young children (*3*,*4*,*12*–*16*).

Increase in Multistate Outbreaks of Turtle-Associated Salmonellosis

Turtle-associated salmonellosis outbreaks were defined as ≥2 culture-confirmed human *S. enterica* infections with a combination of epidemiologic, laboratory, or trace-back evidence linking the illnesses to turtles. During 2006–2014, a total of 15 multistate turtle-associated salmonellosis outbreaks were reported to and investigated by the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA); this number represents an average of 2 cases per year. The outbreaks accounted for 921 illnesses, 156 hospitalizations, and the death of a 3.5-week-old infant (Table) (*6*,*12*–*15*,*17*). Outbreaks ranged in size from 4 to 135 (median 44) laboratory-confirmed cases. In all 15 outbreaks, the median age of ill persons was ≤10 years, indicating that children are still the most affected by turtle-associated salmonellosis.

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Figure. Small turtle with a shell length of <4 in (<10.16 cm). Photo credit: Casey Barton Behravesh.

The 8 multistate outbreaks reported in 2012 alone accounted for 473 reported illnesses; total estimated medical costs were ≈US $2,800,000 (*17*). Among 191 persons for whom information was available, 85 (45%) reported Hispanic ethnicity. Most reported turtles were small: 124 (88%) of 141 ill persons with turtle exposure reported that the implicated turtles had shell lengths of <4 in. Of 35 patients specifically asked their reason for purchasing a small turtle, all reported purchasing the turtle as a pet (*17*).

Patient interviews conducted during these recent outbreak investigations indicated that knowledge of the connection between reptiles and salmonellosis was lower than in previous turtle-associated outbreaks. Only 14 (15%) of 95 patients who reported turtle exposure during the 2012 outbreaks were aware that reptiles could carry *Salmonella* bacteria (*17*). By comparison, 20% of patients in the 2007–2008 *Salmonella* Paratyphi B var. Java outbreak investigation and 27% in the 2008 *Salmonella* Typhimurium outbreak investigation knew of the connection (*13*,*14*). This observation is concerning because numerous risky behaviors were reported in the 2012 outbreaks, including kissing turtles, letting them roam on kitchen countertops and tabletops where food and drink was prepared or consumed, and cleaning turtle habitats in kitchen sinks, all of which can lead to transmission of *Salmonella* bacteria. Frequency of reported turtle contact behavior and knowledge of the connection between reptiles and salmonellosis did not vary by reported ethnicity (*17*).

As part of the 2012 investigations, multiple federal and state public health and regulatory agencies collaborated to trace small turtles, which had been illegally sold in Florida beachside souvenir shops, back to 2 turtle farms in Louisiana. Two different outbreak strains were isolated from 1 farm's breeding pond: *Salmonella* Pomona (PFGE *Xba*I restriction enzyme pattern POMX01.0004) and Poona (PFGE *Xba*I restriction enzyme pattern JLX6X01.0104) (*16*). Because turtle farms in Louisiana are regulated by the Louisiana Department of Agriculture and Forestry (*18*), cease and desist orders were issued on domestic shipments of turtles from the implicated farms, thereby stopping, at the source, distribution of the turtles causing human illness. The Florida Department of Health and the Florida Wildlife Conservation Commission stopped the sale of small turtles at the souvenir shops, highlighting the effectiveness of state agency actions in these investigations (*17*).

Trends in Turtle Ownership and Sources of Turtles

The increase in turtle-associated salmonellosis may be related to the growing popularity of turtles as pets in the United States over the past 15 years. The proportion of US households that own pet turtles increased from 0.5% in 1996 to 1.1% in 2011 (*19*). Turtles are the most common reptile species owned as pets in the United States; approximately twice as many households own turtles than own pet snakes (0.5%) or lizards (0.6%) (*19*). No national data indicate what proportion of pet turtles have shell lengths <4 in, although a resurgence in the illegal sale and distribution of small pet turtles was reported by the FDA in 2003–2004 (*20*,*21*).

Small turtles can be purchased from retail pet stores, discount stores, flea markets, swap meets, roadside vendors, street vendors, beachside souvenir shops, and online merchants (*6*,*17*). In addition, small turtles are often available for sale at fairs, outside of sporting events, or at parks. Because small turtles are being sold illegally, it is difficult to quantify how many are purchased as pets in the United States; however, it appears they come primarily from domestic farmers and distributors. According to the US Fish and Wildlife Service, ≈1.6 million turtles of any size were imported into the United States during 2000–2011 (US Fish and Wildlife Service, pers. comm., 2012 May 15); this number represents a small fraction of the ≈151 million turtles exported to other countries during the same period. Furthermore, during 2006–2012, US quarantine stations detained and denied US entry only 7 times to shipments of turtles with shell lengths of <4 in that were imported for commercial purposes.

Table. Characteristics of 15 multistate outbreaks of human *Salmonella enterica* infections linked to turtle exposure, United States, 2006–2014*

Outbreak no., year†	Duration, mo	Serotype(s)	Outbreak strain(s)‡	No. cases	No. states	No. hosp.	No. deaths	Median patient age, y (range)
1, 2006	1	I 4,[5],12:i-	JPXX01.0621, JPXX01.1056	4	2	1	0	10.0 (7–45)
2, 2007	7	Pomona	POMX01.004, POMX01.002	20	11	1	1	3.0 (<1–59)
3, 2007	4	Paratyphi B var. Java	JKXX01.0014, JKXX01.0015, JKXX01.0038	107	34	26	0	7.0 (1–87)
4, 2008	8	Typhimurium	JPXX01.0416, JPXX01.0006	135	25	29	0	7.0 (1–94)
5, 2009	5	Muenchen	JJ6X01.0063	10	8	0	0	10.0 (<1–60)
6, 2011	14	Paratyphi B var. Java	JKXX01.0116	132	18	13	0	6.0 (1–75)
7, 2012	30	Sandiego Newport	JLXX01.0053 JJPX01.1253	124	22	15	0	6.0 (<1–85)
8, 2012	25	Pomona	POMX01.0004	23	14	5	0	5.5 (<1–89)
9, 2012	27	Poona Sandiego	JLX6X01.0104 JLXX01.0002	58	22	16	0	3.5 (<1–84)
10, 2012	8	Sandiego	JLXX01.0051	7	3	1	0	10.0 (<1–65)
11, 2012	32	Pomona	POMX01.0002	120	29	19	0	2.0 (<1–94)
12, 2012	20	Poona	JL6X01.0055	78	13	8	0	3.0 (<1–83)
13, 2012	4	I 4,[5],12:i-	JPXX01.1056	19	5	3	0	2.0 (<1–33)
14, 2012	8	Typhimurium	JPXX01.1048	44	11	11	0	3.0 (<1–70)
15, 2014	7	Poona	JL6X01.0055	40	12	8	0	5.0 (<1–75)

*Hosp., hospitalizations.
†Outbreaks are depicted in the year they were reported to the Centers for Disease Control and Prevention. For some, onset of illness may have occurred in preceding years.
‡Defined by pulsed-field gel electrophoresis *Xba*I restriction enzyme pattern.

Together, those 7 shipments totaled 66 turtles (CDC, Division of Global Migration and Quarantine, pers. comm., 2013 Mar 23).

Special Risk of Small Turtles

The regulatory size restriction for turtles (i.e., length <4 in) was designed to protect children without interfering with the desire of turtle fanciers to obtain larger turtles (*6*). Small turtles are inexpensive to purchase and may seem to be a safe and attractive pet for young children. Indeed, they are more likely to be given as pets to children compared with other reptiles, such as snakes and iguanas (*6*,*22*), because small turtles are perceived as harmless, slow-moving pets that are safe for children. Hatchlings are small enough to fit in a young child's mouth and are also kissed and held in close physical contact by their young owners (*6*,*17*). Small turtles are often housed in a small pool of water in a plastic turtle bowl, which can become heavily contaminated with *Salmonella* spp. (*4*,*11*). Illnesses have also been attributed to swimming with turtles in an unchlorinated pool (*12*). Cleaning turtle habitats in a kitchen sink or bathtub can lead to cross-contamination with *Salmonella* bacteria and indirect transmission to persons who never had direct contact with the small turtle; this scenario is common for infants who become infected (*17*). Even diligent caregivers can have difficulty ensuring that young children wash their hands immediately and properly after handling a pet turtle or its habitat.

Changes in Turtle-Farming Practices

The US turtle-farming industry has supported research into methods to reduce the carriage rate of *Salmonella* spp. in pet turtles. These efforts have been driven primarily by Louisiana, the only state that currently licenses and regulates its turtle farms (*18*). Farms in Louisiana that sell turtles domestically or internationally must meet certain sanitary conditions, and they are required to treat turtle eggs with a surface disinfectant wash followed by a treatment with a bactericidal solution delivered through the egg pores via a pressure-differential process. Louisiana turtle farms are also required to undergo routine facility and equipment inspections at least once a year. In addition, state inspectors from the Louisiana Department of Agriculture and Forestry collect a 1-time random sample of 60 eggs or hatchling turtles from each lot (≈20,000 eggs or hatchlings) intended for sale. These samples are then tested for *Salmonella* spp. at a state-approved laboratory. If a *Salmonella* sp. is identified in the eggs or turtles sampled, that lot is removed from commerce (*18*). This process has prompted some farmers who followed this protocol and passed state inspections to incorrectly claim they are selling certified *Salmonella*-free turtles.

Academic researchers have found bactericidal pressure-differential egg treatment methods reduce but do not eliminate the frequency and quantity of *Salmonella* spp. in turtle hatchlings (*23*–*25*). Little is known about how those efforts affect *Salmonella* spp. carriage rates in turtles at points of sale or in household aquariums. In a recent study, researchers following *Salmonella*-free turtle hatchlings for 1 year in a laboratory setting found they did not shed *Salmonella* bacteria during that time (*26*). That study was conducted using turtle hatchlings acquired directly from a turtle farm and then housed in controlled aquarium environments with ideal husbandry practices (e.g., using an in-tank water circulator

and bioscrubber, providing consistent lighting, and feeding a consistent commercial diet). Similar long-term studies using treated egg–hatched turtles that are sold and raised in homes are lacking; in such a study, the turtles would be shipped to retail stores in a box with many other turtles and then held in store tanks before being purchased by consumers who use a variety of housing environments and feeding practices. This information gap is critical because multiple outbreaks of human illness have been attributed to turtles that were claimed to be certified *Salmonella*-free, including the 2012 multistate outbreak of serotypes Poona and Sandiego (Table) (*17*,*27*).

Although the turtle farming industry has changed since the 1960s, in light of recent investigation findings, we caution against the overreliance on egg treatment methods alone to reduce turtle-associated salmonellosis. Advertisement of a *Salmonella*-free turtle could give consumers a false sense of security, making them less likely to wash their hands or think other precautions are necessary after handling a turtle or its habitat. Even if turtles do not carry *Salmonella* spp. at the time of sale, they might not remain *Salmonella*-free throughout their lives (*6*). *Salmonella* bacteria are ubiquitous in the environment and are natural inhabitants of the turtle gastrointestinal tract. Turtles could acquire *Salmonella* spp. through several routes, including from other turtles through cross-contamination during shipping or comingling in holding tanks or through contaminated food. Turtles from multiple sources are often kept in high-density conditions in store tanks, which may not be regularly cleaned or maintained (*28*). No market controls or industrywide guidance promote humane and proper housing of turtles in stores after they leave the farm; this is another area for improvement because rates of *Salmonella* spp. shedding are probably higher among turtles housed in stressful conditions (*24*,*29*).

Ongoing Federal Ban Enforcement Challenges

Since the federal ban against the sale of small turtles was enacted, turtle producers have sought to repeal the ban, including through proposed federal legislation and lawsuits in federal court (*30*,*31*). Although the federal ban remains in place, its enforcement continues to be challenging. Consumer demand for baby turtles has led to a veritable black market of small turtles. Many of these turtles are purchased with cash from transient, untraceable vendors, such as sellers in flea markets and unmarked vans or roadside vendors. Therefore, any subsequent regulatory action by state or federal agencies would be difficult or impossible to conduct (*6*,*12*–*15*,*17*). Turtles sold via the Internet and shipped through the mail may also be difficult to trace.

Some merchants routinely exploit a regulatory exemption allowing for the purchase of small turtles for "bona fide scientific, exhibition, or educational purposes, other than use as pets." This is done by asking customers to sign a waiver stating they are purchasing a small turtle for educational purposes only (*21*,*27*,*28*). Some vendors on the Internet provide information on the illegality of selling small turtles as pets and a person's risk for *Salmonella* infection buried in the fine print of the website's terms and conditions of use (*6*). Customers are asked to check a box indicating they have read and agree to the terms of use when purchasing turtles online; this effort is dubious because <10% of customers on the Internet read terms of use agreements when purchasing products online (*32*). It seems that few persons in the United States who purchase small turtles over the Internet are likely to know that they are purchasing an illegally sold product that could make them sick. A bona fide market for turtles purchased for scientific and educational purposes may exist, but in outbreak after outbreak, ill persons reported acquiring their small turtles specifically as pets, an act prohibited under the federal ban (*6*,*12*–*15*,*17*,*27*).

Lack of regulatory authority at the state or local level creates another hurdle in stopping the sale of small turtles. A review of state laws in March 2014 identified 10 states with regulations prohibiting or restricting the sale of turtles with shells <4 in long that enable those states to pursue enforcement activities against the sale of small turtles in their jurisdictions (*33*). States that have not enacted such laws are reliant upon the FDA to enforce regulations, but federal resource limitations mean the FDA must prioritize which turtle suppliers to investigate and prosecute. States may wish to develop their own regulations limiting or banning the sale of small turtles, including requirements that merchants display signage on the human health risks of reptile ownership and barring all turtles from nursing homes and schools and daycare facilities serving young children (*6*,*34*,*35*). Some states have enacted new laws regulating small turtles (*6*,*33*,*34*), but others have encountered challenges (J. Scheftel, pers. comm., 2014 Mar 31), indicating a need to identify other ways to empower state and local jurisdictions to prevent illegal turtle sales.

In states without laws prohibiting the sale of small turtles, investigators have asked retail merchants to voluntarily stop illegal turtle sales in response to outbreaks of human salmonellosis (*17*,*27*). Public health investigators have partnered with other state agencies (e.g., Departments of Agriculture or Fish and Wildlife) with enforcement authorities over the sale of animals (e.g., prohibitions against the sale of endangered or invasive species) (*17*,*27*). In addition, the pet industry has a role to play in confronting this public health issue.

Retail Pet Industry

Small turtles implicated in outbreaks were often purchased from small, independently owned retail pet stores whose

proprietors often claimed to have no knowledge of the federal ban (*12*,*13*,*17*,*27*). By contrast, national pet store chains typically do not sell turtles with shell lengths <4 in because doing so is illegal and because hatchlings have poor survival rates in store tanks and tanks in customers' homes (T. Edling, pers. comm., 2014 Mar 20). An opportunity exists to educate small pet store owners and engage their help in FDA ban compliance. Public health agencies can send letters to licensed pet store owners, informing them of turtle-associated *Salmonella* infections reported in their area and of the ongoing federal ban against the sale and distribution of turtles with shell lengths <4 in. In addition, public health agencies can inform pet store owners of any applicable state laws or local laws and ask them to prominently post education materials on the risk of *Salmonella* infection from reptiles.

Conclusions and Recommendations

The long-standing public health issue of turtle-associated salmonellosis is reemerging in the United States, where multistate outbreaks have increased since 2006. These illnesses have most often occurred after exposure to small pet turtles with shell lengths <4 in, the sale and distribution of which is illegal in the United States. Further efforts to prevent salmonellosis from pet turtles will take an integrated One Health approach involving human, animal, and environmental health officials as well as the turtle industry and the retail pet industry.

Public health partners can help spread awareness, in English and Spanish, of the risk of turtles as a source of salmonellosis in humans and the particular hazard small turtles pose for young children. Pediatricians and family practice physicians are in a unique position to educate families about the risk for turtle-associated salmonellosis during wellness examinations for young children. Veterinarians can reinforce these messages by recommending reptiles as pets only for households with children ≥5 years of age and by providing detailed instruction to clients on proper reptile care and practices to prevent zoonoses. Healthcare providers for humans and animals can make educational literature available in waiting rooms and provide information on websites and in newsletters (*36*,*37*). Suitable educational materials are available in multiple formats and languages on the CDC Zoonotic Diseases (Diseases from Animals) website (http://www.cdc.gov/zoonotic/gi). If pediatricians have a young patient with salmonellosis, they should consider reptiles in the differential of exposures and inform the local health department if small turtles appear to be involved.

In accordance with federal law, turtle farmers, pet store owners, souvenir shop owners, and others who sell turtles should not sell or distribute those with shells <4 in long. Collaboration between human and animal health officials at state- and federal-level public health and regulatory agencies is often necessary to identify and stop merchants and suppliers who illegally sell small turtles. When state and local authorities are able to investigate suppliers, any regulatory action can be facilitated by the collection of water and environmental samples for culture as well as affidavits, bills of lading and invoices, photos to verify turtle size and breed (e.g., turtle pictured next to a ruler or quarter), and receipts that show purchase of small turtles.

Merchants who legally sell or display turtles (i.e., turtles with shell lengths ≥4 in and that are not endangered or otherwise prohibited from sale) can serve as positive role models in the effort to reduce the incidence of turtle-associated salmonellosis. Merchants should use good turtle husbandry practices to reduce in-store stress to minimize *Salmonella* spp. shedding and spread among turtles in the store. These practices could include maintaining a low turtle density in tanks, using a reputable turtle supplier, avoiding the mixing of turtles from different sources, using a water recirculator and filter, and feeding with a *Salmonella*-free food (T. Edling, pers. comm., 2014 Apr 15). In addition, merchants can prominently display information in stores and online about the risk of acquiring salmonellosis from turtles (as well as other reptiles and amphibians) and their tanks or aquariums and instructions for proper cleaning of the turtle habitat. Pet store staff educated about the risk of salmonellosis can direct customers to a more appropriate pet if persons at high risk for severe illness are in the household. This information should be provided to customers well before the point of purchase, not at the cash register or buried in terms of use agreements. CDC and other public health officials are partnering with reptile hobbyist and tradeshow groups and representatives from the pet industry to engage their participation in developing an integrated approach for keeping illegal turtles out of the marketplace.

Turtle-associated salmonellosis remains a preventable and costly public health problem almost 50 years after it was first recognized in the United States. Enhanced efforts to minimize the risk associated with small turtles are needed, including novel One Health partnerships and approaches for prevention.

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responses to requests for epidemiologic assistance. Her main research interests are outbreak response, especially to enteric infections, and building public health workforce capacity in the United States.

References

1. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis. 2011;17:7–15. http://dx.doi.org/10.3201/eid1701.P11101
2. Hale CR, Scallan E, Cronquist AB, Dunn J, Smith K, Robinson T, et al. Estimates of enteric illness attributable to contact with animals and their environments in the United States. Clin Infect Dis. 2012;54:S472–9. http://dx.doi.org/10.1093/cid/cis051
3. Williams LP Jr, Helsdon HL. Pet turtles as a cause of human salmonellosis. JAMA. 1965;192:347–51. http://dx.doi.org/10.1001/jama.1965.03080180005001
4. Lamm SH, Taylor A Jr, Gangarosa EJ, Anderson HW, Young W, Clark MH, et al. Turtle-associated salmonellosis. I. an estimation of the magnitude of the problem in the United States, 1970–1971. Am J Epidemiol. 1972;95:511–7.
5. US Food and Drug Administration. CFR – Code of Federal Regulations Title 21. Turtles intrastate and interstate requirements. 21 C.F.R. Sect. 1240.62 (revised April 1, 2015) [cited 2015 Apr 1]. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=1240.62&SearchTerm=turtles
6. Harris JR, Neil KP, Barton Behravesh C, Sotir MJ, Angulo FJ. Recent multistate outbreaks of human *Salmonella* infections acquired from turtles: a continuing public health challenge. Clin Infect Dis. 2010;50:554–9. http://dx.doi.org/10.1086/649932
7. Tauxe RV, Rigau-Perez JG, Wells JG, Blake PA. Turtle-associated salmonellosis in Puerto Rico: hazards of the global turtle trade. JAMA. 1985;254:237–9. http://dx.doi.org/10.1001/jama.1985.03360020069026
8. Cohen ML, Potter M, Pollard R, Feldman RA. Turtle-associated salmonellosis in the United States: effect of public health action 1970 to 1976. JAMA. 1980;243:1247–9. http://dx.doi.org/10.1001/jama.1980.03300380027016
9. Mermin J, Hutwagner L, Vugia D, Shallow S, Daily P, Bender J, et al. Reptiles, amphibians, and human *Salmonella* infection: a population-based, case-control study. Clin Infect Dis. 2004;38 (Suppl 3):S253–61. http://dx.doi.org/10.1086/381594
10. Mann PH, Bjotvedt G. *Salmonella* organisms isolated from water used for storage of pet turtles. Can J Comp Med Vet Sci. 1967; 31:43–5.
11. Clegg FC, Heath PJ. *Salmonella* excretion by terrapins and the associated hazard to human health. Vet Rec. 1975;96:90–1. http://dx.doi.org/10.1136/vr.96.4.90
12. Centers for Disease Control and Prevention. Turtle-associated salmonellosis in humans—United States, 2006–2007. MMWR Morb Mortal Wkly Rep. 2007;56:649–52.
13. Centers for Disease Control and Prevention. Multistate outbreak of human *Salmonella* infections associated with exposure to turtles—United States, 2007–2008. MMWR Morb Mortal Wkly Rep. 2008;57:69–72.
14. Centers for Disease Control and Prevention. Multistate outbreak of human *Salmonella* Typhimurium infections associated with pet turtle exposure—United States, 2008. MMWR Morb Mortal Wkly Rep. 2010;59:191–6.
15. Centers for Disease Control and Prevention. Outbreak of salmonellosis associated with pet turtle exposures—United States, 2011. MMWR Morb Mortal Wkly Rep. 2012;61:79.
16. American Academy of Pediatrics. *Salmonella* infections. In: Pickering LK, Baker CJ, Kimberlin DW, Long SS, editors. Red book: 2012 report of the Committee on Infectious Diseases. Elk Grove Village (IL): American Academy of Pediatrics; 2012. p. 635.
17. Walters MS, Simmons L, Anderson TC, DeMent J, Van Zile K, Matthias LP, et al. Outbreaks of salmonellosis from small turtles. Pediatrics. 2016;137:e20151735. http://dx.doi.org/10.1542/peds.2015-1735
18. Louisiana administrative code. Title 7, Agriculture and animals. Part XXI. Animals and animal health. Chapter 19. Turtles. Revised March, 2016 [cited 2016 Apr 6]. http://www.doa.la.gov/pages/osr/lac/books.aspx
19. American Veterinary Medical Association. US pet ownership and demographic sourcebook. Schaumberg (IL): American Veterinary Medical Association; 2012.
20. Hampton T. *Salmonella* risk from turtles. JAMA. 2004;291:415.
21. Marchione M. States, FDA probe illegal sale of turtles. Associated Press. Aug 6, 2004 [cited 2015 Apr 1]. http://www.apnewsarchive.com/2004/States-FDA-Probe-Illegal-Sale-of-Turtles/id-c3a6bb329359b129bfe5aef8e7e86f2b
22. de Jong B, Andersson Y, Ekdahl K. Effect of regulation and education on reptile-associated salmonellosis. Emerg Infect Dis. 2005;11:398–403. http://dx.doi.org/10.3201/eid1103.040694
23. Siebeling RJ, Caruso D, Neuman S. Eradication of *Salmonella* and *Arizona* species from turtle hatchlings produced from eggs treated on commercial turtle farms. Appl Environ Microbiol. 1984;47:658–62.
24. Mitchell MA, Bauer R, Nehlig R, Holley MC. Evaluating the efficacy of Baquacil against *Salmonella* spp. in the aquatic habitat of the red-eared slider, *Trachemys scripta elegans* [cited 2015 Apr 1] http://www.turtlefarms.com/images/Baquacil-report-Mitchell.pdf.
25. Mitchell MA, Adamson TW, Singleton CB, Roundtree MK, Bauer RW, Acierno MJ. Evaluation of a combination of sodium hypochlorite and polyhexamethylene biguanide as an egg wash for red-eared slider turtles (*Trachemys scripta elegans*) to suppress or eliminate *Salmonella* organisms on egg surfaces and in hatchlings. Am J Vet Res. 2007;68:158–64. http://dx.doi.org/10.2460/ajvr.68.2.158
26. Coleson C, Gayda R. Farm-raised "*Salmonella*-free" turtle hatchlings do not excrete *Salmonella* in home aquariums. Herpetol Rev. 2012;43:547–50.
27. Centers for Disease Control and Prevention. Salmonellosis associated with pet turtles—Wisconsin and Wyoming, 2004. MMWR Morb Mortal Wkly Rep. 2005;54:223–6.
28. Ballester K, Vazquez E, Saeed M, Bartlett PC. Evaluation of prevalence, transmission, and prevention methods of salmonellosis from pet turtles in Michigan. 2010;4:20–8 [cited 2015 Apr 1]. https://www.mipha.org/pdf/mjph/MJPH_V4_1_2010.pdf
29. DuPonte MW, Nakamura RM, Chang EM. Activation of latent *Salmonella* and *Arizona* organisms by dehydration in red-eared turtles, *Pseudemys scripta elegans*. Am J Vet Res. 1978;39:529–30.
30. Independent Turtle Farmers of Louisiana Inc v. USA et al. Ruling of the United States District Court Western District of Louisiana, Alexandria Division. March 30, 2010 [cited 2015 Apr 1]. https://cases.justia.com/federal/district-courts/louisiana/lawdce/1:2007cv00856/103949/74/0.pdf?ts=1377153152
31. Govtrack.us. H.R. 924 (110th): Domestic Pet Turtle Market Access Act of 2007, 2008 [cited 2015 Feb 1]. http://www.govtrack.us/congress/bills/110/hr924
32. Nielsen J. How little do users read? Real-life reading behavior [cited 2015 Feb 1]. http://www.nngroup.com/articles/how-little-do-users-read/
33. Centers for Disease Control and Prevention. Menu of state turtle-associated salmonellosis laws. Four-inch rule. p. 2 [cited 2016 Apr 6]. http://www.cdc.gov/phlp/docs/turtle-menu.pdf

34. Vora NM, Smith KM, Machalaba CC, Karesh WB. Reptile- and amphibian-associated salmonellosis in childcare centers, United States [letter]. Emerg Infect Dis. 2012;18:2092–4. http://dx.doi.org/10.3201/eid1812.120784
35. National Association of State Public Health Veterinarians. Compendium of measures to prevent disease associated with animals in public settings, 2013. J Am Vet Med Assoc. 2013;243:1270–88. http://dx.doi.org/10.2460/javma.243.9.1270
36. Pickering LK, Marano N, Bocchini JA, Angulo FJ; Committee on Infectious Diseases. Exposure to nontraditional pets at home and to animals in public settings: risks to children. Pediatrics. 2008;122:876–86. http://dx.doi.org/10.1542/peds.2008-1942
37. Mettee Zarecki SL, Bennett SD, Hall J, Yaeger J, Lujan K, Adams-Cameron M, et al. US outbreak of human *Salmonella* infections associated with aquatic frogs, 2008–2011. Pediatrics. 2013;131:724–31. http://dx.doi.org/10.1542/peds.2012-2031

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Pregnancy, Labor, and Delivery after Ebola Virus Disease and Implications for Infection Control in Obstetric Services, United States

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Learning Objectives

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

- Assess the potential for Ebola virus to complicate pregnancy
- Analyze obstetric interventions and outcomes of a case of a pregnant woman with a history of Ebola virus diseases (EVD)
- Evaluate precautions taken during labor and delivery involving this patient with a history of EVD
- Distinguish laboratory testing results for Ebola virus among the mother and infant in the current case.

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Many of the survivors of the 2014–2015 epidemic of Ebola virus disease (EVD) in West Africa were women of childbearing age. Limited clinical and laboratory data exist that describe these women's pregnancies and outcomes. We report the case of an EVD survivor who became pregnant and delivered her child in the United States, and we discuss implications of this case for infection control practices in obstetric services. Hospitals in the United States must be prepared to care for EVD survivors.

The 2014–2015 epidemic of Ebola virus disease (EVD), which was centered in West Africa, is the largest EVD epidemic in history. Vertical transmission of Ebola virus from mother to fetus can occur during acute Ebola infection, leading to intrauterine fetal death, stillbirth, or neonatal death (*1*–*5*). Little is known about the risk for vertical transmission of Ebola virus from women to their neonates outside of the acute infectious period. Ebola virus (EBOV) has been found in breast milk during acute disease (*6*), and a study documenting 2 discordant mother–child pairs postulated that breast feeding of 1 infant may have led to infection of the infant (*7*). EBOV has been found in immune-privileged sites, ocular fluid and semen, many months after onset of infection (*8*–*13*), and it is possible that other immune-privileged sites, such as the central nervous system (CNS), may also contain EBOV many months after onset of infection. In addition, acutely infected pregnant women have had high amounts of Ebola viral nucleic acid persist in the amniotic fluid following clearance of viremia; however, it is not known whether this amniotic fluid is infectious (*2*). Some theoretical concern exists that during labor and delivery or obstetric anesthetic procedures (e.g., spinal anesthesia), contact with products of conception or cerebrospinal fluid from EVD survivors may pose an infectious risk (*6*,*14*–*18*).

As of March 9, 2016, an estimated 17,323 persons worldwide have survived EVD, and among them are ≈5,000 women of childbearing age (*19*). Survivors will require medical care for routine illnesses, surgical services, dental work, and management of disease sequelae (*20*,*21*). In addition, many of the female survivors who are of reproductive age will require obstetric care. Some of these survivors may come to the United States, and hospitals and healthcare workers must be prepared to provide care in a manner that promotes patient dignity and comfort, prevents stigmatization, and ensures that all patients receive appropriate, high-quality medical care (*22*–*24*). However, limited preparations have been made for follow-up care for EVD survivors, including those needing obstetric care. We describe the case of an EVD survivor who delivered a healthy neonate in a community hospital in the United States 14 months after acute EBOV infection, and we discuss the implications of the findings from this case for infection control in obstetric services.

Clinical Course

Ebola Virus Disease Course

A 29-year-old physician from West Africa became ill with EVD in late July 2014. She had contracted the virus from an EVD patient whom she had cared for from July 20th until his death on July 25. On July 29, the woman began feeling unwell, noting arthralgia and myalgia, which she self-treated with antimalarial medications. On August 1, she had fever, and on August 3, she began vomiting and had diarrhea. The woman was admitted to an Ebola treatment center (ETC) and isolated after results of an EBOV real-time reverse transcription PCR (rRT-PCR) were positive for EBOV RNA (cycle threshold unknown). According to the woman, she spent 13 days in the ETC, where she was treated with oral rehydration fluids, acetaminophen, and a second course of antimalarial medications. She was discharged from the ETC on August 16, after showing negative results on 2 EBOV rRT-PCRs. After her recovery, the woman noted some fatigue, anorexia, arthralgia, and alopecia; she did not report any sleep disturbances, headaches, or vision problems. Symptoms resolved 2–3 months later.

Pregnancy, Labor, and Delivery

Eight months before her EVD diagnosis, the patient had had a spontaneous abortion at 10 weeks' gestation. In January 2015, twenty-two weeks after her last negative EBOV rRT-PCR, she became pregnant again. For this second pregnancy, the estimated date of delivery was established on the basis of an 11.5-week ultrasound that was consistent with the patient's last menstrual period. The patient received routine prenatal care in West Africa, and at 25 weeks' gestation, she traveled to Kern County, California, USA, and a detailed anatomy ultrasound was performed in Los Angeles County, California, and demonstrated normal fetal development.

The hospital identified staff members who were willing to assist during labor and delivery for the patient, and at 40 weeks and 1 day of gestation, labor was induced to ensure that those staff members were present. The patient was given 2 vaginal doses of misoprostol, and oxytocin was administered, and labor progressed normally. The patient was given epidural anesthesia for pain control and had a normal vaginal delivery of a female neonate (weight 4,128 g) with Apgar scores of 8 and 9 at 1 and 5 min of age, respectively. The patient had a second-degree perineal laceration, which was repaired.

The patient and her neonate were discharged from the hospital at 36 h postpartum. They returned for routine follow-up 7 days postpartum and were monitored for 6 weeks following delivery, after which they traveled home to West Africa.

Infection Control and Personal Protective Equipment, Public Health Response

Two weeks before the patient's delivery date, her US obstetrician contacted the California Department of Public Health (DPH; Richmond, CA, USA) and the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) to determine if there were any special precautions needed for infection control; the California DPH notified the Los Angeles County DPH (Los Angeles, CA, USA). Because the patient was healthy and had fully recovered from EVD ≈4 months before becoming pregnant, all public health agencies agreed that she presented an extremely low risk for transmission of Ebola virus. Nevertheless, it was deemed appropriate that public health officials play an active role assessing and guiding management of the patient. The Los Angeles County DPH and CDC collaborated with the hospitals healthcare providers, nursing directors, laboratory director, environmental services staff, anesthesiologists, and hospital administration to address concerns and review the care plan, including plans for any complications, such as the need for cesarean delivery or the development of peripartum fever.

Hospital infection control procedures were reviewed in person with hospital staff. In review of these policies, no additional precautions were recommended above the standard precautions and policies currently used for all deliveries at the hospital. Several hospital staff members not directly involved in patient care expressed discomfort about working while an EVD survivor was admitted. To reassure these staff members, the patient was kept in 1 room during labor and delivery and after delivery. No changes were made to the policies for environmental cleaning or waste disposal.

Hospital staff raised concerns about the possibility of EBOV being harbored in immune-privileged sites (e.g., cerebrospinal fluid) in EVD survivors and, thus, expressed their concerns about a theoretical risk for EBOV transmission (*6*,*14*–*17*). This patient did not show signs or symptoms of CNS involvement during her acute illness or during her pregnancy, which likely indicated a decreased risk of any latent EBOV reservoir in her CNS; thus, it was considered likely that epidural or spinal anesthesia for this patient would not pose an infectious risk to staff. Hospital staff also noted the often imperfect adherence to use of personal protective equipment (PPE) during labor and delivery; thus, they voiced concern over this patient's history of EVD because large volumes of blood and amniotic fluid are often encountered in typical, uncomplicated vaginal deliveries (*25*). As a result of these concerns, many discussions were held regarding what PPE should be used during labor and delivery. Standard precautions should always be applied in all medical settings, including labor and delivery; however, neither CDC nor the American College of Obstetricians and Gynecologists had tailored recommendations for PPE specifically for vaginal or cesarean deliveries for any patients. Thus, CDC and Los Angeles County DPH developed a preliminary set of recommendations for the patient's providers regarding the use of PPE (Tables 1, 2) during and after labor and delivery to ensure that standard precautions were implemented. These PPE recommendations were discussed with the providers in the days before the delivery, and staff members were able to ask for clarification and ensure that materials were readily available. These PPE recommendations did not differ from standard precautions, but they explicitly discussed which PPE to use for casual contact, vaginal examinations, labor and delivery, anesthesia, and postpartum care. Routine hand hygiene, the use of barriers for mucous membrane protection, and the use of double gloves for procedures that involve sharps were emphasized.

Table 1. Recommendations for use of personal protective equipment by healthcare workers during labor and delivery for a woman who became pregnant after surviving Ebola virus disease, United States, 2015*

	Personal protective equipment						
			Gown		Gloves		Fluid-resistant, midcalf boot covers
Potential exposure	Face mask	Face shield	Isolation	Fluid-resistant or impermeable†	Single	Double	
Casual contact with patient							
Performing duties for patient with intact membranes (e.g., delivering food or water, talking with patient, adjusting external monitors)	No	No	No	No	No	No	No
Performing duties for patient with ruptured membranes; no touching of patient or bedding	No	No	No	No	No	No	No
Noncasual contact with patient							
Touching patient with ruptured membranes or bedding of patient with ruptured membranes	No	No	Yes	No	Yes	No	No
Administering epidural	Yes	Yes	Yes	No	No	Yes	Yes‡
Performing vaginal examination	Yes	Yes	No	Yes	Yes	No	Yes‡
Performing obstetric procedures§	Yes	Yes	No	Yes	Yes	Yes	Yes

*These personal protective equipment recommendations were developed for this particular patient and do not represent a formal recommendation.
†Impermeable indicates that the material and construction have demonstrated resistance to synthetic blood and simulated bloodborne pathogens; fluid-resistant indicates demonstrated resistance to water (http://www.cdc.gov/niosh/npptl/topics/protectiveclothing/default.html).
‡To be used if membranes were ruptured.
§Procedures include placement of fetal scalp electrode or intrauterine pressure catheter; manual removal of placenta; bimanual massage of uterine.

Table 2. Recommendations for use of personal protective equipment by healthcare workers during postpartum care of a woman who became pregnant after surviving Ebola virus disease and during care of her neonate, United States, 2015*

Level of care	Face mask	Face shield	Gown		Gloves		Fluid-resistant, midcalf boot covers
			Isolation	Fluid-resistant or impermeable†	Single	Double	
While caring for mother							
Before bedding/gown change	Yes	Yes	No	Yes	Yes	No	Yes
After bedding/gown change (vaginal exam, perineal care)	No, unless splash likely	No, unless splash likely	Yes	No	Yes	No	No
While caring for neonate							
Before bathing	Yes	Yes	No	Yes	Yes	No	Yes
After bathing	No	No	No	No	Yes‡	No	No

*These personal protective equipment recommendations were developed for this particular patient and do not represent a formal recommendation.
†Impermeable indicates that the material and construction have demonstrated resistance to synthetic blood and simulated bloodborne pathogens; fluid-resistant indicates demonstrated resistance to water (http://www.cdc.gov/niosh/npptl/topics/protectiveclothing/default.html).
‡To be used if exposure to fluids is likely.

Laboratory Assessment

One week before delivery, EBOV rRT-PCR testing was performed on the patient's blood by the Los Angeles County DPH laboratory and the CDC Viral Special Pathogens Branch; both results were negative. As expected, Ebola serum antibodies were detected by ELISA (IgG ≥1:1600, IgM negative).

After obtaining written informed consent from the patient, healthcare staff obtained the following during and after delivery: vaginal secretions, amniotic fluid (vaginal pool), cord blood, placenta, umbilical cord, breast milk (colostrum collected 16 h after delivery), and oral and ear swab samples from the neonate. Cord blood, colostrum, amniotic fluid, and swab samples were kept refrigerated until processed or frozen on dry ice for shipment to CDC. A placental sample was frozen in a sterile specimen cup and samples of placenta and umbilical cord were placed in buffered formalin and shipped at room temperature to CDC. EBOV rRT-PCR testing was performed on all of these specimens at the Los Angeles County DPH and CDC laboratories by using assays specific for nucleoprotein and viral protein 40 genes.

Placenta, amniotic fluid, and cord blood samples and ear and oral swab samples from the neonate were negative by EBOV rRT-PCR. Attempts were made to recover virus from placenta, amniotic fluid, cord blood, and colostrum at CDC, but no virus was recovered (Table 3). Amniotic fluid, cord blood, and colostrum were tested by ELISA for IgM and IgG against Ebola virus antigens (*26*). Cord blood was negative for IgM and had an IgG titer of ≥1:1600. Amniotic fluid and colostrum were negative for IgM and IgG. The placenta and umbilical cord were histologically normal, and no Ebola virus antigen was detected by immunohistochemistry (*27*), including in maternal and fetal endothelial cells and leukocytes.

Conclusions

We describe the delivery of a healthy baby to an EVD survivor who became pregnant 22 weeks after clearance of viremia and resolution of post-EVD sequelae (i.e., fatigue, anorexia, arthralgia). At 6 weeks follow-up, before returning to West Africa, the mother and baby were doing well. Given that the mother did not exhibit any signs or symptoms of post-EVD sequelae during her pregnancy, we did not expect to find any EBOV by rRT-PCR in any specimens obtained, and none was detected. It is somewhat surprising that we did not detect Ebola IgG in the colostrum; however, studies of antibodies for other infections have found that levels of IgG and IgM in colostrum are much lower than in serum (*28*), and this might also be true for antibodies against EBOV.

Although we did not detect EBOV RNA in this patient during pregnancy, women who are pregnant during acute EBOV infection usually transmit virus to the fetus and may pose an infectious risk to healthcare providers and others during delivery or abortion (*3*). EBOV can readily cross the placenta, and pathologic examination of placental tissues of patients with confirmed EVD have demonstrated EBOV antigen in the trophoblasts, syncytiotrophoblasts, and circulating maternal macrophages (*4*). EBOV RNA has been demonstrated in amniotic fluid; fetal meconium; vaginal secretions; umbilical cord; buccal swab samples from neonates; and peripheral blood samples from neonates, including those of mothers with cleared viremia (*29*,*30*).

The immune effects of pregnancy in the context of EVD have not been well documented (*3*); however, alterations in the immune system do occur during pregnancy (*31*), which during acute EBOV infection likely increases the risk for a poor outcome, including spontaneous abortion and neonatal death. Unlike the CNS, eye, and male testis, the genital tract of a nongravid female is not traditionally considered an immune-privileged site (*32*–*34*). Laboratory data that demonstrate the absence of EBOV or the presence of antibodies in post-EVD pregnancies are lacking; however, on the basis of epidemiological evidence in the field of multiple uneventful deliveries in West Africa and of the laboratory-analyzed case reported here, no evidence currently exists that Ebola virus can persist in the female genital tract. Any perceived risk must be mitigated to ensure that patients are not stigmatized and

Table 3. Laboratory test results for a woman who became pregnant after surviving Ebola virus disease and for her neonate, United States, 2015*

Source	Time of sample collection	rRT-PCR	Ebola antibodies	Immunohistochemistry
Maternal blood	1 week before delivery	Negative	IgG (1:1,600); IgM not detected	NA
Cord blood	At delivery	Negative	IgG (1:1,600); IgM not detected	NA
Amniotic fluid	At delivery	Negative	IgG; IgM not detected	NA
Vaginal swab sample	At delivery	Negative	NA	NA
Neonate ear swab sample	At delivery	Negative	NA	NA
Neonate oral swab sample	At delivery	Negative	NA	NA
Placenta	At delivery	Negative	NA	Negative for Ebola antigen
Umbilical cord	At delivery	NA	NA	Negative for Ebola antigen
Colostrum	1 day after delivery	Negative	IgG and IgM not detected	NA

*NA, not applicable; rRT-PCR, real-time reverse transcription PCR.

receive appropriate care. The authors concur with current guidelines by the World Health Organization, which state that women who have recovered from EVD are not infectious and should receive routine prenatal care, and their labor and delivery should be performed using standard PPE for protection against blood and body fluids (*35*).

The normal pregnancy for the patient described in this study and her delivery of a healthy neonate offer reassurance that women who become pregnant after recovery from EVD pose little risk for transmission of EBOV to the baby or others. Many more EVD survivors will become pregnant and deliver, and some may do so in the United States. Many other survivors will require routine medical care, including care for post-EVD syndrome. Lessons learned from this patient, specifically those addressing concerns about potential risks for virus transmission, may be applied to future patients. However, each survivor who seeks medical care will likely need to be assessed individually to determine possible risks for transmitting virus (*16,18*). Over the course of the public health involvement in this case, it became evident that, although standard precautions should routinely be used in all labor and delivery settings, written guidelines for labor and delivery may be useful, given the heightened concern for a theoretical disease transmission risk. We hope that the preliminary recommendations for PPE use during labor and delivery in the case discussed here will provide a template for other professional organizations to create guidelines for use in all labor and delivery settings.

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References

1. Akerlund E, Prescott J, Tampellini L. Shedding of Ebola virus in an asymptomatic pregnant woman. N Engl J Med. 2015;372:2467–9. http://dx.doi.org/10.1056/NEJMc1503275
2. Baggi FM, Taybi A, Kurth A, Van Herp M, Di Caro A, Wolfel R, et al. Management of pregnant women infected with Ebola virus in a treatment centre in Guinea, June 2014. Euro Surveill. 2014;19:20983. http://dx.doi.org/10.2807/1560-7917.ES2014.19.49.20983
3. Black BO, Caluwaerts S, Achar J. Ebola viral disease and pregnancy. Obstet Med. 2015;8:108–13. http://dx.doi.org/10.1177/1753495X15597354
4. Muehlenbachs A, de la Rosa Vazquez O, Bausch DG, Schafer IJ, Paddock CD, Bergeron E, et al. Ebola virus disease in pregnancy: histopathologic and immunohistochemical findings. Presented at: Abstracts of the American Society of Tropical Medicine and Hygiene 64th Annual Meeting; 2015 Oct 25–29; Philadelphia, PA, USA. Abstract LB-5108.
5. Mupapa K, Mukundu W, Bwaka MA, Kipasa M, De Roo A, Kuvula K, et al. Ebola hemorrhagic fever and pregnancy. J Infect Dis. 1999;179(Suppl 1):S11–2. http://dx.doi.org/10.1086/514289
6. Bausch DG, Towner JS, Dowell SF, Kaducu F, Lukwiya M, Sanchez A, et al. Assessment of the risk of Ebola virus transmission from bodily fluids and fomites. J Infect Dis. 2007;196(Suppl 2):S142–7. http://dx.doi.org/10.1086/520545
7. Moreau M, Spencer C, Gozalbes JG, Colebunders R, Lefevre A, Gryseels S, et al. Lactating mothers infected with Ebola virus: EBOV RT-PCR of blood only may be insufficient. Euro Surveill. 2015;20:21017. http://dx.doi.org/10.2807/1560-7917.ES2015.20.3.21017
8. Deen GF, Knust B, Broutet N, Sesay FR, Formenty P, Ross C, et al. Ebola RNA persistence in semen of Ebola virus disease survivors—preliminary report. N Engl J Med. Epub 2015 Oct 14.
9. Mate SE, Kugelman JR, Nyenswah TG, Ladner JT, Wiley MR, Cordier-Lassalle T, et al. Molecular evidence of sexual transmission of Ebola virus. N Engl J Med. 2015. Epub 2015 Oct 14.

10. Varkey JB, Shantha JG, Crozier I, Kraft CS, Lyon GM, Mehta AK, et al. Persistence of Ebola virus in ocular fluid during convalescence. N Engl J Med. 2015;372:2423–7. http://dx.doi.org/10.1056/NEJMoa1500306
11. Fischer WA II, Wohl DA. Confronting Ebola as a sexually transmitted infection. Clin Infect Dis. Epub 2016 Mar 1.
12. Howlett P, Brown C, Helderman T, Brooks T, Lisk D, Deen G, et al. Ebola virus disease complicated by late-onset encephalitis and polyarthritis, Sierra Leone. Emerg Infect Dis. 2016;22:150–2. http://dx.doi.org/10.3201/eid2201.151212
13. Rowe AK, Bertolli J, Khan AS, Mukunu R, Muyembe-Tamfum JJ, Bressler D, et al. Clinical, virologic, and immunologic follow-up of convalescent Ebola hemorrhagic fever patients and their household contacts, Kikwit, Democratic Republic of the Congo. Commission de Lutte contre les Epidémies à Kikwit. J Infect Dis. 1999;179(Suppl 1):S28–35. http://dx.doi.org/10.1086/514318
14. Barkhordarian A, Thames AD, Du AM, Jan AL, Nahcivan M, Nguyen MT, et al. Viral immune surveillance: toward a TH17/TH9 gate to the central nervous system. Bioinformation. 2015;11:47–54. http://dx.doi.org/10.6026/97320630011047
15. Brainard J, Pond K, Hooper L, Edmunds K, Hunter P. Presence and persistence of Ebola or Marburg virus in patients and survivors: a rapid systematic review. PLoS Negl Trop Dis. 2016;10:e0004475. http://dx.doi.org/10.1371/journal.pntd.0004475
16. Sonnenberg P, Field N. Sexual and mother-to-child transmission of Ebola virus in the postconvalescent period. Clin Infect Dis. 2015;60:974–5. http://dx.doi.org/10.1093/cid/ciu981
17. Farge E, Giahyue JH. Female survivor may be cause of Ebola flare-up in Liberia. Reuters. 2015 Dec 17. In: Kaye D. 15 March news. Clin Infect Dis. 2016;62:i–ii. http://dx.doi.org/10.1093/cid/civ1211
18. Centers for Disease Control and Prevention. Interim guidance for management of survivors of Ebola virus disease in US healthcare settings. 2016 [cited 2016 Mar 18]. http://www.cdc.gov/vhf/ebola/healthcare-us/evaluating-patients/guidance-for-management-of-survivors-ebola.html
19. World Health Organization. Ebola data and statistics: situation summary 09, March 2016. 2016 Mar 11 [cited 2016 Mar 18]. http://apps.who.int/gho/data/view.ebola-sitrep.ebola-summary-20160309?lang=en
20. Bausch DG. Sequelae after Ebola virus disease: even when it's over it's not over. Lancet Infect Dis. 2015;15:865–6. http://dx.doi.org/10.1016/S1473-3099(15)70165-9
21. Clark DV, Kibuuka H, Millard M, Wakabi S, Lukwago L, Taylor A, et al. Long-term sequelae after Ebola virus disease in Bundibugyo, Uganda: a retrospective cohort study. Lancet Infect Dis. 2015;15:905–12. http://dx.doi.org/10.1016/S1473-3099(15)70152-0
22 Kupferschmidt K. Infectious diseases. Surviving Ebola survival. Science. 2015;348:1406–7. http://dx.doi.org/10.1126/science.348.6242.1406
23. Minkoff H, Ecker J. Physicians' obligations to patients infected with Ebola: echoes of acquired immune deficiency syndrome. Am J Obstet Gynecol. 2015:212;456.e1–4.
24. Sprecher A. Handle survivors with care. N Engl J Med. Epub 2015 Oct 14.
25. Magann EF, Bass JD, Chauhan SP, Young RA, Whitworth NS, Morrison JC. Amniotic fluid volume in normal singleton pregnancies. Obstet Gynecol. 1997;90:524–8. http://dx.doi.org/10.1016/S0029-7844(97)00351-7
26. Ksiazek TG, West CP, Rollin PE, Jahrling PB, Peters CJ. ELISA for the detection of antibodies to Ebola viruses. J Infect Dis. 1999;179(Suppl 1):S192–8. http://dx.doi.org/10.1086/514313
27. Martines RB, Ng DL, Greer PW, Rollin PE, Zaki SR. Tissue and cellular tropism, pathology and pathogenesis of Ebola and Marburg viruses. J Pathol. 2015;235:153–74. http://dx.doi.org/10.1002/path.4456
28. Wheeler TT, Hodgkinson AJ, Prosser CG, Davis SR. Immune components of colostrum and milk—a historical perspective. J Mammary Gland Biol Neoplasia. 2007;12:237-47.
29. Bower H, Grass JE, Veltus E, Brault A, Campbell S, Basile AJ, et al. Delivery of an Ebola virus–positive stillborn infant in a rural community health center, Sierra Leone, January 2015. Am J Trop Med Hyg. 2016;94:417–9. http://dx.doi.org/10.4269/ajtmh.15-0619
30. Oduyebo T, Pineda D, Lamin M, Leung A, Corbett C, Jamieson DJ. A pregnant patient with Ebola virus disease. Obstet Gynecol. 2015;126:1273–5. http://dx.doi.org/10.1097/AOG.0000000000001092
31. Kourtis AP, Read JS, Jamieson DJ. Pregnancy and infection. N Engl J Med. 2014;371:1077.
32. Clark GF, Schust DJ. Manifestations of immune tolerance in the human female reproductive tract. Front Immunol. 2013;4:26. http://dx.doi.org/10.3389/fimmu.2013.00026
33. Mital P, Hinton BT, Dufour JM. The blood–testis and blood–epididymis barriers are more than just their tight junctions. Biol Reprod. 2011;84:851–8. http://dx.doi.org/10.1095/biolreprod.110.087452
34. Muldoon LL, Alvarez JI, Begley DJ, Boado RJ, Del Zoppo GJ, Doolittle ND, et al. Immunologic privilege in the central nervous system and the blood–brain barrier. J Cereb Blood Flow Metab. 2013;33:13–21. http://dx.doi.org/10.1038/jcbfm.2012.153
35. World Health Organization. Interim guidance: Ebola virus disease in pregnancy: screening and management of Ebola cases, contacts and survivors. 2015 Sep 4 [cited 2015 Nov 11]. http://apps.who.int/iris/bitstream/10665/184163/1/WHO_EVD_HSE_PED_15.1_eng.pdf?ua=1

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Response to Emergence of Middle East Respiratory Syndrome Coronavirus, Abu Dhabi, United Arab Emirates, 2013–2014

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In January 2013, several months after Middle East respiratory syndrome coronavirus (MERS-CoV) was first identified in Saudi Arabia, Abu Dhabi, United Arab Emirates, began surveillance for MERS-CoV. We analyzed medical chart and laboratory data collected by the Health Authority–Abu Dhabi during January 2013–May 2014. Using real-time reverse transcription PCR, we tested respiratory tract samples for MERS-CoV and identified 65 case-patients. Of these patients, 23 (35%) were asymptomatic at the time of testing, and 4 (6%) showed positive test results for >3 weeks (1 had severe symptoms and 3 had mild symptoms). We also identified 6 clusters of MERS-CoV cases. This report highlights the potential for virus shedding by mildly ill and asymptomatic case-patients. These findings will be useful for MERS-CoV management and infection prevention strategies.

Middle East respiratory syndrome coronavirus (MERS-CoV) was first identified in October 2012 in Saudi Arabia (*1*). By November 6, 2015, the World Health Organization (WHO) had received reports of 1,611 cases and at least 575 deaths caused by MERS-CoV. Index cases that occurred outside the Arabian Peninsula all had a link to the region, such as recent travel (*2*–*11*). Most contact investigations outside the Arabian Peninsula have identified limited spread to additional persons (*5*,*6*,*10*), with the exception of the recent South Korea outbreak, in which 186 persons were infected (*11*). Within the Arabian Peninsula, clusters have been identified among extended families, households, and healthcare settings (*12*,*13*).

Abu Dhabi is the largest emirate in the United Arab Emirates (UAE), with a diverse and mostly expatriate population of 2.3 million persons. The Health Authority–Abu Dhabi (HAAD) is the healthcare sector regulatory body and the custodian of public health in the emirate. In this role, HAAD directs the public health response for MERS-CoV, in conjunction with the UAE Ministry of Health. The Emirate of Abu Dhabi has 3 regions: the Western Region, which borders Saudi Arabia; Abu Dhabi, which includes a large, mostly urban population; and the Eastern Region, which borders Oman.

Given the emirate's proximity to Saudi Arabia, HAAD created a standardized public health response protocol for MERS-CoV in January 2013 with the following objectives: 1) educate physicians by circulating regular instructions to all healthcare facilities, which contained WHO definitions for confirmed and probable cases and the mechanism for handling suspected cases and reporting to HAAD; 2) ensure laboratory capacity to test for MERS-CoV; and 3) add reporting options for MERS-CoV to existing electronic surveillance systems or create new surveillance systems to detect MERS-CoV. This article describes the public health response to the emergence of MERS-CoV in the region and epidemiologic characteristics of patients with laboratory-confirmed MERS-CoV infection in Abu Dhabi during January 1, 2013–May 9, 2014.

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Methods

Definitions

A case was defined as laboratory-confirmed MERS-CoV infection in a person as determined by a PCR test using 2 gene targets (*14*). Cases were further classified into epidemiologic categories. A household case was illness in a person who spent ≥1 night or 8 continuous hours in the same home with a case-patient while that person was infected. A healthcare-associated case (HCA) was illness in a case-patient who had been exposed to another case-patient exclusively in a healthcare setting in the 14 days before their own onset of symptoms or specimen collection date. A work (or other) setting–related case was illness in a person who was exposed to a case-patient but not in a healthcare or household setting. An epidemiologically unlinked case was defined as illness in a person without any documented link to a patient with suspected or confirmed MERS-CoV infection. Contacts were persons who provided care for the patient, including healthcare personnel or family members; had close physical contact with the patient; or stayed in the same place with a laboratory-confirmed case.

A household cluster was defined as ≥2 cases in the same household. An HCA cluster was ≥2 HCA cases in the same healthcare setting or healthcare interaction (e.g., hospital transport). A work cluster was ≥2 cases in the same workplace. To be considered part of the same cluster, secondary case-patients must have had a positive laboratory test result and share an epidemiologic link, such as a workplace, hospital room, or household. Suspected case-patients who had a negative laboratory test result for MERS-CoV were classified as test-negative suspected case-patients.

Asymptomatic case-patients were those who had no reported symptoms at the time of a positive test recorded by a healthcare provider in the medical chart. Mildly symptomatic case-patients reported symptoms, such as sore throat, rhinorrhea, or cough, and did not require oxygen during their hospital stay. Severely symptomatic case-patients required supplemental oxygenation during their hospitalization, ranging from nasal cannula to intubation.

Surveillance System

HAAD began surveillance for MERS-CoV in January 2013. Three surveillance systems were used to identify case-patients who tested positive for the virus: 1) Infectious Disease Electronic Notification System, in which physicians identified suspected case-patients and completed a basic form on the basis of patients' clinical features; 2) Sheikh Khalifa Medical Center Laboratory surveillance, at the laboratory responsible for processing MERS-CoV specimens, which also records patient demographic characteristics, dates of collection, and results; and 3) Operations Center, which runs an active surveillance system that contacts 42 public and private hospitals in Abu Dhabi and records information regarding patients that were admitted or transferred to the intensive care unit because of primary respiratory failure or acute respiratory distress syndrome.

Because suspected case-patients may be admitted for primary respiratory failure or acute respiratory distress syndrome from multiple etiologies, HAAD authorities conducted investigations to determine whether an identified patient should be tested for MERS-CoV, if the patient had not already been captured by 1 of the surveillance systems. In addition, during April and May 2014, screenings for MERS-CoV were conducted, most under the supervision of HAAD, in various locations where MERS-CoV cases had been identified, including, but not limited to, hospitals and workplace settings.

For each case-patient, HAAD conducted detailed contact investigations within 24 hours of notification. HAAD officials interviewed case-patients if available. If the case-patient was unable to be interviewed, the patient's epidemiologic information was collected from family members or other proxies. Staff at hospitals or local Disease and Prevention Screening Centers tested a sputum (preferred) or nasopharyngeal sample from all case-patient contacts for MERS-CoV. Samples from contacts were tested regardless of whether the person experienced symptoms. For each test-positive case-patient identified, we collected clinical information using the International Severe Acute Respiratory and Emerging Infection Consortium form, which was filled out in real time by healthcare providers and later verified by retrospective chart review.

All persons who tested positive for MERS-CoV, including asymptomatic persons, were admitted to a hospital. Before they could be discharged, confirmed case-patients were required to show negative results on 2 MERS-CoV PCR tests conducted at least 48 hours apart.

Laboratory Diagnostic Testing

The laboratory analyzed upper (e.g., nasopharyngeal, oropharyngeal) and lower respiratory tract samples (e.g., sputum, bronchoalveolar lavage fluid, tracheal aspirates) and serum. If hospital staff were not able to collect sputum spontaneously, the clinician could order that sputum be induced in a negative pressure room or that nasopharyngeal aspirates be obtained. Specimens were tested by using real-time reverse transcription PCR (rRT-PCR), upE, and open reading frame 1 assays in the Sheikh Khalifa Medical Center laboratory (*15*,*16*). A laboratory team from the US Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) used a

nucleocapsid-based rRT-PCR assay to verify infection in a random sample of 23 specimens from 2014 (*17*). To calculate the length of positivity for each MERS-CoV case-patient, we calculated the difference between the date of the first positive test and the date the virus was last detected.

Data Management and Analysis

We merged results from Sheikh Khalifa Medical Center laboratory and HAAD's epidemiologic databases, which contained information on patients' demographic characteristics, symptoms, and exposure, using a combination of unique individual identifiers. We also retrospectively reviewed medical charts to collect additional clinical information about case-patients with positive test results.

Because changes occurred in the surveillance system during the study period, we compared demographic characteristics of MERS-CoV case-patients and those of suspected case-patients who were tested and had negative results during January 1, 2013–April 17, 2014. Suspected case-patients with negative test results identified during April 18, 2014–May 9, 2014, were not available for comparison. We included all MERS-CoV case-patients identified during January 1, 2013–May 9, 2014, in analyses of demographic characteristics, clusters, disease severity, sample type, and PCR positivity. Differences in proportions were contrasted by using Mantel-Haenszel χ^2 test. We defined statistical significance as a p value <0.05. Data were analyzed by using SAS version 9.3 (SAS Institute, Cary, NC, USA).

Ethics

Because these data were collected as part of a public health response, HAAD and CDC determined that data were nonresearch and not subject to review by an institutional review board. Secondary data analysis was then carried out for operational purposes.

Results

Comparison of Laboratory-Confirmed Case-Patients and Test-Negative Suspected Case-Patients

For January 1, 2013–April 17, 2014, HAAD surveillance systems contained records for 1,586 unique persons, including 41 (3%) with confirmed MERS-CoV infection, 1,467 (92%) suspected case-patients with negative test results, and 78 (5%) whose test results were unknown or no test had been performed. Most case-patients were male (61%), Asian (54%), and 20–59 years of age (76%) (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/7/16-0040-Techapp1.pdf). Case-patients more frequently reported exposure to a known or suspected MERS-CoV case-patient (30, 73%) and exposure to an animal (10, 24%) within the previous 2 weeks of illness onset or specimen collection than did suspected case-patients with negative test results. Fifty-one percent of MERS-CoV cases were healthcare associated.

Emiratis and nationals from other Gulf Cooperation Council countries (i.e., Saudi Arabia, United Arab Emirates, Qatar, Bahrain, and Oman) were the groups most often tested (855, 57%), whereas Asians had the highest proportion of positive test results (22/187, 12%). Of all reported and evaluated case-patients, 15% (6/41) had traveled internationally (online Technical Appendix Table 1).

All MERS-CoV Cases

During April 17–May 9, 2014, surveillance activities identified an additional 24 MERS-CoV cases, for a total of 65 cases during the January 1, 2013–April 17, 2014, study period. We retrospectively reviewed charts of 64/65 (98%) case-patients; 1 case-patient was transferred to a hospital outside Abu Dhabi, and medical records were unavailable.

The first case-patient with confirmed MERS-CoV infection in Abu Dhabi was identified in March 2013, and the number of case-patients identified (n = 41) peaked in April 2014 (Figure). During April 2014, the highest number of tests was performed, with 24 positive results, 323 negative results, and no test result available for 64 tests. Through contact tracing, HAAD identified 2,372 contacts for 56 case-patients, an average of 56 contacts (range 2–199 contacts) per positive case-patient.

Most case-patients were male (43/65, 66%) and 20 to 59 years of age (51/65, 78%) (online Technical Appendix Table 2). All but 3 expatriate case-patients had been in the UAE for >1 month before diagnosis. However, those 3 had traveled to or lived in the Arabian Peninsula in the month before diagnosis. Forty-two percent of cases were healthcare associated, 34% were epidemiologically unlinked, 19% were household associated, and 6% were related to work or another setting. Eight (12%) cases resulted in death.

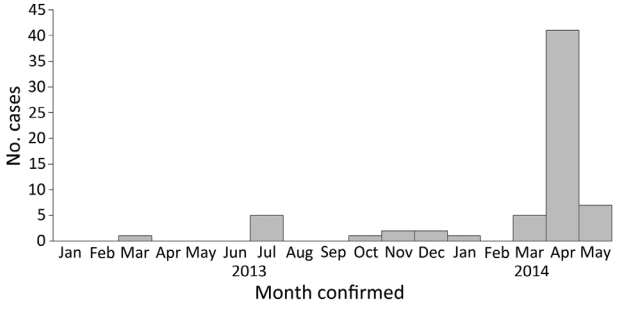

Figure. Epidemiologic curve showing confirmed cases of Middle East respiratory syndrome coronavirus (MERS-CoV) infection, Abu Dhabi, United Arab Emirates, January 1, 2013–May 9, 2014 (N = 65). Most cases were reported during April 2014.

Cluster Identification

We identified 6 clusters. The number of MERS-CoV cases detected by surveillance in 2014 was greater than the number detected in 2013 because of a large cluster that involved both healthcare and household settings in the Eastern Region of UAE during April 2014 (Figure) (*18*). This resulted in 28 epidemiologically-linked MERS-CoV cases. All other clusters in Abu Dhabi—2 healthcare-associated clusters, 1 household-associated cluster, and 2 work-related clusters—involved ≤5 persons.

Epidemiologically Unlinked Cases

We identified 22 (34%) epidemiologically unlinked cases in Abu Dhabi (online Technical Appendix Table 2); 86% were diagnosed in 2014, and the same percentage occurred in men. Most (59%) epidemiologically unlinked case-patients were 20–59 years of age; median age was 45 years (interquartile range [IQR] 36–66 years). Eight (36%) had traveled outside UAE within 14 days of initial symptoms (3 to Saudi Arabia, 3 to Oman, 1 to Bahrain, and 1 to Thailand). Six (27%) case-patients had documented contact with camels within 14 days before symptom onset or, if asymptomatic, before the date of specimen collection. Eight (36%) case-patients had visited a healthcare setting, such as a dialysis clinic, health clinic, or emergency department, before onset of illness, but these cases were not connected with a known healthcare-associated cluster.

Disease Severity

A higher proportion of case-patients were male in all 3 disease severity categories (Table 1). The gender disparity appeared to be lower among mildly symptomatic case patients; it was not significantly different for asymptomatic and for severely symptomatic case-patients (p = 0.25; Table 1). Patients who had mild or no symptoms were of similar ages, whereas those patients with severe symptoms were older (median age 37, 42, and 60 years, respectively) (Table 1). Of the 31 healthcare-personnel case-patients, 12 (39%) were asymptomatic. Among case-patients with mild and severe symptoms, 46% and 73%, respectively, had symptoms 1 to 7 days before being hospitalized (Table 1). Duration of patients' PCR-positivity lengthened as disease severity increased (p = 0.03; Table 2). Of 15 case-patients who had positive test results for >2 weeks, 1 (7%) was asymptomatic.

Sample Type and Positivity

Samples from case-patients were tested, at physician's discretion, while patients were in isolation. Case-patients with severe symptoms had a median of 4 (IQR 2–8, total 103) MERS-CoV rRT-PCR tests, patients with mild symptoms a median of 7 (IQR 3–14, total 214) tests, and patients with no reported symptoms a median of 5 (IQR 4–8, total 147) tests. Most (249/297, 84%) positive results were from lower respiratory tract specimens. Across all disease severity categories, 34% (85/249) of lower respiratory tract samples were positive ≥14 days after initial positive test for

Table 1. Demographic information, symptom duration, and length of positivity by disease severity among MERS-CoV case-patients, Abu Dhabi, United Arab Emirates, January 1, 2013–May 9, 2014*

Characteristic, N = 65	Asymptomatic, n = 23 (35)	With mild symptoms, n = 24 (37)	With severe symptoms, n = 18 (28)	p value†
Sex				0.25
M	16 (70)	13 (54)	14 (78)	
F	7 (30)	11 (46)	4 (22)	
Age, y, median (IQR)	42 (30–54)	37 (30–43)	60 (40–68)	
≤19	0	2 (8)	0	
20–39	10 (44)	13 (54)	4 (22)	
40–59	10 (44)	9 (38)	5 (28)	
≥60	3 (13)	0 (0)	9 (50)	
Healthcare personnel				<0.001
Yes‡	12 (52)	17 (71)	2 (11)	
No	11 (48)	7 (29)	16 (89)	
Symptom duration before hospitalization				<0.001
≥8 d	NA	1 (4)	1 (6)	
4–7 d	NA	6 (25)	10 (56)	
1–3 d	NA	5 (21)	3 (17)	
Same day as admission	NA	9 (38)	2 (11)	
After admission	NA	2 (8)	2 (11)	
Unknown	NA	1 (4)	0	
Length of PCR positivity, d				0.03
<7	15 (65)	12 (50)	12 (67)	
7–14	7 (30)	3 (13)	1 (6)	
>14	1 (4)	9 (38)	5 (28)	

*Values are no. (%) patients except as indicated. Percentages might not sum to 100% due to rounding. IQR, interquartile range; MERS-CoV, Middle East respiratory syndrome coronavirus; NA, not applicable.
†Mantel-Haenszel χ^2 test.
‡Healthcare personnel were not necessarily part of a healthcare-associated cluster.

MERS-CoV, whereas 13% (6/48) of upper respiratory tract samples were positive ≥14 days (p<0.01) (Table 2). Specimens from 6% of case-patients (4/65) remained positive for >3 weeks. These positive specimens included those from 1 case-patient with severe symptoms and from 1 case-patient with mild symptoms whose samples remained positive for 23 days; 1 case-patient with mild symptoms whose samples remained positive for 27 days; and 1 case-patient with mild symptoms whose samples remained positive for 28 days.

Overall, upper respiratory samples were positive less frequently than lower respiratory tract samples (Table 2). In addition, upper respiratory samples were positive less frequently as disease severity lessened (Table 2). Lower respiratory tract samples, however, had a longer duration of PCR positivity than upper respiratory tract samples. For case-patients with severe and mild symptoms, a higher percentage of lower respiratory samples than upper respiratory samples was positive ≥14 days (for patients with severe cases, 39% vs. 8%; for patients with mild symptoms, 37% vs. 1%) (Table 2).

Discussion

We describe the public health response and epidemiologic characteristics, clusters, and laboratory results of MERS-CoV case-patients in the Abu Dhabi Emirate of UAE, the country with the third highest number of cases reported to WHO as of July 15, 2015. The Abu Dhabi cases report a relatively low case-fatality rate of 12% compared to the global case-fatality rate of 36% as reported by WHO (*19*); however, surveillance, contact investigations, and reporting methods may vary over time among countries. This descriptive study has several unique characteristics: a large number of cases, including cases from the 3 regions of the Abu Dhabi Emirate; wide range of ages tested; comprehensive contact investigations, including laboratory results; and test results for asymptomatic case-patients. We also report the lengthy duration of viral detection in some asymptomatic case-patients. These findings have useful implications for MERS-CoV management and prevention strategies.

Consistent with previous reports, men were predominantly infected. However, the age group most commonly affected was 20–59 years of age, and 35% of all case-patients were asymptomatic detected during contact investigations. Those with severe symptoms tended to be ≥60 years of age, whereas asymptomatic case-patients tended to be younger. This finding agrees with prior case series and cluster analyses in which more severe disease tended to develop in older case-patients (*13*); however, younger persons may have been overrepresented during the contact tracing investigations in our study.

Fever and cough were prominent features of MERS-CoV infection in previous case reports (*6*,*20*), and we found that these symptoms were slightly more frequent among case-patients with confirmed MERS-CoV than among test-negative suspected case-patients. The clinical features of MERS-CoV mimic several other more common illnesses; fever, cough, shortness of breath, and odynophagia were most commonly reported in both case-patients and test-negative suspected case-patients. Therefore, clinicians must continue to maintain a high index of suspicion based on epidemiologic risk factors. More than half of case-patients in our study had contact with another MERS-CoV case-patient, and 20% had some type of animal contact within the previous 14 days. The importance of animal contact is unknown but might be an indicator of camel contact, which has been associated with MERS-CoV infection (*21*).

Most samples from case-patients were taken from the lower respiratory tract, which is believed to be the priority source for specimens for the diagnosis of MERS-CoV (rather than the upper respiratory tract) (*3*,*22*,*23*). In a recent study, Poissy et al. suggested that lower respiratory tract samples are valuable for monitoring MERS-CoV infection (*24*); our results from 65 case-patients with 249 lower respiratory tract specimens further supports this hypothesis. Among all case-patients and disease severity categories, a higher proportion of lower respiratory tract samples were positive ≥14 days than were upper respiratory tract samples.

Our study identified case-patients who continued to have positive test results for >3 weeks; the longest length of positivity was 28 days in a person with mild symptoms. This finding aligns with results of recent studies that have found case-patients testing positive for MERS-CoV up to 30 days after their first positive test (*24*–*26*). Of the 15 persons in our study who tested positive for >2 weeks, 1 (7%) was asymptomatic and 9 (60%) were mildly symptomatic, which

Table 2. Number of days samples from MERS-CoV case-patients were positive for the virus by rRT-PCR, stratified by disease severity and type of sample, Abu Dhabi, United Arab Emirates, January 1, 2013–May 9, 2014*

		No. (%) positive LRT samples		No. (%) positive URT samples	
Disease severity	No. samples	<14 d	≥14 d	<14 d	≥14 d
Severely symptomatic	67	23 (34)	26 (39)	13 (19)	5 (8)
Mildly symptomatic	148	81 (55)	54 (37)	12 (8)	1 (1)
Asymptomatic	82	60 (73)	5 (6)†	17 (21)	0
Total	297	164 (55)	85 (29)	42 (14)	6 (2)

*Percentages might not sum to 100% due to rounding. LRT, lower respiratory tract; MERS-CoV, Middle East respiratory syndrome coronavirus; rRT-PCR, real-time reverse transcription PCR; URT, upper respiratory tract.
†All 5 lower respiratory samples that were positive for ≥14 d were from 1 asymptomatic case-patient.

is consistent with a recent case report (*25*). This finding highlights the need to further clarify whether asymptomatic and mildly symptomatic persons play a role in transmitting MERS-CoV to others (*27*).

This study has several imitations. After the merging of 3 independent surveillance systems, some data were incomplete. The case counts increased in April 2014, and HAAD overhauled the surveillance system to meet current epidemiologic needs; this resulted in an inability to compare test-negative suspected case-patients with case-patients after April 17. In addition, characteristics of case-patients may be skewed because most were healthcare personnel from a single hospital cluster. Also, we were unable to correlate timing of reported clinical symptoms with laboratory sample collection in 7 of the symptomatic case-patients, even though multiple data sources were used. Moreover, laboratory specimen collection was not systematic in timing or in type of specimens; laboratory specimens were ordered by physicians at different times during a patient's hospitalization. Because we reviewed medical charts retrospectively, we were unable to verify whether asymptomatic patients were truly asymptomatic, or if they had undocumented mild symptoms. Although PCR testing provides information regarding viral detection, it does not provide information regarding live virus, and its correlation with virus transmission is unknown. Finally, genetic sequence analysis was able to support the epidemiologic links found in the large healthcare-associated cluster, but not all case-patients had specimens available for genetic sequencing.

In summary, our findings of predominance of male MERS-CoV case-patients, development of more severe disease in older case-patients, and clustering in healthcare settings and household settings are consistent with previous reports (*12*,*20*). This descriptive study also highlights demographic, risk factor, and symptom data related to case-patients tested for MERS-CoV in Abu Dhabi. Our study provides further evidence of a long duration of PCR positivity and the value of using lower respiratory tract samples in monitoring MERS-CoV infection. We also identified asymptomatic and mildly ill MERS-CoV case-patients, which informs practicing clinicians that MERS-CoV causes a wide spectrum of disease. Finally, our study provided a detailed overview of the unique and comprehensive surveillance and response model for MERS-CoV in Abu Dhabi, which included screening symptomatic and asymptomatic case-patient contacts and collecting detailed epidemiologic data on MERS-CoV case-patients. Further studies must investigate characteristics of case-patients, the role of virus detected by PCR in virus transmission, and potential MERS-CoV spread from mildly ill or asymptomatic patients to clarify, and ultimately stop, MERS-CoV transmission.

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References

1. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med. 2012;367:1814–20. http://dx.doi.org/10.1056/NEJMoa1211721
2. Puzelli S, Azzi A, Santini MG, Di Martino A, Facchini M, Castrucci MR, et al. Investigation of an imported case of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) infection in Florence, Italy, May to June 2013. Euro Surveill. 2013;18:20564. http://dx.doi.org/10.2807/1560-7917.ES2013.18.34.20564
3. Pebody RG, Chand MA, Thomas HL, Green HK, Boddington NL, Carvalho C, et al. The United Kingdom public health response to an imported laboratory confirmed case of a novel coronavirus in September 2012. Euro Surveill. 2012;17:20292.
4. Kraaij-Dirkzwager M, Timen A, Dirksen K, Gelinck L, Leyten E, Groeneveld P, et al. Middle East respiratory syndrome coronavirus (MERS-CoV) infections in two returning travellers in the Netherlands, May 2014. Euro Surveill. 2014;19:20817. http://dx.doi.org/10.2807/1560-7917.ES2014.19.21.20817
5. Health Protection Agency UK Novel Coronavirus Investigation Team. Evidence of person-to-person transmission within a family cluster of novel coronavirus infections, United Kingdom, February 2013. Euro Surveill. 2013;18:20427.
6. Gulland A. Two cases of novel coronavirus are confirmed in France. BMJ. 2013;346:f3114. http://dx.doi.org/10.1136/bmj.f3114
7. Buchholz U, Muller MA, Nitsche A, Sanewski A, Wevering N, Bauer-Balci T, et al. Contact investigation of a case of human novel coronavirus infection treated in a German hospital, October–November 2012. Euro Surveill. 2013;18:20406.
8. Kapoor M, Pringle K, Kumar A, Dearth S, Liu L, Lovchik J, et al. Clinical and laboratory findings of the first imported case of Middle East respiratory syndrome coronavirus (MERS-CoV) into the United States. Clin Infect Dis. 2014;59:1511–8. http://dx.doi.org/10.1093/cid/ciu635
9. Al-Abdallat MM, Payne DC, Alqasrawi S, Rha B, Tohme RA, Abedi GR, et al. Hospital-associated outbreak of Middle East respiratory syndrome coronavirus: a serologic, epidemiologic, and clinical description. Clin Infect Dis. 2014;59:1225–33. http://dx.doi.org/10.1093/cid/ciu359
10. Abroug F, Slim A, Ouanes-Besbes L, Hadj Kacem MA, Dachraoui F, Ouanes I, et al. Family cluster of Middle East respiratory syndrome coronavirus infections, Tunisia, 2013. Emerg Infect Dis. 2014;20:1527–30. http://dx.doi.org/10.3201/eid2009.140378
11. World Health Organization. Middle East respiratory syndrome coronavirus (MERS-CoV)—Republic of Korea. 2015 Jul 7 [cited 2015 Aug 21]. http://www.who.int/csr/don/07-july-2015-mers-korea/en/
12. Omrani AS, Matin MA, Haddad Q, Al-Nakhli D, Memish ZA, Albarrak AM. A family cluster of Middle East respiratory syndrome coronavirus infections related to a likely unrecognized asymptomatic or mild case. Int J Infect Dis. 2013;17:e668–72. http://dx.doi.org/10.1016/j.ijid.2013.07.001

13. Assiri A, McGeer A, Perl TM, Price CS, Al Rabeeah AA, Cummings DA, et al. Hospital outbreak of Middle East respiratory syndrome coronavirus. N Engl J Med. 2013;369:407–16. http://dx.doi.org/10.1056/NEJMoa1306742
14. World Health Organization. Revised interim case definition for reporting to WHO—Middle East respiratory syndrome coronavirus (MERS-CoV). 2013 Jul 3 [cited 2015 Aug 21]. http://www.who.int/csr/disease/coronavirus_infections/case_definition_03_07_2014/en/
15. Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, et al. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill. 2012;17:20285.
16. Corman VM, Muller MA, Costabel U, Timm J, Binger T, Meyer B, et al. Assays for laboratory confirmation of novel human coronavirus (hCoV-EMC) infections. Euro Surveill. 2012;17:pii:20334.
17. Lu X, Whitaker B, Sakthivel SK, Kamili S, Rose LE, Lowe L, et al. Real-time reverse transcription-PCR assay panel for Middle East respiratory syndrome coronavirus. J Clin Microbiol. 2014;52:67–75. http://dx.doi.org/10.1128/JCM.02533-13
18. Hunter JC, Nguyen D, Aden B, Al Bandar Z, Al Dhaheri W, Abu Elkheir K, et al. Transmission of Middle East respiratory syndrome coronavirus infections in healthcare settings, Abu Dhabi. Emerg Infect Dis. 2016;22:647–56. http://dx.doi.org/10.3201/eid2204.151615
19. World Health Organization. Middle East respiratory syndrome coronavirus (MERS-CoV) [cited 2016 Feb 10]. http://www.who.int/emergencies/mers-cov/en/
20. Assiri A, Al-Tawfiq JA, Al-Rabeeah AA, Al-Rabiah FA, Al-Hajjar S, Al-Barrak A, et al. Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. Lancet Infect Dis. 2013;13:752–61. http://dx.doi.org/10.1016/S1473-3099(13)70204-4
21. Alraddadi BM, Watson JT, Almarashi A, Abedi GR, Turkistani A, Sadran M, et al. Risk factors for primary Middle East respiratory syndrome coronavirus illness in humans, Saudi Arabia, 2014. Emerg Infect Dis. 2016;22:49–55. http://dx.doi.org/10.3201/eid2201.151340
22. Guery B, Poissy J, el Mansouf L, Sejourne C, Ettahar N, Lemaire X, et al. Clinical features and viral diagnosis of two cases of infection with Middle East respiratory syndrome coronavirus: a report of nosocomial transmission. Lancet. 2013;381:2265–72. http://dx.doi.org/10.1016/S0140-6736(13)60982-4
23. Drosten C, Seilmaier M, Corman VM, Hartmann W, Scheible G, Sack S, et al. Clinical features and virological analysis of a case of Middle East respiratory syndrome coronavirus infection. Lancet Infect Dis. 2013;13:745–51. http://dx.doi.org/10.1016/S1473-3099(13)70154-3
24. Poissy J, Goffard A, Parmentier-Decrucq E, Favory R, Kauv M, Kipnis E, et al. Kinetics and pattern of viral excretion in biological specimens of two MERS-CoV cases. J Clin Virol. 2014;61:275–8. http://dx.doi.org/10.1016/j.jcv.2014.07.002
25. Shalhoub S, AlZahrani A, Simhairi R, Mushtaq A. Successful recovery of MERS CoV pneumonia in a patient with acquired immunodeficiency syndrome: a case report. J Clin Virol. 2015;62:69–71. http://dx.doi.org/10.1016/j.jcv.2014.11.030
26. Al-Gethamy M, Corman VM, Hussain R, Al-Tawfiq JA, Drosten C, Memish ZA. A case of long-term excretion and subclinical infection with Middle East respiratory syndrome coronavirus in a healthcare worker. Clin Infect Dis. 2015;60:973–4. http://dx.doi.org/10.1093/cid/ciu1135
27. Corman VM, Albarrak AM, Omrani AS, Albarrak MM, Farah ME, Almasri M, et al. Viral shedding and antibody response in 37 patients with Middle East respiratory syndrome coronavirus infection. Clin Infect Dis. 2016; 62:477–83. http://dx.doi.org/10.1093/cid/civ951

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Current Guidelines, Common Clinical Pitfalls, and Future Directions for Laboratory Diagnosis of Lyme Disease, United States

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Learning Objectives

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

- Distinguish current US testing guidelines for Lyme disease, based on a review
- Determine appropriate use and interpretation of tests for Lyme disease
- Identify recent developments in Lyme disease diagnostics

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In the United States, Lyme disease is caused by *Borrelia burgdorferi* and transmitted to humans by blacklegged ticks. Patients with an erythema migrans lesion and epidemiologic risk can be given a diagnosis without laboratory testing.

[1]These authors contributed equally to this article.

For all other patients, laboratory testing is necessary to confirm the diagnosis, but proper interpretation depends on symptoms and timing of illness. The recommended laboratory test in the United States is 2-tiered serologic analysis consisting of an enzyme-linked immunoassay or immunofluorescence assay, followed by reflexive immunoblotting. Sensitivity of 2-tiered testing is low (30%–40%) during early infection while the antibody response is developing (window period). For disseminated Lyme disease, sensitivity is 70%–100%. Specificity is high (>95%) during all stages of disease. Use of other diagnostic tests for Lyme disease is limited. We review the rationale behind current US testing guidelines, appropriate use and interpretation of tests, and recent developments in Lyme disease diagnostics.

Lyme disease is a tickborne disease caused by spirochetes within the *Borrelia burgdorferi* sensu lato species complex (*1*). In the United States, Lyme disease is caused by *B. burgdorferi* sensu stricto and *B. mayonii* and is transmitted to humans by infected *Ixodes scapularis* or *I. pacificus* ticks (commonly known as blacklegged ticks) (*2*). Lyme disease is the most common vectorborne disease in the United States and causes an estimated 300,000 illnesses annually (*3,4*). Cases occur primarily in the northeast and upper midwest regions (Figure 1); however, ecologic and environmental changes have catalyzed a gradual geographic expansion (*5*).

There are 3 stages of *B. burgdorferi* infection: early localized, early disseminated, and late disseminated. The classic sign of localized infection is erythema migrans (EM), which is defined as a gradually expanding annular lesion ≥5 cm in diameter. Approximately 70%–80% of persons with Lyme disease have EM (*1,6*). Accompanying signs and symptoms might include fever, lymphadenopathy, myalgias, or arthralgias. If the infection is not treated, the bacteria might spread hematogenously and cause early disseminated Lyme disease, which can manifest as multiple EM skin lesions, facial palsy, meningitis, or carditis. Recurrent large-joint arthritis is the hallmark of late disseminated disease. Late neurologic Lyme disease is uncommon in the United States. Symptoms might include peripheral neuropathy, encephalopathy, or encephalomyelitis.

Patients who have a lesion consistent with EM and live in or have traveled to Lyme-endemic areas can be given a diagnosis without laboratory testing (*6*). In the absence of EM, all other manifestations of Lyme disease

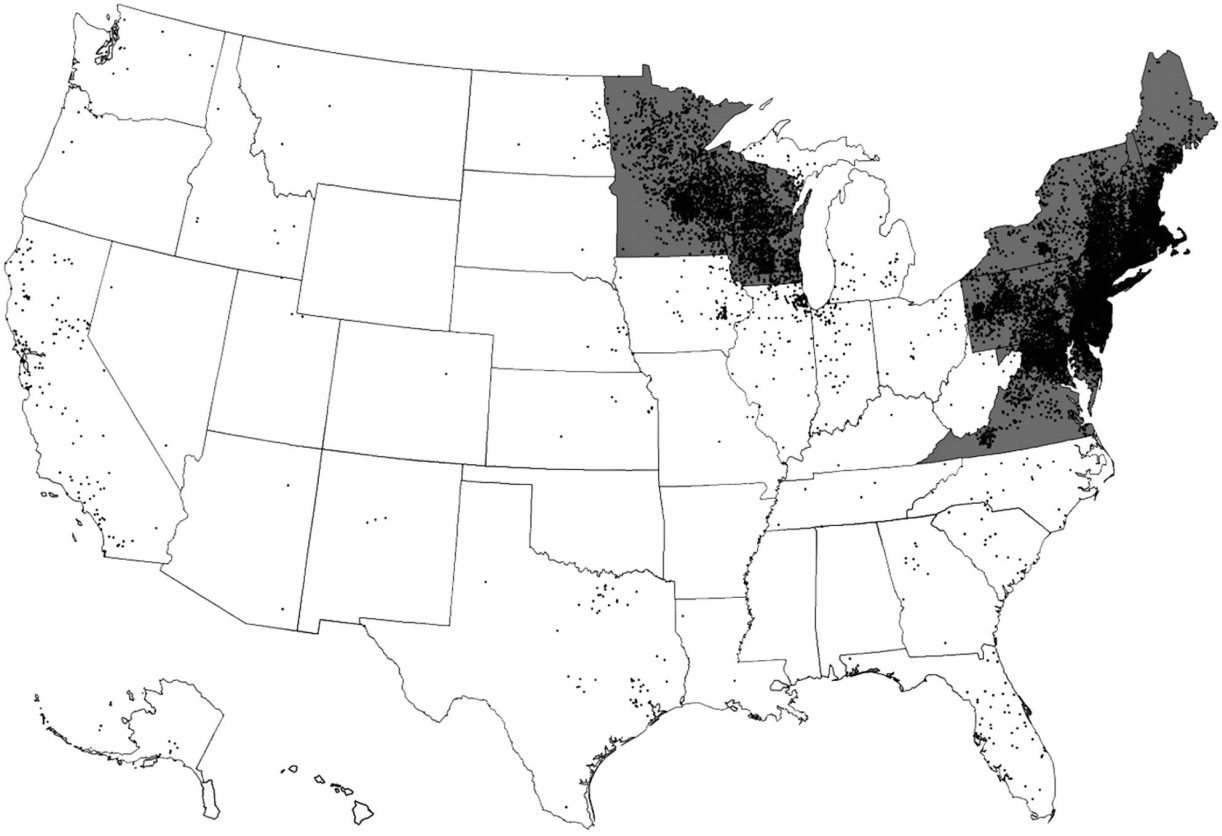

Figure 1. Lyme disease cases (black dots) reported by surveillance, United States, 2005–2010. One dot is placed randomly within the county of residence for each confirmed case. States with the highest incidence of clinician-diagnosed Lyme disease in a large health insurance claims database (gray areas) are also shown. Transmission also occurs in small regions of northern California, Oregon, and Washington. Adapted from (*4*).

require serologic analysis to confirm the diagnosis. The recommended approach for laboratory diagnosis of Lyme disease is a 2-tiered serologic test comprised of an enzyme-linked immunoassay (EIA or ELISA) or immunofluorescence assay (IFA), followed by a reflex Western immunoblot (*7*). When used in accordance with current testing guidelines (*7*), 2-tiered serologic testing is a valuable and highly specific clinical tool for diagnosis of disseminated Lyme disease. Confusion exists, however, among patients and clinicians concerning appropriate use and interpretation of this and other diagnostic tests for Lyme disease (*8,9*). In this article, we review the rationale behind current United States testing guidelines, use and interpretation of 2-tiered serologic analysis and other tests in the clinical setting, and recent developments in the field of Lyme disease diagnostics.

Historical Perspective

The discovery of *B. burgdorferi* as the causative agent of Lyme disease in 1982 prompted development of numerous tests by clinical and private laboratories. Because spirochetes only transiently enter the bloodstream of infected persons in small numbers, direct detection of *B. burgdorferi* by PCR or culture has been challenging (*10*). For this reason, most diagnostic test development has focused on indirect detection of infection by assessing the antibody response of the patient.

Initially, the variety of serologic tests and lack of concordance among different methods necessitated standardization. In 1994, leading experts convened at the Second National Conference on the Serologic Diagnosis of Lyme Disease (Dearborn, Michigan, USA) to review the current evidence and devise a standard testing strategy (*7*). After evaluating the evidence, it became clear that no single test was sufficient on its own. To maximize clinical utility and specificity, the conference diagnostic working group ultimately decided on a 2-tiered serologic testing algorithm (Figure 2). The first tier uses a highly sensitive EIA or IFA that, if the result is positive or equivocal, is followed by a highly specific Western immunoblot as the second-tier test (*7*). Western immunoblot was included in response to a multicenter evaluation of laboratories performing Lyme disease testing, which found that using Western immunoblot in addition to EIA increased specificity to >98%, reducing false-positive results produced by the first-tier EIA (*11*).

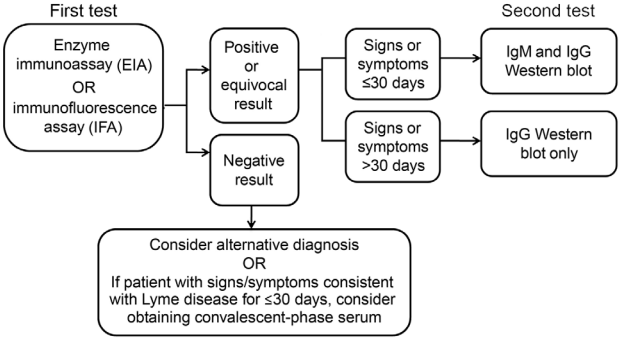

Figure 2. Two-tiered testing for Lyme disease, United States. Adapted from (*7*).

Two-Tiered Serologic Testing

When performed and interpreted in accordance with current guidelines, 2-tiered serologic analysis has a sensitivity of ≈70%–100% and a specificity >95% for disseminated Lyme disease (Table) (*6,12–15*). Thus, this analysis is the standard of care in diagnosing disseminated Lyme disease but requires appropriate clinical judgment when ordering the test and interpreting the results. To this end, understanding the underlying testing procedure is beneficial.

First Tier

The first-tier test involves measuring the overall antibody response (typically IgM and IgG) of a patient to *B. burgdorferi* antigens (*7*). Although both the EIA and IFA have been cleared by the Food and Drug Administration (FDA; Silver Spring, MD, USA) as first-tier tests, laboratories most commonly perform EIA because it is more easily automated. An additional benefit of EIA is that it provides a quantitative value of the relative concentration of antibodies in the serum of a patient compared with that of a control, which enables use of objective cutoff values (*10*).

In the United States, most laboratories use a whole-cell sonicate preparation of *B. burgdorferi* as antigen for the EIA. This test approach has high sensitivity because of multiple antigens in whole-cell sonicate preparation. However, because some of these antigens are cross-reactive with antigens from the host or other pathogens, specificity of the EIA alone is not optimal (*10*).

Additional FDA-cleared EIAs that use as few as 1 to several antigens, which results in a higher specificity and similar sensitivity than that for whole-cell sonicate EIAs, have recently become commercially available. The cell surface variable-major protein-like sequence expressed (VlsE) lipoprotein and its sixth invariable region, the C6 peptide, are 2 FDA-cleared EIA antigens that are gaining popularity (*16,17*). These *Borrelia* antigens are highly conserved and immunogenic among all Lyme borreliosis species and strains, and cause an early antibody response useful for diagnostic testing (*18*).

Second Tier

Similar to EIA, the second-tier immunoblot is a serologic test that detects antibodies produced against *B. burgdorferi* (*10*). Unlike EIA, however, the immunoblot detects

Table. Sensitivity and specificity of serologic tests for patients with Lyme disease, United States*

Variable	Standard 2-tiered algorithm with whole-cell sonicate EIA†			Standard 2-tiered algorithm with C6 EIA,‡	Two-EIA algorithm§	
	Molins et al. (CDC Lyme Repository) (*14*)	Wormser et al. (*15*)	Branda et al. (*12*)	Wormser et al. (*13*)	Branda et al. (*12*)	Wormser et al. (*13,15*)
	% Sensitivity (no. tested)					
Early Lyme disease with EM¶						
Acute phase	40 (40)	38 (298)	42 (114)	38 (298)	53 (114)	58 (298)
Convalescent phase	61 (38)	27 (105)	57 (63)#	26 (105)	89 (63)#	67 (105)
Noncutaneous manifestations	96 (46)	94 (142)	87 (55)	93 (142)	100 (55)	97 (144)
Neuritis or carditis	88 (17)	80 (20)	73 (26)	80 (20)	100 (26)	ND
Early Lyme disease with neuritis or carditis	100 (29)	96 (122)	100 (29)	95 (122)	100 (29)	ND
	% Specificity (no. tested)					
Healthy controls						
Endemic area	98 (101)	99 (1,329)	99 (1,146)	100 (1,329)	99 (1,146)	≥99 (1,329)**
Nonendemic area	100 (102)	99.8 (513)	100 (100)	100 (513)	100 (100)	≥99 (513)**
Controls with selected other diseases						
Syphilis or RPR positive††	95 (20)	95 (20)	ND	95 (20)	ND	≥95 (20)**
Infectious mononucleosis or EBV/CMV positive††	90 (30)	100 (40)	ND	100 (40)	ND	100 (20)
Helicobacter pylori	ND	95 (20)	ND	100 (20)	ND	100 (20)
All nonhealthy controls	97 (144)‡‡	99 (366)§§	100 (54)¶¶	100 (366)§§	100 (54)¶¶	100 (366)

*All percentage values were rounded to the nearest whole number. C6, C6 peptide of *Borrelia burgdorferi*; CDC, Centers for Disease Control and Prevention; CMV, cytomegalovirus; EIA, enzyme immunoassay; EM, erythema migrans; EBV, Epstein-Barr virus; ND, not done; RPR, rapid plasma regain.
†Standard 2-tiered algorithm: whole-cell sonicate EIA, then IgG (+IgM if presenting within 1 month) Western blot if positive or equivocal result.
‡C6+ Western blot algorithm: C6 EIA, then IgG (+IgM if presenting within 1 month) Western blot if positive or equivocal result.
§Two-tiered EIA: whole-cell sonicate EIA, then C6 EIA if positive or equivocal result.
¶Patients with EM and epidemiologic risk can be given a diagnosis without serologic analysis (see Figure 3).
#Branda et al. (*12*) conducted only convalescent-phase serologic analysis on a well-characterized serum set of Lyme disease patients and controls. All other data points from this study include the data from well-characterized serum set and serum samples submitted to Massachusetts General Hospital (Boston, MA, USA) for routine testing.
**Minimum specificity reported by Wormser et al. (*13,15*).
††Molins et al. (*14*) tested samples from patients with syphilis or infectious mononucleosis. Wormser et al. (*13,15*) tested blood samples with positive results for RPR or CMV/EBV.
‡‡In the report by Molins et al. (*14*), 2-tiered testing had 100% specificity for all other diseases not mentioned above. Other conditions tested include fibromyalgia, severe periodontitis, rheumatoid arthritis, and multiple sclerosis.
§§Among patients tested by Wormser et al. (*13,15*) there was a single hemolyzed blood sample that showed positive results for all tests. However, both methods of 2-tiered testing had 100% specificity for all other conditions not mentioned above, including *Mycoplasma pneumoniae* infection; HIV; hepatitis A, B, and C; influenza vaccinations; antinuclear antibodies; lipemia; icterus; systemic lupus erythematosus; rheumatoid arthritis; and positive results for rheumatoid factor.
¶¶Includes 25 patients with chronic fatigue syndrome or fibromyalgia, 14 with rheumatic diseases, 9 with neurologic conditions, 5 with infections, and 1 with T-cell lymphoma.

antibodies against a set of preselected *B. burgdorferi* protein antigens. Antibody reactivity to these antigens (indicated by bands on the Western immunoblot) is considered present if bands are visualized with intensity equal to or greater than a control band (*7*).

The specific Western immunoblot test ordered and its subsequent interpretation is dependent on the time course of illness (Figure 2) (*7*). IgM response appears first and is generally directed at the most immunogenic antigens (*19*). Therefore, IgM Western immunoblot should be performed along with IgG Western immunoblot on a reflex basis for patients with signs and symptoms lasting ≤30 days (*7*). Some patients may require acute-phase and convalescent-phase serologic analysis because of decreased sensitivity during the first weeks of infection (*7,10*).

The IgG response generally follows that of IgM and involves a larger number of antigens. Because most patients have a detectable IgG response beyond 30 days, IgG Western immunoblot as the second-tier test is typically sufficient for diagnosis (*19*). At this stage, IgM Western immunoblot is unnecessary and increases the risk for false-positive results.

A positive IgM Western immunoblot result is indicated by the scored presence of ≥2 of 3 bands (21–24, 39, and 41 kDa), and a positive IgG result is indicated by the scored presence of ≥5 of 10 bands (18, 21–24, 28, 30, 39, 41, 45, 58, 66, and 93 kDa) (*7*). The 21–24-kDa band represents OspC, an outer surface protein with variable length and amino acid sequence.

It is imperative to avoid interpreting fewer bands as a positive overall result or evidence of infection because antibodies to several antigens are cross-reactive with non-Borrelial antigens. For example, the 41-kDa band indicates reactive antibody against a *B. burgdorferi* flagellin protein. However, this antibody cross-reacts with other bacterial flagellar proteins and was found in 43% of healthy controls in 1 study, including many persons with little or no exposure risk for Lyme disease (*17*).

Therefore, presence of 1 IgM band or ≤4 IgG bands does not indicate an overall positive result. Overinterpreting a small number of antibody bands leads to reduced specificity and potential misdiagnosis (*9,20*).

Additional Diagnostic Tests

Antibody Testing of Cerebrospinal Fluid

Testing for intrathecal antibody production is integral in the diagnosis of Lyme neuroborreliosis in Europe, where multiple *Borrelia* species and high background seroprevalence limit the usefulness of serologic analysis (*1*). In the United States, the presence of serum antibodies in the appropriate clinical setting is highly sensitive and specific for Lyme neuroborreliosis, making 2-tiered serologic analysis the diagnostic test of choice in most instances (*6,10*). Adjunctive testing for intrathecal antibody production is highly specific and might be helpful in confirming the diagnosis, particularly in regions of high seroprevalence. However, a negative result is insufficient to rule out Lyme neuroborreliosis except in cases of encephalomyelitis.

When testing for intrathecal antibodies, it is essential to note that antibodies in serum are passively transferred to cerebrospinal fluid (CSF) in some patients with Lyme disease (*10,21*). To control for this transfer, CSF and serum should be collected on the same day and diluted to match the total protein or IgG concentration. A CSF/serum IgG EIA optical density ratio >1.0 indicates active intrathecal antibody production.

PCR and Culture

PCR can provide highly specific evidence of *B. burgdorferi* nucleic acid in a variety of samples, including synovial fluid, skin biopsy tissue, blood, and CSF (*10,22*). However, its clinical utility is limited by low sensitivity (particularly for blood and CSF samples) and its potential for contamination (*10,23*).

Synovial fluid PCR is >75% sensitive for Lyme arthritis and might be useful in conjunction with other synovial fluid analyses to differentiate Lyme arthritis from other arthritides (*10,22*). Comparatively, PCR of CSF is substantially less sensitive, which limits its clinical utility. In 1 US study, PCR testing of CSF yielded positive results for only 38% of patients with early neuroborreliosis and was even less sensitive for late neuroborreliosis (*24*).

Studies of PCR on blood have found that its high specificity is outweighed by its lack of clinical sensitivity and potential for contamination (*10,22*). Thus, PCR has not been universally standardized or optimized for diagnosis of Lyme disease. Nevertheless, some clinical laboratories offer PCR testing for *Borrelia* spp., and PCR of blood has shown utility in detection of the novel genospecies *B. miyamotoi* and *B. mayonii* (*25*).

Because *B. burgdorferi* is a slow-growing organism, current culturing methods are labor-intensive and have poor sensitivity. Culturing is generally not recommended for purposes other than research or for corroboration of disease acquired in regions previously unrecognized for risk of infection (*10*).

Clinical Considerations and Common Pitfalls

Timing of Testing—Window Period

As with all serologic tests, clinicians must consider the timing of a patient's illness when ordering and interpreting Lyme disease tests (*6*). Serologic analysis has low sensitivity during the first few weeks of infection while the antibody response is still developing (*10*). This period is known as the window period and is common to all serologic testing. Patients with illnesses suspicious for early Lyme disease but lacking typical EM can present a diagnostic dilemma because serologic test results might be negative at this point (*6*). In these cases, treatment can be administered at the discretion of the clinician, but serologic analysis is necessary to confirm the diagnosis (Figures 2,3).

Background Seropositivity

Background seropositivity is a major consideration when testing for Lyme disease. In a seroepidemiologic study conducted in New York, 5% of study participants were found to have antibodies against *B. burgdorferi* (*26*). Seropositivity can result from previous exposure because IgM and IgG against *B. burgdorferi* can remain for many years after initial infection (which, incidentally, is why serologic testing is not useful as a test of cure) (*26,27*). However, in the seroepidemiologic study in New York, 59% of seropositive patients denied a prior diagnosis of Lyme disease (*26*). In such persons, seropositivity might indicate a false-positive result or be due to a prior undiagnosed infection that either resolved spontaneously or was treated incidentally with antimicrobial drugs prescribed for another indication.

Reinfection

Because of antibody persistence, serologic diagnosis of patients with possible reinfection poses a major dilemma for clinicians (*28*). In cases of suspected reinfection, a detailed history and physical examination, including a thorough skin examination, are essential because most patients will have EM. For patients without EM, serologic analysis is still recommended but results should be interpreted with caution. In these cases, it might be helpful to conduct acute-phase and convalescent-phase serologic analysis to detect an increase in EIA titer or an increase in the number of antibody bands that might indicate active infection (*10,28*).

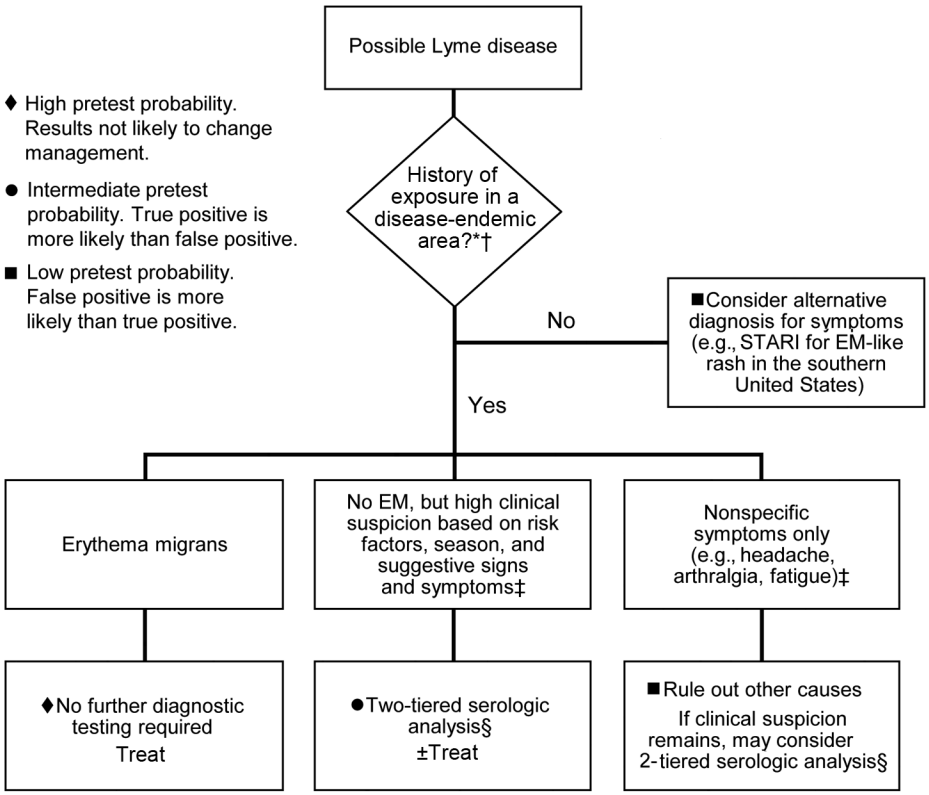

Figure 3. Clinical approach to diagnosis of early Lyme disease, United States. STARI; Southern tick-associated rash illness; EM, erythema migrans. *See Figure 1. †Given the gradual geographic expansion of Lyme disease, testing may be warranted for patients with signs and symptoms of Lyme disease who were exposed in areas that border known disease-endemic regions. ‡For a more detailed discussion of symptoms as they relate to pretest probability, see section on exposure and pretest probability. §For recommended 2-tiered testing protocol, see Figure 2.

Exposure and Pretest Probability

When determining whether to test for Lyme disease, clinicians must consider a patient's pretest probability (Figure 3) (*8*). Even highly specific tests can show false-positive results when performed for patients with low pretest probability.

The most crucial factor governing pretest probability for Lyme disease is exposure history. A recent retrospective cohort study by Lantos et al. reported a positive predictive value for Lyme disease serologic analysis in the Duke University hospital system in North Carolina (a low-incidence state) of only 10% for patients with no history of recent travel to a disease-endemic region (*29*). In addition, only 0.7% of patients without recent travel history who had potential signs of disseminated infection (arthritis, cranial neuropathies, or meningitis) were ultimately given a diagnosis of Lyme disease, which indicated that even clinical signs considered consistent with Lyme disease have poor predictive value in low-incidence regions. Furthermore, even EM-like lesions—once considered pathognomonic for Lyme disease—can be caused by other conditions, such as Southern tick-associated rash illness, a tick-borne illness found primarily in the southeastern United States for which an infectious etiology has not been identified (*30*).

For these reasons, positive results for Lyme serologic analysis provide little diagnostic value for patients in areas to which this disease is not endemic and with no history of recent travel to disease-endemic areas (Figure 1) (*8,31*). When assessing whether an area is endemic for Lyme disease, it is essential to note that surveillance guidelines classify cases on the basis of the patient's permanent residence, rather than location of exposure (National Notifiable Disease Surveillance System, http://wwwn.cdc.gov/nndss/conditions/lyme-disease/). A recent study of Lyme disease in low-incidence states found that 84% of infected patients reported recent travel to high-incidence regions (*31*). Thus, although cases have been reported in all 50 states, this finding does not indicate that Lyme disease is endemic to all states.

In addition to exposure history, patient signs and symptoms provide useful information regarding pretest probability (*6*). Patients with EM who live in or have traveled to Lyme disease–endemic areas can be given a diagnosis without serologic testing. For patients without EM, headache and arthralgias are the most common symptoms of early Lyme disease (*32*). However, such symptoms are nonspecific and do not justify serologic testing unless clinical suspicion is high. Signs such as cranial nerve palsy, meningitis, carditis, and migratory large joint arthritis are more suggestive of Lyme disease and improve pretest probability for patients with epidemiologic risk for Lyme disease (*29*). Such signs in at-risk patients generally justify serologic testing. Conversely, gastrointestinal or upper respiratory symptoms are rarely seen in Lyme disease and suggest an alternative diagnosis (*32*).

Surveillance versus Clinical Diagnostic Testing

One misconception is that 2-tiered serologic analysis is intended only for surveillance, rather than patient diagnosis. This misconception is inaccurate and is an apparent conflation of clinical serologic testing recommendations for Lyme disease and the surveillance case definition of the Council of State and Territorial Epidemiologists (*7*) (http://wwwn.cdc.gov/nndss/conditions/lyme-disease/). Recommendations for 2-tiered testing are meant to aid the diagnosis of individual patients in the clinical setting. Serologic test results might be used by public health officials to determine whether a given illness meets the surveillance case definition, but the methods themselves were not developed for this purpose. Furthermore, for practical reasons, serologic results might be used slightly differently in surveillance than is recommended in the clinical setting. For example, although it is not recommended to perform Western immunoblot without a first-tier EIA for laboratory diagnosis, a positive IgG result by Western immunoblot alone is accepted as laboratory evidence of infection for surveillance purposes (http://wwwn.cdc.gov/nndss/conditions/lyme-disease/). This operational definition enables simplification of reporting practices because it can be difficult to track down records of the first-tier test. However, it does not represent best clinical practice.

Unvalidated Tests and Interpretation Criteria

Several alternative testing centers use laboratory-developed tests, also known as home brew tests, that are not currently subject to FDA regulations and might not be clinically validated (*9,33*). Alternative laboratories might also use standard Western immunoblot techniques but apply nonstandard interpretation criteria or fail to perform the recommended first-tier EIA. Unfortunately, many of these alternative laboratories have appealed to patients because they often claim to specialize in testing for tickborne diseases and assert that their tests have better sensitivity than standardized 2-tiered serologic analysis.

False-positive results for alternative tests or unvalidated interpretation criteria can lead to patient confusion and misdiagnosis (*9,20,33*). A recent evaluation of laboratories by Fallon et al. reported an alarming false-positive rate of 58% for samples from healthy control patients submitted to an alternative testing center that used unvalidated criteria to interpret IgM and IgG immunoblots (*34*). Moreover, evaluation of published results from a laboratory claiming to have a new *Borrelia* culture method demonstrated that results were highly suspicious for laboratory contamination (*33,35*). Additional alternative tests, such as urine antigen tests and CD57 tests, have also been shown to be inaccurate (*36,37*).

It is recommended that clinicians only use Lyme disease tests that have been clinically validated and cleared by the FDA (*16,33*). If there is ever any question regarding testing protocols or interpretation, clinicians should consult an infectious disease specialist.

Future Directions in Diagnostic Testing

Novel 2-Tiered Algorithms

A great deal of research has focused recently on improving early diagnosis of Lyme disease and reducing subjectivity inherent in Western immunoblot techniques. When used as a stand-alone test, the C6 EIA is more sensitive than the current 2-tiered test for patients with early Lyme disease (64% vs. 48%) but is hampered by decreased specificity (98.4% vs 99.5%) and thus is more prone to false-positive results (*12,17*). To address this issue, Branda et al. proposed a 2-tiered EIA approach consisting of 2 FDA-cleared EIAs: whole-cell sonicate EIA followed by reflex C6 EIA. This approach provided a higher sensitivity for early Lyme disease (61% vs. 48% for 2-tiered testing) and equivalent specificity (99.5%) to the current approach (Table) (*12*). A 2-tiered EIA with VlsE EIA followed by reflex C6 EIA has also been proposed. The ease of automation and straightforward results of 2-EIA approaches make them particularly appealing because they would be easier to perform and eliminate the subjectivity of Western immunoblot. Further research is still needed, but in the future, the 2-tiered EIA approach might prove to be a valid alternative for diagnosis of Lyme disease.

Additional Novel Diagnostic Approaches

Another approach to improve sensitivity for detection of early Lyme disease involves identifying diagnostic proteins and metabolites in serum of patients with Lyme disease. These methods, referred to as proteomics and metabolomics, respectively, are particularly appealing because they also have the potential to identify biomarkers indicative of cure (*38,39*). Researchers have also reported promising results using immuno-PCR, which combines the sensitivity of PCR with EIA-based antibody detection (*40*).

Lyme Serum Repository for Validation of Novel Diagnostic Tests

When developing new tests or assessing their performance, researchers must have access to well-characterized positive and negative controls. Moreover, it is essential to include samples from patients with diseases that have overlapping clinical features and that are known to be serologically cross-reactive because sensitivity and specificity are heavily dependent on the types of patient samples used. However, collecting and characterizing a wide variety of clinical samples for this purpose can be challenging, costly, and time-consuming.

To improve availability of serum sample sets to evaluate novel diagnostic tests, the Centers for Disease Control and Prevention (Fort Collins, CO, USA) and the National Institutes of Health (Bethesda, MD, USA) have developed a repository of well-characterized serum samples from patients with Lyme disease (*14*). The repository includes samples from patients with various stages of Lyme disease; patients with cross-reactive conditions, such as multiple sclerosis and infectious mononucleosis; and healthy controls from both disease-endemic and non–disease-endemic areas. Panels of serum, along with accompanying clinical and laboratory testing results, are now available to researchers for validation of novel diagnostic tests.

Conclusions

In the United States, 2-tiered serologic analysis is currently the diagnostic test of choice for all patients with signs of extracutaneous Lyme disease. When considering testing, clinicians must take into account the patient's history, timeline of symptoms, and pretest probability to accurately order the test and interpret the test result. Moreover, clinicians should understand the hazards of alternative laboratory tests and only use FDA-cleared diagnostic tests. Ongoing and published research promises to improve diagnosis of early Lyme disease and reduce subjectivity of the second-tier Western immunoblotting.

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References

1. Stanek G, Wormser GP, Gray J, Strle F. Lyme borreliosis. Lancet. 2012;379:461–73. http://dx.doi.org/10.1016/S0140-6736(11)60103-7
2. Pritt BS, Mead PS, Johnson DK, Neitzel DF, Respicio-Kingry LB, Davis JP, et al. Identification of a novel pathogenic *Borrelia* species causing Lyme borreliosis with unusually high spirochataemia: a descriptive study. Lancet Infect Dis. 2016;Feb 5:pii: S1473-3099(15)00464-8.
3. Adams DA, Jajosky RA, Ajani U, Kriseman J, Sharp P, Onwen DH, et al. Summary of notifiable diseases—United States, 2012. MMWR Morb Mortal Wkly Rep. 2014;61:1–121.
4. Nelson CA, Saha S, Kugeler KJ, Delorey MJ, Shankar MB, Hinckley AF, et al. Incidence of clinician-diagnosed Lyme disease, United States, 2005–2010. Emerg Infect Dis. 2015;21:1625–31. http://dx.doi.org/10.3201/eid2109.150417
5. Kugeler KJ, Farley GM, Forrester JD, Mead PS. Geographic distribution and expansion of human Lyme disease, United States. Emerg Infect Dis. 2015;21:1455–7. http://dx.doi.org/10.3201/eid2108.141878
6. Wormser GP, Dattwyler RJ, Shapiro ED, Halperin JJ, Steere AC, Klempner MS, et al. The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clin Infect Dis. 2006;43:1089–134. http://dx.doi.org/10.1086/508667
7. Centers for Disease Control and Prevention. Recommendations for test performance and interpretation from the Second National Conference on serologic diagnosis of Lyme disease. MMWR Morb Mortal Wkly Rep. 1995;44:590–1.
8. Seltzer EG, Shapiro ED. Misdiagnosis of Lyme disease: when not to order serologic tests. Pediatr Infect Dis J. 1996;15:762–3. http://dx.doi.org/10.1097/00006454-199609000-00003
9. Centers for Disease Control and Prevention. Notice to readers: caution regarding testing for Lyme disease. MMWR Morb Mortal Wkly Rep. 2005;54:125 [cited 2016 Mar 14]. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5405a6.htm
10. Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP. Diagnosis of Lyme borreliosis. Clin Microbiol Rev. 2005;18:484–509. http://dx.doi.org/10.1128/CMR.18.3.484-509.2005
11. Craven RB, Quan TJ, Bailey RE, Dattwyler R, Ryan RW, Sigal LH, et al. Improved serodiagnostic testing for Lyme disease: results of a multicenter serologic evaluation. Emerg Infect Dis. 1996;2:136–40. http://dx.doi.org/10.3201/eid0202.960211
12. Branda JA, Linskey K, Kim YA, Steere AC, Ferraro MJ. Two-tiered antibody testing for Lyme disease with use of 2 enzyme immunoassays, a whole-cell sonicate enzyme immunoassay followed by a VlsE C6 peptide enzyme immunoassay. Clin Infect Dis. 2011;53:541–7. http://dx.doi.org/10.1093/cid/cir464
13. Wormser GP, Schriefer M, Aguero-Rosenfeld ME, Levin A, Steere AC, Nadelman RB, et al. Single-tier testing with the C6 peptide ELISA kit compared with two-tier testing for Lyme disease. Diagn Microbiol Infect Dis. 2013;75:9–15. http://dx.doi.org/10.1016/j.diagmicrobio.2012.09.003
14. Molins CR, Sexton C, Young JW, Ashton LV, Pappert R, Beard CB, et al. Collection and characterization of samples for establishment of a serum repository for Lyme disease diagnostic test development and evaluation. J Clin Microbiol. 2014;52:3755–62. http://dx.doi.org/10.1128/JCM.01409-14
15. Wormser GP, Levin A, Soman S, Adenikinju O, Longo MV, Branda JA. Comparative cost-effectiveness of two-tiered testing strategies for serodiagnosis of Lyme disease with noncutaneous manifestations. J Clin Microbiol. 2013;51:4045–9. http://dx.doi.org/10.1128/JCM.01853-13
16. Food and Drug Administration. Database: search on product code LSR [cited 2015 Jul 17]. http://www.accessdata.fda.gov/scripts/cdrh/devicesatfda/index.cfm
17. Bacon RM, Biggerstaff BJ, Schriefer ME, Gilmore RD Jr, Philipp MT, Steere AC, et al. Serodiagnosis of Lyme disease by kinetic enzyme-linked immunosorbent assay using recombinant VlsE1 or peptide antigens of *Borrelia burgdorferi* compared with 2-tiered testing using whole-cell lysates. J Infect Dis. 2003;187:1187–99. http://dx.doi.org/10.1086/374395
18. Liang FT, Steere AC, Marques AR, Johnson BJ, Miller JN, Philipp MT. Sensitive and specific serodiagnosis of Lyme disease by enzyme-linked immunosorbent assay with a peptide based on an immunodominant conserved region of *Borrelia burgdorferi* VlsE. J Clin Microbiol. 1999;37:3990–6.
19. Aguero-Rosenfeld ME, Nowakowski J, Bittker S, Cooper D, Nadelman RB, Wormser GP. Evolution of the serologic response to *Borrelia burgdorferi* in treated patients with culture-confirmed erythema migrans. J Clin Microbiol. 1996;34:1–9.
20. Nelson C, Elmendorf S, Mead P. Neoplasms misdiagnosed as "chronic Lyme disease." JAMA Intern Med. 2015;175:132–3. http://dx.doi.org/10.1001/jamainternmed.2014.5426
21. Steere AC, Berardi VP, Weeks KE, Logigian EL, Ackermann R. Evaluation of the intrathecal antibody response to *Borrelia burgdorferi* as a diagnostic test for Lyme neuroborreliosis. J Infect Dis. 1990;161:1203–9. http://dx.doi.org/10.1093/infdis/161.6.1203
22. Babady NE, Sloan LM, Vetter EA, Patel R, Binnicker MJ. Percent positive rate of Lyme real-time polymerase chain reaction in blood, cerebrospinal fluid, synovial fluid, and tissue. Diagn Microbiol

Infect Dis. 2008;62:464–6. http://dx.doi.org/10.1016/j.diagmicrobio.2008.08.016
23. Molloy PJ, Persing DH, Berardi VP. False-positive results of PCR testing for Lyme disease. Clin Infect Dis. 2001;33:412–3. http://dx.doi.org/10.1086/321911
24. Nocton JJ, Bloom BJ, Rutledge BJ, Persing DH, Logigian EL, Schmid CH, et al. Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in cerebrospinal fluid in Lyme neuroborreliosis. J Infect Dis. 1996;174:623–7. http://dx.doi.org/10.1093/infdis/174.3.623
25. Molloy PJ, Telford SR III, Chowdri HR, Lepore TJ, Gugliotta JL, Weeks KE, et al. *Borrelia miyamotoi* disease in the northeastern United States: a case series. Ann Intern Med. 2015;163:91–8. http://dx.doi.org/10.7326/M15-0333
26. Hilton E, DeVoti J, Benach JL, Halluska ML, White DJ, Paxton H, et al. Seroprevalence and seroconversion for tick-borne diseases in a high-risk population in the northeast United States. Am J Med. 1999;106:404–9. http://dx.doi.org/10.1016/S0002-9343(99)00046-7
27. Kalish RA, McHugh G, Granquist J, Shea B, Ruthazer R, Steere AC. Persistence of immunoglobulin M or immunoglobulin G antibody responses to *Borrelia burgdorferi* 10–20 years after active Lyme disease. Clin Infect Dis. 2001;33:780–5. http://dx.doi.org/10.1086/322669
28. Nadelman RB, Wormser GP. Reinfection in patients with Lyme disease. Clin Infect Dis. 2007;45:1032–8. http://dx.doi.org/10.1086/521256
29. Lantos PM, Branda JA, Boggan JC, Chudgar SM, Wilson EA, Ruffin F, et al. Poor positive predictive value of Lyme disease serologic testing in an area of low disease incidence. Clin Infect Dis. 2015;61:1374–80. http://dx.doi.org/10.1093/cid/civ584
30. Masters EJ, Grigery CN, Masters RW. STARI, or Masters disease: Lone Star tick–vectored Lyme-like illness. Infect Dis Clin North Am. 2008;22:361–76. http://dx.doi.org/10.1016/j.idc.2007.12.010
31. Forrester JD, Brett M, Matthias J, Stanek D, Springs CB, Marsden-Haug N, et al. Epidemiology of Lyme disease in low-incidence states. Ticks Tick Borne Dis. 2015;6:721–3. http://dx.doi.org/10.1016/j.ttbdis.2015.06.005
32. Steere AC, Dhar A, Hernandez J, Fischer PA, Sikand VK, Schoen RT, et al. Systemic symptoms without erythema migrans as the presenting picture of early Lyme disease. Am J Med. 2003;114:58–62. http://dx.doi.org/10.1016/S0002-9343(02)01440-7
33. Nelson C, Johnson B, Petersen J, Schriefer M, Beard CB, Petersen L, et al. Concerns regarding a new culture method for *Borrelia burgdorferi* not approved for the diagnosis of Lyme disease. MMWR Morb Mortal Wkly Rep. 2014;63:333.
34. Fallon BA, Pavlicova M, Coffino SW, Brenner C. A comparison of Lyme disease serologic test results from 4 laboratories in patients with persistent symptoms after antibiotic treatment. Clin Infect Dis. 2014;59:1705–10. http://dx.doi.org/10.1093/cid/ciu703
35. Johnson BJ, Pilgard MA, Russell TM. Assessment of new culture method for detection of *Borrelia* species from serum of Lyme disease patients. J Clin Microbiol. 2014;52:721–4. http://dx.doi.org/10.1128/JCM.01674-13
36. Klempner MS, Schmid CH, Hu L, Steere AC, Johnson G, McCloud B, et al. Intralaboratory reliability of serologic and urine testing for Lyme disease. Am J Med. 2001;110:217–9. http://dx.doi.org/10.1016/S0002-9343(00)00701-4
37. Marques A, Brown MR, Fleisher TA. Natural killer cell counts are not different between patients with post-Lyme disease syndrome and controls. Clin Vaccine Immunol. 2009;16:1249–50. http://dx.doi.org/10.1128/CVI.00167-09
38. Molins CR, Ashton LV, Wormser GP, Hess AM, Delorey MJ, Mahapatra S, et al. Development of a metabolic biosignature for detection of early Lyme disease. Clin Infect Dis. 2015;60:1767–75. http://dx.doi.org/10.1093/cid/civ185
39. Schnell G, Boeuf A, Westermann B, Jaulhac B, Lipsker D, Carapito C, et al. Discovery and targeted proteomics on cutaneous biopsies infected by *Borrelia* to investigate Lyme disease. Mol Cell Proteomics. 2015;14:1254–64. http://dx.doi.org/10.1074/mcp.M114.046540
40. Halpern MD, Molins CR, Schriefer M, Jewett MW. Simple objective detection of human Lyme disease infection using immuno-PCR and a single recombinant hybrid antigen. Clin Vaccine Immunol. 2014;21:1094–105. http://dx.doi.org/10.1128/CVI.00245-14

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Two Linked Enteroinvasive *Escherichia coli* Outbreaks, Nottingham, UK, June 2014

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Enteroinvasive *Escherichia coli* (EIEC) outbreaks are uncommon in Europe. In June 2014, two EIEC outbreaks occurred in Nottingham, UK, within 2 days; outbreak A was linked to a takeaway restaurant and outbreak B to a wedding party. We conducted 2 analytical studies: a case–control study for outbreak A and a cohort study for outbreak B. We tested microbiological and environmental samples, including by using whole-genome sequencing. For both outbreaks combined, we identified 157 probable case-patients; 27 were laboratory-confirmed as EIEC O96:H19–positive. Combined epidemiologic, microbiological, and environmental findings implicated lettuce as the vehicle of infection in outbreak A, but the source of the organism remained unknown. Whole-genome sequencing identified the same organism in cases from both outbreaks, but no epidemiologic link was confirmed. These outbreaks highlight that EIEC has the capacity to cause large and severe gastrointestinal disease outbreaks and should be considered as a potential pathogen in foodborne outbreaks in Europe.

Enteroinvasive *Escherichia coli* (EIEC) bacteria are human enteric pathogens that have been identified worldwide. EIEC has been found to be endemic to developing countries, particularly where sanitation is poor, and causes illness in both adults and children (*1*–*3*). EIEC are genetically similar to *Shigella*; both genera contain the *ipaH* invasive gene (*4*) and cause invasive disease that may result in severe illness in otherwise healthy persons (*5*). Transmission of EIEC is by the fecal–oral route, and contaminated food or water are the usual vehicles of infection.

EIEC outbreaks are rare in Europe; cases are typically sporadic and travel-related (*6*,*7*). EIEC outbreaks have been reported in Hungary in 1959 (*8*), Czechoslovakia in 1982 (*9*), and Israel in 1990 (*10*). The only recently reported EIEC outbreak in western Europe was in Italy in 2012 (*5*), and no outbreaks have been reported in the United Kingdom or other parts of northern Europe.

In June 2014, Public Health England (PHE) (East Midlands) was notified of 2 suspected gastroenteritis outbreaks within 2 days of each other. On June 26, 2014, PHE received a report of 7 patients admitted to an emergency department with diarrhea, vomiting, and fever 24 hours after consuming food purchased at a local takeaway restaurant in Nottingham (outbreak A). An outbreak control team was convened and Environmental Health Officers issued a Hygiene Emergency Prohibition Notice to close the restaurant. On June 27, 2014, PHE received a report of another outbreak of gastrointestinal illness characterized by diarrhea and vomiting after a wedding party on June 24 at a second restaurant in Nottingham (outbreak B), located within 0.1 miles of the restaurant implicated in outbreak A. Initial culture-based methods used to test the fecal specimens from both outbreaks had negative results for enteric organisms routinely tested for at the local laboratory; specimens were then sent to the Gastrointestinal Bacterial Reference Unit at PHE London (GBRU).

The 2 outbreaks were considered potentially linked in time, person, and place and were investigated to identify their potential sources. We report the findings of the investigations into these EIEC outbreaks.

Methods

Epidemiologic

We conducted 2 separate analytical epidemiologic studies to investigate the outbreaks: a case–control study with case-nominated controls for outbreak A, and a cohort study for outbreak B. We created 2 separate questionnaires for the outbreaks to collect data on basic demographics, symptoms and onset dates, contact with healthcare services, travel, contact with persons with diarrhea and vomiting in the 10 days before illness, and food consumed in each restaurant. PHE staff interviewed eligible study participants by telephone.

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Outbreak A Investigation

A probable case-patient was defined as a person who consumed food from the restaurant during June 12–26, 2014, and within 7 days of exposure had diarrhea or ≥2 of the following symptoms: vomiting, nausea, abdominal pain, fever, muscle ache or influenza–like symptoms, or headache; and who had no history of travel abroad or contact with anyone who had diarrhea or vomiting during the 10 days before onset, whether or not PCR assay detected *ipaH* gene from a fecal sample. Confirmed case-patients were defined as above plus EIEC O96:H19 isolated from a fecal sample.

Cases were identified through laboratory surveillance, notifications from clinicians in healthcare settings, and calls to the environmental health team. Healthcare providers in the area were alerted to notify any persons with suspected cases of food poisoning who had recently eaten at the restaurants. Restaurant staff were investigated separately and excluded from the analytical study.

The restaurant did not keep records of customers, so case-patients were asked to nominate controls by providing details of persons they knew who had eaten at the restaurant. A control was defined as a person who had consumed food from the restaurant during the same time period (June 12–26, 2014) but who did not have diarrhea, vomiting, nausea, abdominal pain, or fever and muscle ache or influenza–like symptoms since then.

Outbreak B Investigation

Case definitions for outbreak B were the same as for outbreak A, but case-patients consumed food at a wedding party, in a different restaurant from the one associated with outbreak A, on June 24. A list of persons who had attended the wedding was compiled by the Environmental Health Officers by consulting one of the wedding party organizers.

Statistical Analyses

The sample size for both outbreaks was not calculated a priori, but was determined by the number of available case-patients and controls. We retrospectively calculated the power of the studies on the basis of the final sample size.

Descriptive analysis was undertaken for each outbreak by time, person, and place. Univariable analysis was undertaken to calculate odds ratios (case–control) and relative risks (cohort) with 95% CIs. Variables that had a p value ≤0.25 in the univariable analysis were included in the multivariable model. We conducted multivariable analysis using logistic regression (case–control) and Poisson regression with robust SEs (cohort), using a backward stepwise elimination process for both. We used Stata version 12 (StataCorp LP, College Station, TX) for analysis.

Microbiological

Fecal samples from case-patients and food handlers were submitted to the GBRU for PCR testing for a range of pathogenic markers associated with *Shigella* spp. and the 5 diarrheagenic *E. coli* groups. Primers and conditions were as previously described, including the enteroinvasive ipaH gene associated with *Shigella* spp. and EIEC (*11*), the EAEC regulation gene aggR (*12*), the ETEC LT/ST toxin genes (*13*), Shiga toxin genes stx1 and stx2 for STEC, and the effacement and attachment gene eae for EPEC and the O157rfb gene (*14*). Additionally, the first 59 fecal samples underwent multiplex PCR testing for other bacterial and viral pathogens, as previously described at the regional laboratory (*15*).

We selected isolates from the outbreak for whole genome sequencing and phylogenetic analysis as described (*16*). Short reads were quality trimmed (*17*) and mapped to the Spades version 2.5.1 (*18*) de novo assembly of 1 EIEC genome isolated by using BWA-MEM (*19*). Single nucleotide polymorphisms (SNPs) were identified by using GATK2 (*20*) in unified genotyper mode. Genome positions that had a high quality SNP (>90% consensus, minimum depth ×10, GQ≥30) in ≥1 isolate were extracted. We used pseudosequences of polymorphic positions to create maximum-likelihood trees by using RAxML (The Exelixis Lab, Heidelberg, Germany) (*21*) and calculated pairwise SNP distances between each pseudosequence. We deposited FASTQ sequences in the National Center for Biotechnology Information Short Read Archive under the BioProject PRJNA248042.

Environmental

Environmental health officers inspected both restaurants and collected food and environmental samples. The food items sampled from the restaurant in outbreak A were targeted on the basis of food histories from initial case-patients and included brown rice with chickpeas, chicken curry, spicy chicken dish with bullet chili peppers, sauces, and salad items. Environmental samples were taken from cutting boards, blenders, water, and taps. No specific food samples remained from the wedding party in outbreak B, so samples were taken from the restaurant. Samples of food items similar to those from outbreak A were collected and included mixed salad, fresh coriander, carrot topping, green chutney, and fresh green chili peppers. Environmental samples were taken from salad tongs, a tea towel, a cutting board and knife used in salad preparation, a blender, and a hot water tap.

We initially sent all food and environmental samples to the PHE Food, Water and Environment laboratory in York to test for enteric pathogens. *E. coli*–positive isolates were then sent to the GBRU for PCR testing for *ipaH*, culture and serotyping.

Environmental health officers investigated food handlers working at the restaurants and in the food supply chain by interviewing the restaurants' proprietors. Details from identified food suppliers were used to trace the source of the food items and to identify any commonality between the restaurants.

Results

Epidemiologic

Outbreak A

For outbreak A, PHE was notified of 142 persons with gastrointestinal illness; 108 (76%) were successfully interviewed, resulting in 19 confirmed cases, 88 probable cases, and 1 excluded case due to foreign travel. We recruited and interviewed 28 controls.

The onset of symptoms for case-patients ranged from the evening of June 22 to the evening of June 27; peak onset occurred on June 26 (Figure 1). Case-patients reported having eaten in or eaten takeaway from the restaurant during June 18–26 (premises closed on the evening of June 26). Among those with available information (n = 85), the median incubation period was 24 hours (interquartile range [IQR] 17–35, range 6–168 hours). The median age of case-patients was 30 years (IQR 15–39, range 1–75 years); 56 (52%) of the case-patients were male.

The sex of controls and the dates that controls reported eating from the restaurant were the same as those of case-patients. However, controls were significantly younger (p = 0.038), at a median age of 19 years (IQR 8–33, range <1–63 years).

Most (n = 106, 99%) case-patients reported having diarrhea plus a combination of other symptoms. A total of 55 (51.4%) case-patients sought healthcare from general practice medical doctors; in addition, 21 case-patients sought care in a hospital (19.6%), of whom 14 were admitted. When interviewed again ≈30 days after onset of illness, 3 case-patients were still symptomatic. Among case-patients who recovered and whose information was available (n = 87), the median duration of illness was 7 days (IQR 3–10, range 1–21 days).

All items from the restaurant menu were included in the univariable analysis (n = 71). Food items with the highest percentage of case-patients exposed were lettuce (80.4%), cucumber (74.8%), tomatoes (71.0%), and onions (68.2%). Univariable analysis showed that consumption of any of these 4 salad items was positively associated with being a case-patient. A total of 11 food items were included in the multivariable model, but only consumption of lettuce remained a statistically significant risk factor (Table 1). Case-patients were 5 times more likely to have consumed lettuce than were controls (OR 4.99, 95% CI 2.01–12.42). Consumption of lamb donner, a ground meat comprising cuts from various parts of the lamb, also remained in the model but was negatively associated with being a case-patient (OR 0.35, 95% CI 0.14–0.90).

Outbreak B

From a list of 60 persons who attended the wedding, we obtained information related to outbreak B for 41 (68%). Of those, 15 persons met the outbreak case definition (3 confirmed and 12 probable cases), 24 had no signs or symptoms of illness, and 2 were excluded because they did not consume food at the wedding. The median age of case-patients was 34 years (IQR 12–36, range 3–64 years); 10 (67%) were male.

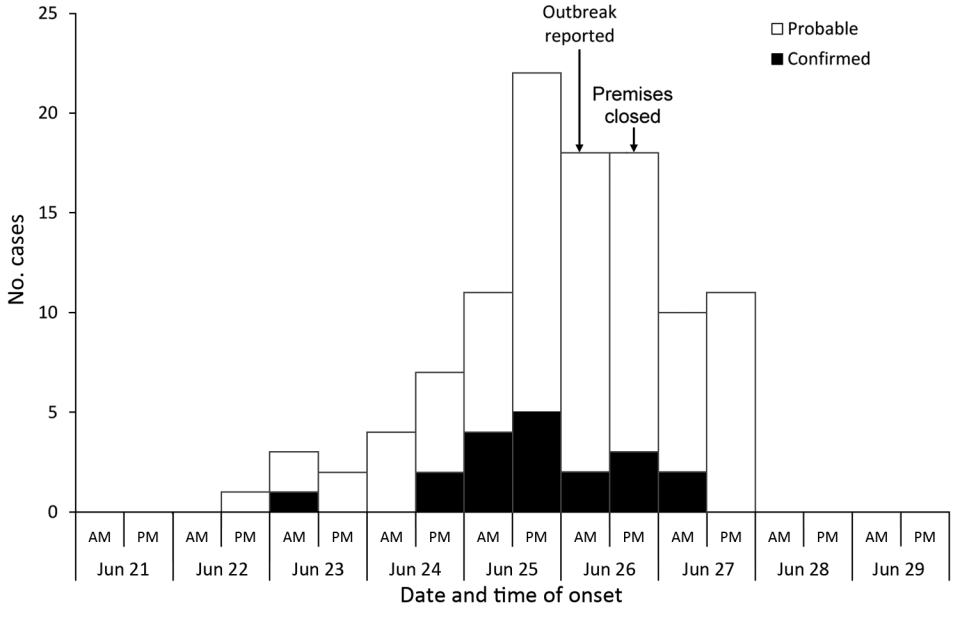

Figure 1. Distribution of cases by symptom onset and case status (n = 107), outbreak A, Nottingham, UK, June 2014.

Table 1. Multivariable model of exposures associated with EIEC outbreak A, Nottingham, United Kingdom, June 2014*

Exposure	Odds ratio	95% CI	p value
Lettuce	4.99	2.01–12.42	0.001
Lamb donner	0.35	0.14–0.90	0.030

*EIEC, enteroinvasive *Escherichia coli*.

The symptom onset date ranged from the evening of June 24 to the morning of June 26; peak onset was on the morning of June 25 (Figure 2). Among those for whom information was available, the median incubation period was 11 hours (IQR 10–19, range 9–37 hours) (Table 2), which was significantly shorter than the incubation period in outbreak A (p = 0.002).

All case-patients reported diarrhea plus a combination of other symptoms. A total of 8 (53%) case-patients sought healthcare for their illness from their general practitioner, but none was admitted to a hospital. Among recovered case-patients whose information was available (n = 11), the median duration of illness was 4 days (IQR 2–10, range 2–25 days). When interviewed ≈30 days after onset of illness, 1 case-patient was still symptomatic.

The overall attack rate varied by sex: male patients were ≈2 times more likely to have a case than were female patients (risk ratio [RR] 2.33, 95% CI 0.98–5.57, p = 0.042). However, because of the small size of the cohort, it was not possible to meaningfully stratify the analysis by sex.

Univariable analysis showed that drinking tap water was positively associated with being a case-patient (RR 2.29, 95% CI 1.06–4.91), whereas lentil curry was negatively associated (RR 0.21, 95% CI 0.03–1.38). Of the 7 menu items included in the multivariable model, 6 were independently associated with being a case-patient (Table 3). Multivariable analysis showed the risk for illness was ≈5 times higher among those who ate salad (RR 4.79, 95% CI 1.97–11.62), 6 times higher among those who drank tap water (RR 5.73, 95% CI 1.85–17.76), and 4 times higher among those who ate chicken curry (RR 3.94, 95% CI 1.52–10.19) compared with those who did not consume these items. The consumption of naan bread (RR 0.16, 95% CI 0.05–0.51), milk pudding (RR 0.36, 95% CI 0.14–0.90), or green chutney (RR 0.26, 95% CI 0.77–0.86) was negatively associated with illness.

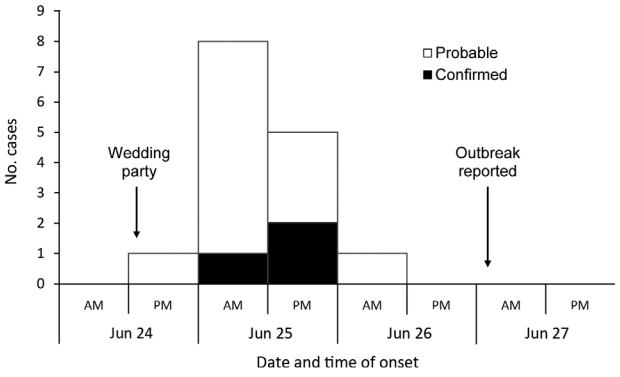

Figure 2. Distribution of cases by symptom onset and case status (n = 15), outbreak B, Nottingham, UK, June 2014.

Microbiological

Fecal samples from 44 case-patients and 17 food handlers in outbreaks A and B were submitted for microbiological testing (Table 4). Across both outbreaks, EIEC O96:H19 was isolated from 23 case-patient samples, and the *ipaH* gene was detected in samples from 14 other case-patients; 2 case-patients from outbreak B also tested positive for *Campylobacter jejuni* by multiplex PCR.

Fecal samples from all 12 food handlers in outbreak A were tested; 4 were culture-positive for EIEC O96:H19. All 4 persons were asymptomatic, but 1 reported travel to Pakistan during May 2014 and was ill for 3 days on return to the United Kingdom. The *ipaH* gene was detected in samples from 5 food handlers, of whom 2 were symptomatic, with onset dates of June 25 and 26, 2014. Samples from 2 food handlers who were PCR-positive for EIEC tested positive for verocytotoxin-producing *Escherichia coli* by using multiplex PCR. Of 6 food handlers in outbreak B, samples from 5 were tested and were negative for EIEC.

Environmental

A total of 41 food and environmental samples taken from the 2 restaurants were sent to the GBRU. Of these, EIEC O96:H19 was isolated from 1 lettuce sample taken from the restaurant in outbreak A, which was the only lettuce sample taken from the restaurant. The lettuce had been washed, cut, and then stored in a container in a chilled display unit. No other organisms were detected by multiplex PCR from these samples.

Inspections of the restaurant in outbreak A identified potential opportunities for cross-contamination between raw meats and ready-to-eat foods during storage, washing, and cooking; chilled food items being stored above the temperature required by law; and inadequate handwashing facilities and practices. No commonalities were identified among food handlers, the food suppliers, or brands of lettuce in the 2 restaurants.

Whole Genome Sequencing

We sequenced 9 isolates from samples in outbreaks A and B: from 4 case-patients, 1 food handler, and the lettuce from outbreak A, and from 3 case-patients in outbreak B. Phylogenetic analysis showed that all isolates from case-patients and the food handler were either identical or differed by a single SNP from that sequenced from the lettuce sample.

Discussion

We describe investigations into 2 outbreaks of EIEC infections that affected 157 persons in Nottingham, UK. The epidemic curves were indicative of 2 common-source outbreaks linked to a restaurant and a wedding party in another

Table 2. Characteristics of case-patients from EIEC outbreaks A and B, Nottingham, United Kingdom, June 2014*

Characteristics	Outbreak A, n = 107	Outbreak B, n = 15
Male sex, %	52	67
Median age, y (IQR)	30 (15–39)	34 (12–36)
Dates exposed	June 18–26	June 24
Onset dates	June 22–27	June 24–26
Median incubation period, hours (IQR)	24 (17–34)	11 (10–19)
Contacted GP, no. (%)	55 (51.4)	8 (53)
Contacted hospital, no. (%)	21 (19.6)	0 (0)
Median duration of illness, d (IQR)	7 (3–10)	4 (2–10)

*EIEC, enteroinvasive *Escherichia coli*; GP, general practice medical doctor; IQR, interquartile range.

restaurant within 0.1 miles of one another. Although whole-genome sequencing showed that the organisms isolated from case-patients in both outbreaks were genetically related, no specific epidemiologic link was identified.

In Europe, reports of EIEC outbreaks have previously been uncommon. However, these 2 large outbreaks and the 2012 outbreak in Italy (*5*) suggest a possible undocumented increase in this pathogen in Europe. Analyses of isolates from these outbreaks plus a sporadic case in Spain found all to be the rare serotype O96:H19 and belong to an EIEC clone not seen before the 2012 outbreak in Italy (*22*).

Difficulties surround the surveillance and diagnosis of EIEC, possibly resulting in underreporting. Clinicians, as well as pathologists based in laboratories, may be unaware of EIEC as a pathogen for diarrheal illness, especially when case-patients appear to have acquired their infection within the United Kingdom, and frontline diagnostic tests are not usually able to distinguish EIEC from nonpathogenic *E. coli* (*5*). In England, the prevalence of this organism is currently unknown. An intestinal infectious disease study in England during 1993–1996 did not identify any cases of EIEC (*23*), but it was not tested for in a repeat study during 2008–2009, so it is unknown if this status remained unchanged (*24*). In the outbreaks we investigated, the prompt notification and referral of samples to the reference laboratory enabled us to quickly identify and microbiologically confirm EIEC in several cases. The symptom profile and incubation period of cases from outbreaks A and B are consistent with those reported for EIEC (*5*,*25*). Based on the proportion of case-patients admitted to hospitals, it appears that case-patients in outbreak A experienced more severe illness than those in outbreak B; however, the reason for this is unknown.

In outbreak A, the combined epidemiologic, microbiological, and environmental findings implicated lettuce as the vehicle of infection. Lettuce and other salad items requested were either served directly onto the food or were placed in a small plastic bag to accompany takeaway dishes. EIEC foodborne outbreaks have previously been documented (*26*–*28*), and in an outbreak in Italy, EIEC infection was found to be associated with vegetables, although EIEC was not isolated from the food (*5*).

Table 3. Multivariable model of exposures associated with EIEC outbreak B, Nottingham, United Kingdom, June 2014*

Exposure	Risk ratio	95% CI	p value
Salad	4.79	1.97–11.62	0.001
Tap water	5.73	1.85–17.76	0.003
Naan bread	0.16	0.05–0.51	0.002
Milk pudding	0.36	0.14–0.90	0.029
Chicken curry	3.94	1.52–10.19	0.005
Green chutney	0.26	0.77–0.86	0.027

*EIEC, enteroinvasive *Escherichia coli*.

The source of the organism in this outbreak is less clear: of the 12 food handlers associated with outbreak A, 9 (75%) tested positive for EIEC, but most reported they were asymptomatic, so we are unable to ascertain how or when they acquired their infection. However, 1 food handler who was asymptomatic at the time of the outbreaks but who tested positive for EIEC reported becoming ill with gastrointestinal symptoms on return from Pakistan in May 2014. Although the food handler reported not working while symptomatic, there have been reports in the literature of asymptomatic persons shedding EIEC up to 1 year after infection (*25*), so it is plausible that this food handler may have introduced the organism into the restaurant. Poor food hygiene standards identified at the restaurant may have facilitated cross-contamination among the other food handlers through person-to-person transmission or consumption of contaminated food items.

A second hypothesis for the source of infection is that contaminated lettuce was introduced into the restaurant. However, we found no commonality with the lettuce supplier for outbreaks A and B, and we were not notified of any further outbreaks of EIEC, which might have been expected if there was an issue with the supplier. Considering the challenges in diagnosis and surveillance of EIEC detailed above, isolated cases that were not part of a localized cluster would have been difficult to identify.

The choice of case-nominated controls may have introduced selection bias to our study. Our assessment showed that controls were significantly younger than case-patients, and the high attack rate among those who ate at the restaurant resulted in only a small number of suitable controls being identified. The restaurant had no daily records of customers; therefore, the choice of case-nominated controls was the most pragmatic and timely way of recruiting controls. Power and sample size calculations showed that our study was adequately powered to

Table 4. Summary of EIEC fecal sample test results by outbreak, Nottingham, United Kingdom, June 2014 (n = 61)*

	Outbreak A, no. (%)		Outbreak B, no. (%)	
Fecal sample test and result	Case-patients	Food handlers	Case-patients	Food handlers
EIEC O96:H19, culture positive	20 (57.1)	4 (33.3)	3 (33.3)	0
EIEC PCR positive, *ipaH* gene	9 (25.7)	5 (41.7)	5 (55.6)	0
EIEC-negative, PCR and culture	6 (17.1)	3 (25.0)	0	5 (100)
Leaked sample not processed	0	0	1 (11.1)	0
Total samples tested	35 (100)	12 (100)	9 (100)	5 (100)

*EIEC, enteroinvasive *Escherichia coli*.

detect lettuce as a vehicle of infection, but any food items with smaller effect sizes may not have been identified. However, we believe our epidemiologic findings are valid because they are supported by environmental and microbiological findings.

For outbreak B, we were unable to identify a definite source and route of EIEC infection at the wedding party. Power calculations found the study to be underpowered, and we did not have any microbiological evidence to identify the true source or vehicle of infection. Salad was a food item associated with the risk for illness, but no links could be found between the 2 restaurants related to food handlers, customers, or suppliers, despite its close proximity to the restaurant in outbreak A. Some wedding party guests chose not to participate in the study; therefore, the study cohort may not be representative of the outbreak cohort.

Prompt control measures seemed to be effective in limiting further transmission of EIEC. Outbreak A stopped after the restaurant was closed, and in outbreak B, no cases were identified outside of the wedding party. We found little in the literature on the management of EIEC cases to prevent secondary transmission. In both outbreaks, guidelines for preventing *Shigella* infections (*29*) were used because of the genetic similarity of EIEC to *Shigella*. Case-patients and contacts in high risk groups were excluded from working or attending high-risk settings such as eating establishments, day nurseries, and healthcare facilities until microbiological clearance, defined as 2 negative fecal specimens taken at intervals of not less than 48 hours, had been achieved. Case-patients who were not in a high-risk group were provided with an information sheet detailing advice on enteric precautions they should take to prevent the spread of the infection.

These 2 outbreaks of EIEC in Nottingham during June 2014 were uncommon for England, but highlight that EIEC has the capacity to cause large and potentially severe gastrointestinal outbreaks in Europe and should be considered as a potential pathogen in foodborne outbreaks.

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References

1. Vieira N, Bates SJ, Solberg OD, Ponce K, Howsmon R, Cevallos W, et al. High prevalence of enteroinvasive *Escherichia coli* isolated in a remote region of northern coastal Ecuador. Am J Trop Med Hyg. 2007;76:528–33.
2. Domah CG, Paul VK, Balaya S, Agarwal R, Mohapatra LN, Ghai OP. Enteroinvasive *Escherichia coli* (EIEC) in sporadic diarrhea in Delhi. Indian J Pediatr. 1985;52:529–31. http://dx.doi.org/10.1007/BF02751029
3. Taylor DN, Echeverria P, Sethabutr O, Pitarangsi C, Leksomboon U, Blacklow NR, et al. Clinical and microbiologic features of *Shigella* and enteroinvasive *Escherichia coli* infections detected by DNA hybridization. J Clin Microbiol. 1988;26:1362–6.
4. Kaper JB, Nataro JP, Mobley HL. Pathogenic *Escherichia coli*. Nat Rev Microbiol. 2004;2:123–40 http://dx.doi.org/10.1038/nrmicro818
5. Escher M, Scavia G, Morabito S, Tozzoli R, Maugliani A, Cantoni S, et al. A severe foodborne outbreak of diarrhoea linked to a canteen in Italy caused by enteroinvasive *Escherichia coli*, an uncommon agent. Epidemiol Infect. 2014;142:2559–66 http://dx.doi.org/10.1017/S0950268814000181
6. Beutin L, Gleier K, Kontny I, Echeverria P, Scheutz F. Origin and characteristics of enteroinvasive strains of *Escherichia coli* (EIEC) isolated in Germany. Epidemiol Infect. 1997;118:199–205 http://dx.doi.org/10.1017/S0950268897007413
7. Wanger AR, Murray BE, Echeverria P, Mathewson JJ, DuPont HL. Enteroinvasive *Escherichia coli* in travelers with diarrhea. J Infect Dis. 1988;158:640–2 http://dx.doi.org/10.1093/infdis/158.3.640
8. Lanyi B, Szita J, Ringelhann B, Kovach K. Waterborne epidemic caused by *Escherichia coli* serotype 124:72-32 in Paradsasvar [in Hungarian]. Orv Hetil. 1959;100:1075–7.

9. Neubauer M, Aldová E, Duben J. Outbreak of enterocolitis caused by an enteroinvasive *E. coli* (serotype O 164, synonym serotype 147). Zentralbl Bakteriol Mikrobiol Hyg A. 1982;252:507–13.
10. Ozbonfil D, Cohen D, Ohad E, Sechter I. An outbreak of enteritis associated with enteroinvasive *E. coli* in an Israeli military base. Public Health Rev. 1990-1991;18:171–7.
11. Wang SM, Ma JC, Hao ZY, Zhang ZY, Mason C, Sethabutr O, et al. Surveillance of shigellosis by real-time PCR suggests underestimation of shigellosis prevalence by culture-based methods in a population of rural China. J Infect. 2010;61:471–5. http://dx.doi.org/10.1016/j.jinf.2010.10.004 PMID: 20951728
12. Chattaway MA, Jenkins C, Ciesielczuk H, Day M, DoNascimento V, Day M, et al. Evidence of evolving extraintestinal enteroaggregative *Escherichia coli* ST38 clone. Emerg Infect Dis. 2014;20:1935–7. http://dx.doi.org/10.3201/eid2011.131845 PMID: 25340736
13. EU Reference Laboratory for E.coli Department of Veterinary Public Health and Food Safety. Detection of enterotoxigenic *Escherichia coli* in food by real time PCR amplification of the lt, sth, and stp genes, encoding the heat-labile and heat-stable enterotoxins. Istituto Superiore di Sanità; 2013. Epub 2013 May 7 [cited 2015 Dec 10]. http://www.iss.it/binary/vtec/cont/EU_RL_VTEC_Method_08_Rev_0.pdf
14. Jenkins C, Lawson AJ, Cheasty T, Willshaw GA. Assessment of a real-time PCR for the detection and characterization of verocytotoxigenic *Escherichia coli*. J Med Microbiol. 2012;61:1082–5. http://dx.doi.org/10.1099/jmm.0.041517-0
15. Moran-Gilad J, Chand M, Brown C, Shetty N, Morris G, Green J, et al.; HPA Microbiology Services Olympics Planning Group. Microbiological aspects of public health planning and preparedness for the 2012 Olympic Games. Epidemiol Infect. 2012;140:2142–51. http://dx.doi.org/10.1017/S0950268812001835
16. Jenkins C, Dallman TJ, Launders N, Willis C, Byrne L, Jorgensen F, et al. Public health investigation of two outbreaks of Shiga toxin–producing *Escherichia coli* O157 associated with consumption of watercress. Appl Environ Microbiol. 2015;81:3946–52. http://dx.doi.org/10.1128/AEM.04188-14
17. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30:2114–20. http://dx.doi.org/10.1093/bioinformatics/btu170
18. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19:455–77. http://dx.doi.org/10.1089/cmb.2012.0021
19. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics. 2010;26:589–95. http://dx.doi.org/10.1093/bioinformatics/btp698
20. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res. 2010;20:1297–303. http://dx.doi.org/10.1101/gr.107524.110
21. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30:1312–3. http://dx.doi.org/10.1093/bioinformatics/btu033
22. Michelacci V, Prosseda G, Maugliani A, Tozzoli R, Sanchez S, Herrera-Leon S, et al. Characterization of an emergent clone of enteroinvasive *Escherichia coli* circulating in Europe. Clin Microbiol Infect. 2016;22:287 e11–9. Epub 2015 Nov 10. http://dx.doi.org/10.1016/j.cmi.2015.10.025
23. Tompkins DS, Hudson MJ, Smith HR, Eglin RP, Wheeler JG, Brett MM, et al. A study of infectious intestinal disease in England: microbiological findings in cases and controls. Commun Dis Public Health. 1999;2:108–13.
24. Tam CC, O'Brien SJ, Tompkins DS, Bolton FJ, Berry L, Dodds J, et al.; IID2 Study Executive Committee. Changes in causes of acute gastroenteritis in the United Kingdom over 15 years: microbiologic findings from 2 prospective, population-based studies of infectious intestinal disease. Clin Infect Dis. 2012;54:1275–86. http://dx.doi.org/10.1093/cid/cis028
25. Kétyi I. Epidemiology of the enteroinvasive *Escherichia coli*. Observations in Hungary. J Hyg Epidemiol Microbiol Immunol. 1989;33:261–7.
26. Yamamura K, Sumi N, Egashira Y, Fukuoka I, Motomura S, Tsuchida R. Food poisoning caused by enteroinvasive *Escherichia coli* (O164:H-)—a case in which the causative agent was identified [in Japanese]. Kansenshogaku Zasshi. 1992;66:761–8. http://dx.doi.org/10.11150/kansenshogakuzasshi1970.66.761
27. Tulloch EF Jr, Ryan KJ, Formal SB, Franklin FA. Invasive enteropathic *Escherichia coli* dysentery: an outbreak in 28 adults. Ann Intern Med. 1973;79:13–7. http://dx.doi.org/10.7326/0003-4819-79-1-13
28. Snyder JD, Wells JG, Yashuk J, Puhr N, Blake PA. Outbreak of invasive *Escherichia coli* gastroenteritis on a cruise ship. Am J Trop Med Hyg. 1984;33:281–4.
29. PHLS Advisory Committee on Gastrointestinal Infections. Preventing person-to-person spread following gastrointestinal infections: guidelines for public health physicians and environmental health officers. Commun Dis Public Health. 2004;7:362–84.

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A Literature Review of Zika Virus

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Zika virus is a mosquitoborne flavivirus that is the focus of an ongoing pandemic and public health emergency. Previously limited to sporadic cases in Africa and Asia, the emergence of Zika virus in Brazil in 2015 heralded rapid spread throughout the Americas. Although most Zika virus infections are characterized by subclinical or mild influenza-like illness, severe manifestations have been described, including Guillain-Barre syndrome in adults and microcephaly in babies born to infected mothers. Neither an effective treatment nor a vaccine is available for Zika virus; therefore, the public health response primarily focuses on preventing infection, particularly in pregnant women. Despite growing knowledge about this virus, questions remain regarding the virus's vectors and reservoirs, pathogenesis, genetic diversity, and potential synergistic effects of co-infection with other circulating viruses. These questions highlight the need for research to optimize surveillance, patient management, and public health intervention in the current Zika virus epidemic.

Zika virus is a flavivirus that was first isolated in 1947 from a febrile rhesus macaque monkey in the Zika Forest of Uganda and later identified in *Aedes africanus* mosquitoes from the same forest (*1*). In 1954, the first 3 cases of human infection were reported in Nigeria (*2*). Serosurveillance studies in humans suggest that Zika virus is widespread throughout Africa, Asia, and Oceania (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/7/15-1990-Techapp1.pdf). However, these studies may overestimate the virus's true prevalence, given serologic overlap between Zika virus and related flaviviruses, such as dengue virus (DENV) and West Nile virus (WNV) (*3,4*).

Historically, symptomatic Zika virus infections were limited to sporadic cases or small clusters of patients (online Technical Appendix Table 2). This pattern changed in 2007, when the first major outbreak of Zika virus infection occurred in Yap (Federated States of Micronesia), where ≈73% of the population were infected and symptomatic disease developed in ≈18% of infected persons (*5*). Since then, Zika virus infection has spread rapidly. Outbreaks have occurred in French Polynesia (*6*), Cook Islands (*6*), Easter Island (*7*), New Caledonia (*8*), and, most recently, the Americas (*9*), with sporadic exportations to Europe (Figures 1–3; online Technical Appendix Table 2).

Zika virus was first reported in May 2015 in continental South America in Brazil, where ≈440,000–1,300,000 persons have subsequently been infected (as of February 16, 2016). Furthermore, 29 other countries in the Americas have reported autochthonous Zika virus transmission, including Puerto Rico and US Virgin Islands (Figure 3; online Technical Appendix Table 2) (*13*). Except for 2 sexually acquired cases, Zika virus in the United States, Canada, and Europe has been restricted to travelers from affected areas (Figure 1; online Technical Appendix Table 2); a patient who delivered an infant with microcephaly in Hawaii had spent part of her pregnancy in Brazil (*14*).

Given the wealth of new information about Zika virus, we conducted a literature review to summarize the published findings. This review contextualizes the ongoing Zika virus epidemic in the Americas and identifies knowledge gaps that must be addressed to combat Zika virus successfully.

The Review

Search Strategy and Selection Criteria

Using the keywords "Zika," "ZIKV," "ZIKAV," and "Zika virus," we searched Google, PubMed, Web of Science, Scopus, and ProMed Mail. We reviewed all literature published through February 16, 2016, including peer-reviewed journal articles, infectious disease reporting system broadcasts, and public health agency information (e.g., US Centers for Disease Control and Prevention [CDC] and European Centre for Disease Prevention and Control [ECDC]). To ensure the capture of all information, we cross-referenced the bibliographies of reviewed articles. The search included English-language and foreign-language articles, which were computer translated.

Virology and Pathogenesis

Zika virus is a positive-sense single-stranded RNA virus in the family *Flaviviridae*, which includes several other mosquitoborne viruses of clinical importance (e.g., DENV, WNV, and yellow fever virus [YFV]) (*15*). Its closest relative is Spondweni virus, the only other member of its clade (*15,16*). The Zika virus genome contains 10,794 nt encoding 3,419 aa (*16*). Like other flaviviruses, Zika virus is composed of 2 noncoding regions (5′ and 3′) that flank an open reading frame (*16*), which encodes a polyprotein

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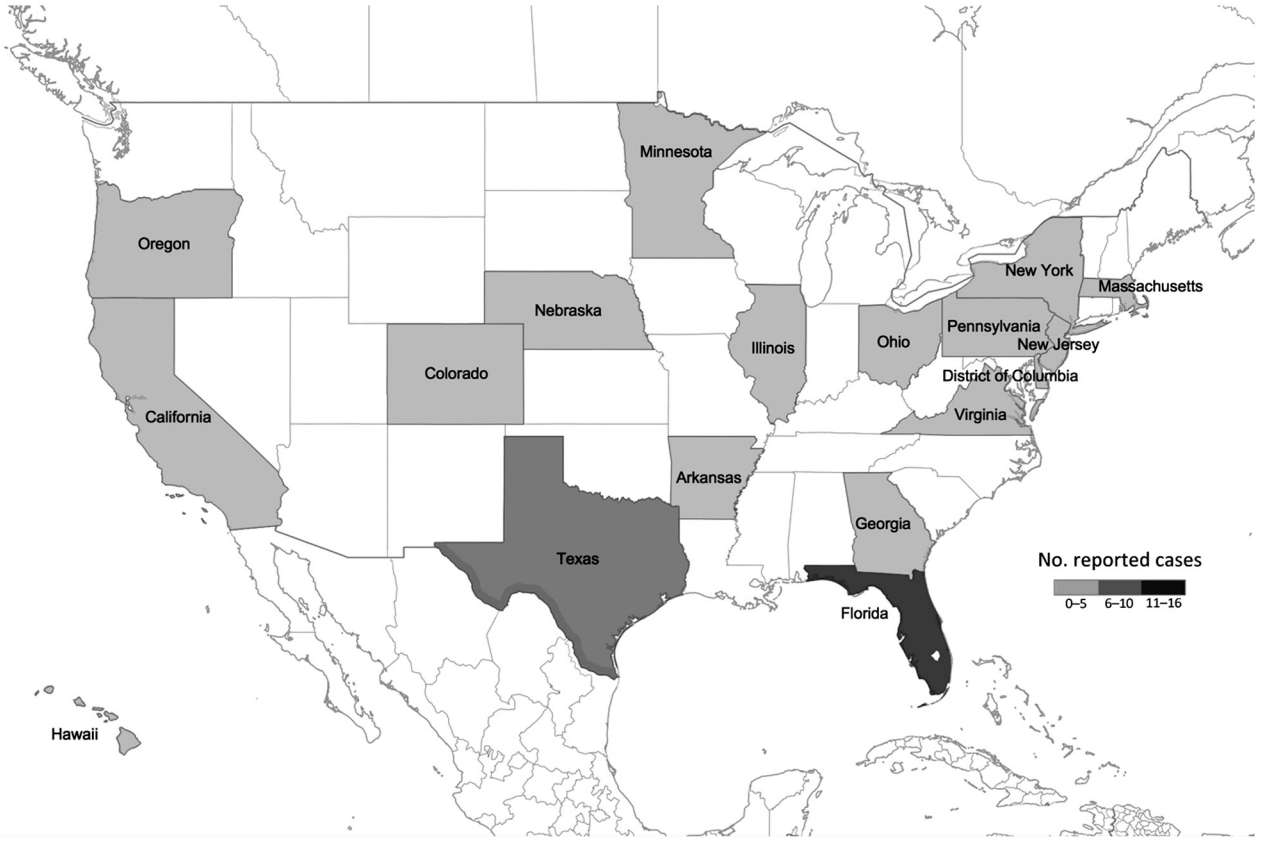

Figure 1. Cases of laboratory-confirmed, imported Zika virus infections in the United States, by state, January 1, 2015–February 10, 2016 (*10*). All cases are imported, with the exception of 2 sexually acquired autochthonous cases (*11,12)*.

cleaved into the capsid, precursor of membrane, envelope, and 7 nonstructural proteins (*16*).

Phylogenetic analysis shows that Zika virus can be classified into distinct African and Asian lineages; both emerged from East Africa during the late 1800s or early 1900s (*17*). The Asian lineage originated during the virus's migration from Africa to Southeast Asia, where it was first detected in Malaysia. From there, Zika virus spread to the Pacific Islands, separately to Yap and French Polynesia, and then to New Caledonia, Cook Islands, Easter Island, and the Americas (*17*).

A study of Zika virus's molecular evolution, based on viral strains collected from 4 countries in West Africa during 1947–2007, identified several sites within the Zika viral genome that were under strong negative selection pressure. This finding suggests frequent purging of deleterious polymorphisms in functionally important genes and the possibility of recombination, which occurs rarely among flaviviruses (*18*). The implications of this finding require further evaluation with respect to viral spread, zoonotic maintenance, and epidemiologic potential.

After mosquito inoculation of a human host, cellular entry likely resembles that of other flaviviruses, whereby the virus enters skin cells through cellular receptors, enabling migration to the lymph nodes and bloodstream. Few studies have investigated the pathogenesis of Zika virus infection. One study showed that human skin fibroblasts, keratinocytes, and immature dendritic cells allow entry of Zika virus (*19*). Several entry and adhesion factors (e.g., AXL receptor tyrosine kinase) facilitate infection, and cellular autophagy, needed for flaviviral replication, enhances Zika virus replication in skin fibroblasts (*19*). After cellular entry, flaviviruses typically replicate within endoplasmic reticulum-derived vesicles. However, Zika virus antigens were found exclusively in the nuclei of infected cells; this finding suggests a location for replication that differs from that of other flaviviruses and merits further investigation (*20*).

Transmission

Zika virus, like other flaviviruses, is transmitted by mosquitoes, primarily of the *Aedes* (*Stegomyia*) genus. Several *Aedes* spp. have been implicated, including *Ae. aegypti*, *Ae. africanus*, *Ae. hensilli*, *and Ae. albopictus* (*1,21–23*). The *Ae. aegypti* mosquito appears to be the major vector in Asia (*24*) and was the suspected primary vector for the French Polynesia outbreak (*25*). Zika virus has been detected in wild-caught

Figure 2. All countries and regions reporting laboratory-confirmed autochthonous Zika virus cases, January 1, 2015–February 10, 2016 (online Technical Appendix Table 2, http://wwwnc.cdc.gov/EID/article/22/7/15-1990-Techapp1.pdf). Data represent outbreaks and case reports for all reported autochthonous laboratory-confirmed cases of Zika virus infection, including those reported in the peer-reviewed literature; public health agency Web sites, bulletins, and broadcasts; and media reports for selected dates.

Ae. aegypti mosquitoes, which laboratory experiments have shown to be capable of transmitting Zika virus (*26,27*). *Ae. hensilli* mosquitoes were implicated in the Yap outbreak, yet Zika virus has never been isolated from these mosquitoes (*28,29*). In Africa, the predominant *Aedes* species vector has not been definitively identified, although viral isolation studies suggest that *Ae. albopticus* was the likely vector in a 2007 Zika virus outbreak in Gabon (*23*).

Aedes mosquitoes are widely distributed globally, and native habitats of most species are warm tropical and subtropical regions (*29–31*). Some species show a limited distribution (e.g., *Ae. luteocephalus* in Africa and *Ae. hensilli* in the Pacific Islands); others have a broad geographic span (e.g., *Ae. aegypti* and *Ae. albopictus*) (*29–31*). *Ae. albopictus* does not yet appear to be a major vector of Zika virus. However, its role in the 2007 Gabon outbreak, its wide distribution throughout the United States, and Zika virus's lack of restriction to a specific *Aedes* sp. indicate that this species could serve as a vector in the United States (*9*).

Mosquito acquisition of the virus likely occurs during a blood meal; after uptake, the virus replicates and is transmitted to a reservoir animal at the next blood meal (*32*). Isolation of the virus or of anti-Zika virus antibodies from various nonhuman primates and other wild and domestic animals suggests multiple animal reservoirs (*33*). One study examined the kinetics of Zika virus infectivity in *Ae. aegypti* mosquitoes by using blood-feeding membranes (*27*); viral content was high on the day of feeding (inoculation), decreased to undetectable levels through day 10, increased by day 15, and remained high on days 20–60. These findings suggest an incubation period in mosquitoes of ≈10 days.

Other nonvector modes of Zika virus transmission include congenital (*34*), perinatal (*35*), and sexual (*11,36*). Possible transmission by blood transfusion (*37,38*), animal bite (*39*), and laboratory exposure (*40*; online Technical Appendix reference *41*) has been described; however, confounding by contemporaneous vectorborne transmission in these instances cannot be excluded. For example, the patient who became infected with Zika virus after a monkey bite had concomitant exposure to mosquitoes, a more plausible route of acquisition (*39*). Similarly, 1 of 2 patients with potentially laboratory-acquired infection (*40*; online Technical Appendix reference *41*) reported recent

Figure 3. South America, Central America, and Caribbean countries and regions reporting laboratory-confirmed autochthonous Zika virus disease cases during January 1, 2015–February 10, 2016 (online Technical Appendix Table 2, http://wwwnc.cdc.gov/EID/article/22/7/15-1990-Techapp1.pdf). Data represent outbreaks and case reports for all reported autochthonous laboratory-confirmed cases of Zika virus infection in these countries and regions during January 1, 1952–February 10, 2016, including those reported in peer-reviewed literature; public health agency Web sites, bulletins, and broadcasts; and media reports.

exposure to mosquitoes (*40*); no definitive mechanism for transmission was described for either patient.

Intrauterine transmission is supported by the finding of Zika virus RNA by reverse transcription PCR (RT-PCR) in amniotic fluid of 2 mothers with symptoms of Zika virus infection during pregnancy; both delivered babies with microcephaly (*34*). Zika virus RNA has also been identified in tissue of fetuses from women infected during pregnancy and in brains of 2 live-born infants with microcephaly who died <20 hours after birth (online Technical Appendix references *42*–*45*). Probable intrapartum transmission has also been described: 2 newborns were found to be viremic with Zika virus ≤4 days after being born to infected mothers (*35*). Viral RNA, but not culturable virus, has been detected in breast milk (*35*), but transmission by breast-feeding has not been reported.

Two cases of possible transfusion-transmitted Zika virus were reported in Brazil (*38*). Furthermore, during the French Polynesia outbreak, a study found that 42 (2.8%) of 1,505 asymptomatic blood donors were positive for Zika virus by RT-PCR; 11 donors described a Zika fever-like syndrome 3–10 days after donation (*37*).

Clinical Manifestations

In humans, the incubation period from mosquito bite to symptom onset is ≈3–12 days. Infection is likely asymptomatic in ≈80% of cases (*5,32*). All ages are susceptible (4 days–76 years), with a slight preponderance of cases in females (online Technical Appendix Table 3). When symptoms occur, they are typically mild, self-limiting, and nonspecific (online Technical Appendix Table 3); similarity to other arbovirus infections (e.g., DENV and chikungunya virus [CHIKV]) may confound the diagnosis (online Technical Appendix reference *46*). Commonly reported symptoms include rash, fever, arthralgia, myalgia, fatigue, headache, and conjunctivitis (online Technical Appendix Table 3). Rash, a prominent feature, is maculopapular and pruritic in most cases; it begins proximally and spreads to the extremities with spontaneous resolution within 1–4 days of onset (*40*). Fever is typically low grade (37.4°C –38.0°C) (*8,36,40*). Symptoms resolve within 2 weeks; accounts of longer persistence are rare (*25*; online Technical Appendix reference *47*).

More severe clinical sequelae have increasingly been associated with Zika virus. During the ongoing outbreak

in Brazil, reports of infants born with microcephaly have markedly increased (>3,800 cases; 20 cases/10,000 live births vs. 0.5/10,000 live births in previous years) (online Technical Appendix reference *48*). However, concern exists that these findings may in part be artifactual, resulting from previous underreporting of cases and confounding by other risk factors for microcephaly (online Technical Appendix reference *49*). Because systematic surveillance for microcephaly was not previously undertaken, the baseline rate of microcephaly in Brazil is unknown, and subsequent reports suggest that a substantial proportion of infants that reportedly have microcephaly do not actually have the condition (online Technical Appendix reference *50*).

Health officials in French Polynesia have reported an apparent increase in congenital central nervous system (CNS) malformations, coinciding with the outbreak occurring during 2013–2014 (online Technical Appendix reference *51*). However, this finding should be cautiously interpreted; reports included only 17 cases, and none were laboratory-confirmed Zika virus cases. In addition, the true baseline rate of such malformations before the outbreak is unknown (online Technical Appendix reference *51*).

A plausible neuropathologic link between Zika virus and CNS anomalies is supported by research showing viral neurotropism in intraperitoneally infected mice (online Technical Appendix reference *52*) and progression of disease in directly infected mouse brains (online Technical Appendix reference *53*). One hypothesis for Zika virus's role in CNS malformations pertains to the virus's hijacking of autophagy during viral replication (online Technical Appendix reference *54*). Some cellular proteins have a dual role in autophagy and centrosome stability; a normal number of centrosomes is important for brain development (online Technical Appendix reference *54*). An increase in centrosomes in mice has been shown to result in microcephaly (online Technical Appendix reference *54*). Therefore, Zika virus's interference in autophagy has been hypothesized to lead to an increase in centrosome number and microcephaly; this potential role in malformations merits further investigation.

Severe neurologic sequelae have also been described in adults, including meningitis, meningoencephalitis, and Guillain-Barre syndrome (online Technical Appendix reference *55*). A surge in Guillain-Barre syndrome cases has been observed in Brazil, Colombia, El Salvador, Suriname, Venezuela, and French Polynesia during outbreaks; however, Zika virus has been laboratory confirmed in only some of these cases (online Technical Appendix reference *55*).

Nonneurologic sequelae include transient hearing loss, hypotension, and genitourinary symptoms (*11,36*; online Technical Appendix references *56,57*). Hematospermia was reported in 2 cases (*11,36*). A 44-year-old man in Tahiti in whom hematospermia developed 2 weeks after symptoms of Zika virus infection was found to have replicative cultured Zika virus particles in his semen (*36*). In addition, a 36-year-old man from the United States contracted Zika virus infection while in Senegal, and subsequently, his wife was infected in the United States; this case supports sexual transmission (*11*). A second sexually acquired case was reported in Texas (online Technical Appendix reference *58*).

Rare deaths have been described in patients infected with Zika virus (online Technical Appendix reference *44*). Besides 1 infant death, 3 other fatalities were reported (2 from Brazil and 1 from Colombia): 1 man with lupus erythematosus, chronic corticosteroid use, rheumatoid arthritis, and alcoholism; and 2 girls 16 years of age, 1 with sickle cell disease (online Technical Appendix reference *59*). (Medical history was not reported for the other girl [online Technical Appendix reference *44*].)

General Laboratory Findings

Information on laboratory findings for Zika virus infection is limited. Complete blood count is often normal; even if blood count is abnormal, changes may be nonspecific (e.g., mild lymphopenia, mild neutropenia, mild-to-moderate thrombocytopenia) (*8*; online Technical Appendix references *46,60–62*). Mild elevations in inflammatory markers (C-reactive protein, fibrinogen, and ferritin), serum lactate dehydrogenase, or liver enzymes have been described (*8,25*; online Technical Appendix reference *57*). These findings are observed in many other viral infections, including the co-circulating viruses DENV and CHIKV, so none of these laboratory alterations reliably distinguish among these infections.

Diagnosis

Clinical evaluation alone is unreliable for a diagnosis of Zika virus infection. Because of clinical overlap with other arboviruses, diagnosis relies on laboratory testing. Evaluation for Zika virus, CHIKV, and DENV should be undertaken concurrently for all patients who have acute fever, rash, myalgia, or arthralgia after recent (previous 2 weeks) travel to an area of ongoing Zika virus transmission (online Technical Appendix reference *63*). Commercial assays have been developed, including a PCR-based assay that has been approved by the Communauté Européenne (RealStar Zika Virus RT-PCR Kit 1.0, altona Diagnostics, Hamburg, Germany) and a serologic assay that has been approved by the US Food and Drug Administration for restricted use in emergency situations (online Technical Appendix reference *64*). Testing has typically been performed by large reference laboratories (e.g., US CDC and US state laboratories) and universities. CDC's typical turnaround time is 4–14 days. Appropriate tests are selected by the laboratory on the basis of clinical information provided by the requesting healthcare provider (online Technical Appendix reference *65*). To coordinate sample collection, providers should contact local public health agencies before testing.

Molecular amplification (e.g., RT-PCR) on serum samples remains the most specific diagnostic approach and is the preferred testing method for Zika virus during the acute phase of illness (<7 days from symptom onset) (online Technical Appendix reference *63*). In contrast, serologic testing is not recommended during the acute phase, when Zika virus IgM may be undetectable (*22*). However, molecular testing must be performed during the viremic period (*15*). Several case reports of negative RT-PCR results but positive IgM results for patients whose samples were tested at ≥5 days after symptom onset indicate a possible viremic period as brief as 5 days (*25,36*; online Technical Appendix reference *61*). Consequently, testing algorithms are based on sampling relative to symptom onset, and serologic testing should be considered if samples are negative for Zika virus by RT-PCR (online Technical Appendix reference *63*).

Serologic testing has limitations. Zika virus IgM and IgG are notoriously cross-reactive with those against other flaviviruses (particularly DENV), limiting specificity (*5,15*; online Technical Appendix reference *46*). Therefore, positive serologic test results should be confirmed with testing that uses an alternative platform such as a seroneutralization assay (e.g., plaque-reduction neutralization test) (*22*). However, flaviviral cross-reactivity can also pose problems in confirmatory assays, especially for patients immunized (e.g., against YFV or Japanese encephalitis virus) or infected with another flavivirus (e.g., WNV or St. Louis encephalitis virus); presence of antibodies confounds diagnosis (online Technical Appendix reference *63*).

The type of sample can also affect the probability of detection. Although diagnostic testing is performed primarily on serum or cerebrospinal fluid, the diagnostic utility of other specimen types (e.g., urine, saliva, amniotic fluid, and tissue) is being evaluated (online Technical Appendix reference *63*). Urine and saliva may offer alternatives, particularly when blood collection is difficult (e.g., in children or remote locations). Viruria may persist longer than viremia. One study reported that Zika virus RNA was detected in urine up to 20 days after viremia had become undetectable (online Technical Appendix reference *62*); therefore, RT-PCR testing of urine should be considered when Zika virus is clinically suspected, despite negative serum testing (*22,33,35,36*; online Technical Appendix reference *62*). Similarly, RT-PCR conducted with saliva has been shown to increase the detection rate during the acute phase of infection but does not extend the window of detection of Zika virus RNA; consequently, blood remains the preferred sample (online Technical Appendix reference *66*).

Management and Prevention

No specific treatment or vaccine is available for Zika virus infection. Management is supportive and includes rest, fluids, antipyretics, and analgesics. Aspirin and other nonsteroidal antiinflammatory drugs should be avoided until dengue is excluded because of the risk for hemorrhage among dengue patients (online Technical Appendix reference *67*).

Other general measures focus on prevention of mosquito bites, including individual protection (e.g., long pants, light-colored clothing, insect repellants, bed nets), particularly during known *Ae. aegypti* peak biting times (early morning and late afternoon) (online Technical Appendix reference *68*). Community-level strategies target mosquito breeding through elimination of potential egg-laying sites (e.g., potted plant saucers, water storage units, used tires) by drying wet environments or using insecticide treatment (online Technical Appendix reference *68*). Pregnant women residing in countries that are not Zika virus–endemic are advised against travel to affected countries (online Technical Appendix reference *69*). Testing should be offered to all pregnant women who have traveled to areas with ongoing Zika virus transmission (online Technical Appendix reference *70*). Serial fetal ultrasounds should be considered to monitor fetal anatomy and growth every 3–4 weeks in pregnant women with positive or inconclusive Zika virus test results, and the infant should be tested at birth (online Technical Appendix reference *70*). Men who reside in or have traveled to an area of active Zika virus transmission and who have a pregnant partner should abstain from sexual activity or use condoms during sex; similar guidelines apply for men with a nonpregnant female sex partner who is concerned about sexual transmission of Zika virus (online Technical Appendix reference *58*).

Discussion

Zika virus has been declared a public health emergency. As many as 1.3 million persons have been affected in Brazil alone (online Technical Appendix Table 2), and 20 countries or territories have reported local transmission of the virus during 2016 (Figures 2,3). Because of the ease of air travel and international trade, further spread into regions where the virus is not endemic is likely, and transmission is probable in locations with competent mosquito vectors. A robust, multifaceted response is underway that involves public health authorities, government agencies, the biomedical industry, medical practitioners, and researchers. However, uncertainty remains regarding aspects of the virus's vectors, epidemiology, and pathogenesis. As the epidemic unfolds, evaluating incoming data critically will be necessary to separate fact from speculation.

Foremost, diagnosis remains suboptimal. Diagnostic guidelines are contingent on laboratory testing that is not widely available. Although commercial tests for Zika virus are limited in number and availability, more are in development, including prototype multiplex molecular assays that concurrently test for Zika virus, CHIKV, and DENV

(M.P. Busch, pers. comm.). However, although not unique to Zika virus, laboratory infrastructure and testing capability is lacking in resource-constrained settings where Zika virus is most prevalent.

Prevention measures (specifically, vector control) are a current priority, pending advances in diagnostics; the World Health Organization and the Pan American Health Organization have issued recommendations (online Technical Appendix reference *44*). In the United States, multiple factors guard against the explosive epidemic occurring throughout Central and South America. Specifically, lower rates of human crowding in urban areas, wider access to air conditioning and mosquito repellants, and waste management limit mosquitoborne transmission, which has been the case for DENV (online Technical Appendix reference *71*). Nonetheless, further entomologic research is needed to define the range of Zika virus vectors and identify new areas where autochthonous transmission could take place to enable early intervention. Investment is also needed in durable control measures such as adaptable vaccine platforms for arboviruses; currently, no Zika virus vaccines are in advanced development (*9*).

Aspects of Zika virus pathogenesis remain unclear. Zika virus's association with neurologic sequelae, including potential neuropathophysiologic mechanisms, is being actively investigated. Continued epidemiologic study, combined with research involving animal models, will offer increased insight, which could spur novel prevention strategies (*9*). If confirmed, insights into the timing of infection relative to gestational outcomes will guide policy. In the interim, new cases of Zika virus infection should be monitored for complications, particularly in babies born to mothers residing in Zika virus–affected areas. The effects of Zika virus in other vulnerable clinical subsets (e.g., those who have concurrent conditions or are immunocompromised) also merit further investigation, as does co-infection or sequential infection by co-circulating viruses.

Given reports of possible transfusion-transmitted Zika virus, the pandemic also has implications for the blood supply within Zika virus–endemic and nonendemic regions. The US Food and Drug Administration recommends 28-day deferral for blood donors with confirmed or suspected Zika virus infection (*38*). Donor screening by nucleic acid testing is being considered but will be challenging to implement because of high costs and regulatory considerations. Pathogen-reduction technology has shown efficacy for treatment of plasma (online Technical Appendix reference *72*); however, absence of a licensed pathogen reduction technology for use in red cells, high incremental cost, and technical barriers render such technology an unlikely short-term solution.

Zika virus has the propensity to infect large numbers of persons with severe consequences in some cases. The epidemic has serious medical, ethical, and economic ramifications, particularly in countries where the resources for early diagnosis are lacking and potential intervention measures (e.g., contraception or termination of pregnancy) are discouraged or illegal (online Technical Appendix reference *73*). Although autochthonous transmission in the United States is unlikely to match the scale of the epidemic in Central and South America, much about Zika virus and the way the pandemic will evolve are unknown. Continued vigilance is warranted, along with a concerted effort toward improving our understanding, management, and prevention of this emerging pathogen.

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References

1. Dick GW, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. Trans R Soc Trop Med Hyg. 1952;46:509–20. http://dx.doi.org/10.1016/0035-9203(52)90042-4
2. MacNamara FN. Zika virus: a report on three cases of human infection during an epidemic of jaundice in Nigeria. Trans R Soc Trop Med Hyg. 1954;48:139–45. http://dx.doi.org/10.1016/0035-9203(54)90006-1
3. Korhonen EM, Huhtamo E, Smura T, Kallio-Kokko H, Raassina M, Vapalahti O. Zika virus infection in a traveller returning from the Maldives, June 2015. Euro Surveill. 2016;21:30107. http://dx.doi.org/10.2807/1560-7917.ES.2016.21.2.30107
4. Baba SS, Fagbami AH, Ojeh CK. Preliminary studies on the use of solid-phase immunosorbent techniques for the rapid detection of Wesselsbron virus (WSLV) IgM by haemagglutination-inhibition. Comp Immunol Microbiol Infect Dis. 1999;22:71–9. http://dx.doi.org/10.1016/S0147-9571(98)00003-4
5. Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009;360:2536–43. http://dx.doi.org/10.1056/NEJMoa0805715
6. Roth A, Mercier A, Lepers C, Hoy D, Duituturaga S, Benyon E, et al. Concurrent outbreaks of dengue, chikungunya and Zika virus infections - an unprecedented epidemic wave of mosquito-borne viruses in the Pacific 2012-2014. Euro Surveill. 2014;19:20929. http://dx.doi.org/10.2807/1560-7917.ES2014.19.41.20929
7. Tognarelli J, Ulloa S, Villagra E, Lagos J, Aguayo C, Fasce R, et al. A report on the outbreak of Zika virus on Easter Island, South Pacific, 2014. Arch Virol. 2016;161:665–8. http://dx.doi.org/10.1007/s00705-015-2695-5
8. Dupont-Rouzeyrol M, O'Connor O, Calvez E, Daurès M, John M, Grangeon JP, et al. Co-infection with Zika and dengue viruses in 2

patients, New Caledonia, 2014. Emerg Infect Dis. 2015;21:381–2. http://dx.doi.org/10.3201/eid2102.141553
9. Fauci AS, Morens DM. Zika virus in the Americas—yet another arbovirus threat. N Engl J Med. 2016;374:601–4. http://dx.doi.org/10.1056/NEJMp1600297
10. Centers for Disease Control and Prevention. Zika virus disease in the United States, 2015–2016. 2016 Feb 10 [cited 2016 Feb 17]. http://www.cdc.gov/zika/geo/united-states.html
11. Foy BD, Kobylinski KC, Chilson Foy JL, Blitvich BJ, Travassos da Rosa A, Haddow AD, et al. Probable non-vector-borne transmission of Zika virus, Colorado, USA. Emerg Infect Dis. 2011;17:880–2. http://dx.doi.org/10.3201/eid1705.101939
12. Dallas County Health and Human Services. DCHHS reports first Zika virus case in Dallas County acquired through sexual transmission. 2016 Feb 2 [cited 2016 Feb 8]. http://www.dallascounty.org/department/hhs/press/documents/PR2-2-16DCHHSReportsFirstCaseofZikaVirusThroughSexualTransmission.pdf
13. Hennessey M, Fischer M, Staples JE. Zika virus spreads to new areas—region of the Americas, May 2015–January 2016. MMWR Morb Mortal Wkly Rep. 2016;65:55–8. http://dx.doi.org/10.15585/mmwr.mm6503e1
14. European Centre for Disease Prevention and Control. Zika virus disease epidemic: potential association with microcephaly and Guillain-Barré syndrome (first update). 2016 Jan 21 [cited 2016 Feb 3]. http://ecdc.europa.eu/en/publications/Publications/rapid-risk-assessment-zika-virus-first-update-jan-2016.pdf
15. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis. 2008;14:1232–9. http://dx.doi.org/10.3201/eid1408.080287
16. Kuno G, Chang GJ. Full-length sequencing and genomic characterization of Bagaza, Kedougou, and Zika viruses. Arch Virol. 2007;152:687–96. http://dx.doi.org/10.1007/s00705-006-0903-z
17. Gatherer D, Kohl A. Zika virus: a previously slow pandemic spreads rapidly through the Americas. J Gen Virol. 2016;97:269–73. http://dx.doi.org/10.1099/jgv.0.000381
18. Faye O, Freire CC, Iamarino A, Faye O, de Oliveira JV, Diallo M, et al. Molecular evolution of Zika virus during its emergence in the 20(th) century. PLoS Negl Trop Dis. 2014;8:e2636. http://dx.doi.org/10.1371/journal.pntd.0002636
19. Hamel R, Dejarnac O, Wichit S, Ekchariyawat P, Neyret A, Luplertlop N, et al. Biology of Zika virus infection in human skin cells. J Virol. 2015;89:8880–96. http://dx.doi.org/10.1128/JVI.00354-15
20. Buckley A, Gould EA. Detection of virus-specific antigen in the nuclei or nucleoli of cells infected with Zika or Langat virus. J Gen Virol. 1988;69:1913–20. http://dx.doi.org/10.1099/0022-1317-69-8-1913
21. Marchette NJ, Garcia R, Rudnick A. Isolation of Zika virus from *Aedes aegypti* mosquitoes in Malaysia. Am J Trop Med Hyg. 1969;18:411–5.
22. Hayes EB. Zika virus outside Africa. Emerg Infect Dis. 2009;15:1347–50. http://dx.doi.org/10.3201/eid1509.090442
23. Grard G, Caron M, Mombo IM, Nkoghe D, Mboui Ondo S, Jiolle D, et al. Zika virus in Gabon (Central Africa)—2007: a new threat from *Aedes albopictus?* PLoS Negl Trop Dis. 2014;8:e2681. http://dx.doi.org/10.1371/journal.pntd.0002681
24. Olson JG, Ksiazek TG, Suhandiman, Triwibowo. Zika virus, a cause of fever in Central Java, Indonesia. Trans R Soc Trop Med Hyg. 1981;75:389–93. http://dx.doi.org/10.1016/0035-9203(81)90100-0
25. Oehler E, Watrin L, Larre P, Leparc-Goffart I, Lastere S, Valour F, et al. Zika virus infection complicated by Guillain-Barre syndrome—case report, French Polynesia, December 2013. Euro Surveill. 2014;19:20720. http://dx.doi.org/10.2807/1560-7917.ES2014.19.9.20720
26. Diallo D, Sall AA, Diagne CT, Faye O, Faye O, Ba Y, et al. Zika virus emergence in mosquitoes in southeastern Senegal, 2011. PLoS One. 2014;9:e109442. http://dx.doi.org/10.1371/journal.pone.0109442
27. Boorman JP, Porterfield JS. A simple technique for infection of mosquitoes with viruses; transmission of Zika virus. Trans R Soc Trop Med Hyg. 1956;50:238–42. http://dx.doi.org/10.1016/0035-9203(56)90029-3
28. Musso D, Nilles EJ, Cao-Lormeau VM. Rapid spread of emerging Zika virus in the Pacific area. Clin Microbiol Infect. 2014;20:O595–6. http://dx.doi.org/10.1111/1469-0691.12707
29. Ledermann JP, Guillaumot L, Yug L, Saweyog SC, Tided M, Machieng P, et al. *Aedes hensilli* as a potential vector of Chikungunya and Zika viruses. PLoS Negl Trop Dis. 2014;8:e3188. http://dx.doi.org/10.1371/journal.pntd.0003188
30. Kraemer MU, Sinka ME, Duda KA, Mylne A, Shearer FM, Brady OJ, et al. The global compendium of *Aedes aegypti* and *Ae. albopictus* occurrence. Sci Data. 2015;2:150035. http://dx.doi.org/10.1038/sdata.2015.35
31. Gaffigan TVWR, Pecor JE, Stoffer JA, Anderson T. Systematic catalog of culicidae. Suitland (MD): Smithsonian Institution; Silver Spring (MD): Walter Reed Army Institute of Research; 2016 [cited 2016 Feb 10]. http://mosquitocatalog.org/
32. Ioos S, Mallet HP, Leparc Goffart I, Gauthier V, Cardoso T, Herida M. Current Zika virus epidemiology and recent epidemics. Med Mal Infect. 2014;44:302–7. http://dx.doi.org/10.1016/j.medmal.2014.04.008
33. Haddow AD, Schuh AJ, Yasuda CY, Kasper MR, Heang V, Huy R, et al. Genetic characterization of Zika virus strains: geographic expansion of the Asian lineage. PLoS Negl Trop Dis. 2012;6:e1477. http://dx.doi.org/10.1371/journal.pntd.0001477
34 Oliveira Melo AS, Malinger G, Ximenes R, Szejnfeld PO, Alves Sampaio S, Bispo de Filippis AM. Zika virus intrauterine infection causes fetal brain abnormality and microcephaly: tip of the iceberg? Ultrasound Obstet Gynecol. 2016;47:6–7. http://dx.doi.org/10.1002/uog.1583135. Besnard M, Lastere S, Teissier A, Cao-Lormeau V, Musso D. Evidence of perinatal transmission of Zika virus, French Polynesia, December 2013 and February 2014. Euro Surveill. 2014;19:20751. http://dx.doi.org/10.2807/1560-7917.ES2014.19.13.20751
36. Musso D, Roche C, Robin E, Nhan T, Teissier A, Cao-Lormeau VM. Potential sexual transmission of Zika virus. Emerg Infect Dis. 2015;21:359–61. http://dx.doi.org/10.3201/eid2102.141363
37. Musso D, Nhan T, Robin E, Roche C, Bierlaire D, Zisou K, et al. Potential for Zika virus transmission through blood transfusion demonstrated during an outbreak in French Polynesia, November 2013 to February 2014. Euro Surveill. 2014;19:20761. http://dx.doi.org/10.2807/1560-7917.ES2014.19.14.20761
38. US Food and Drug Administration. Recommendations for donor screening, deferral, and product management to reduce the risk of transfusion transmission of Zika virus, recommendations for industry. 2016 Feb 16 [cited 2016 Feb 16]. http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/UCM486360.pdf
39. Leung GH, Baird RW, Druce J, Anstey NM. Zika virus infection in Australia following a monkey bite in Indonesia. Southeast Asian J Trop Med Public Health. 2015;46:460–4.
40. Simpson DI. Zika virus infection in man. Trans R Soc Trop Med Hyg. 1964;58:335–8. http://dx.doi.org/10.1016/0035-9203(64)90201-9

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Comparing Characteristics of Sporadic and Outbreak-Associated Foodborne Illnesses, United States, 2004–2011

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Outbreak data have been used to estimate the proportion of illnesses attributable to different foods. Applying outbreak-based attribution estimates to nonoutbreak foodborne illnesses requires an assumption of similar exposure pathways for outbreak and sporadic illnesses. This assumption cannot be tested, but other comparisons can assess its veracity. Our study compares demographic, clinical, temporal, and geographic characteristics of outbreak and sporadic illnesses from *Campylobacter*, *Escherichia coli* O157, *Listeria*, and *Salmonella* bacteria ascertained by the Foodborne Diseases Active Surveillance Network (FoodNet). Differences among FoodNet sites in outbreak and sporadic illnesses might reflect differences in surveillance practices. For *Campylobacter*, *Listeria,* and *Escherichia coli* O157, outbreak and sporadic illnesses are similar for severity, sex, and age. For *Salmonella*, outbreak and sporadic illnesses are similar for severity and sex. Nevertheless, the percentage of outbreak illnesses in the youngest age category was lower. Therefore, we do not reject the assumption that outbreak and sporadic illnesses are similar.

A previous study used outbreak data to determine the relative contributions of 17 different food commodities to the annual prevalence of foodborne illness in the United States (*1*). That work assumed that the exposure pathways of foodborne outbreak illnesses were representative of those pathways for all foodborne illnesses, including outbreak-associated and sporadic (nonoutbreak) illnesses. However, this assumption cannot be tested directly because the food sources of sporadic illnesses typically are unknowable. In fact, despite the availability of multiple cases and controls that might enable examination of the likelihood of illness for different foods consumed, the food sources of outbreaks are identified in only about one half of all foodborne disease outbreaks investigated (*2*).

In lieu of a direct comparison of exposure pathways between outbreak and sporadic foodborne illnesses, we compare selected demographic, clinical, temporal, and geographic characteristics of outbreak and sporadic cases of illness caused by *Campylobacter*, *Escherichia coli* O157, *Listeria*, and *Salmonella* bacteria by using data from the Foodborne Diseases Active Surveillance Network (FoodNet) for 2004–2011. Such an analysis is limited but still useful. Although similarities between outbreak and sporadic cases in terms of disease characteristics would not imply that these cases have identical food exposures, notable differences in disease characteristics might indicate differences in food exposures.

Methods

Data submitted to the Centers for Disease Control and Prevention (CDC) by public health personnel from each FoodNet site indicate whether a case of foodborne illness is an outbreak or nonoutbreak (sporadic) case. We aimed to determine whether differences exist in terms of 6 selected characteristics of outbreak cases of laboratory-confirmed *Campylobacter*, *E. coli* O157, *Listeria*, and *Salmonella* infection reported in FoodNet (*3*) during 2004–2011. The 6 characteristics examined were as follows: 1) the FoodNet site reporting the case; 2) the year in which a case occurred; 3) the season in which a case occurred; 4) the age of patient (generally, the difference between submission date and reported date of birth); 5) the sex of the patient; and 6) the hospitalization status of the patient (i.e., whether the patient was hospitalized within 7 days of specimen collection).

Since 2004, the FoodNet surveillance catchment area has been stable. The FoodNet sites were Connecticut, Georgia, Maryland, Minnesota, New Mexico, Oregon, Tennessee, and selected counties in California, Colorado, and New York. To ensure sufficient data, we determined quintiles for season and age groups. Because the data distributions

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differed between the pathogens, these quintiles were determined for each pathogen separately. Sex and hospitalization status were binary variables.

Other variables of potential interest, such as source of specimen (e.g., stool, blood, or urine), race, ethnicity, and international travel, were not included in the analysis because there were relatively high percentages of missing observations for some pathogens and because percentages were highly variable over time and across other variables in the analysis, possibly introducing an unknown amount of surveillance bias and limiting interpretation of results. For example, the fraction of cases for which information on international travel by the patient was missing ranged from 6% for *E. coli* O157 to 44% for *Campylobacter*. Similarly, the fraction of cases for which information on race was missing ranged from 7% for *E. coli* O157 to 26% for *Campylobacter*. Our summary descriptions and final models are based on the set of FoodNet case reports for which all 6 variables are complete. Missing values for certain variables are described in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/22/7/15-0833-Techapp1.pdf).

To complete the analysis of these characteristics, we used a 2-step approach for each of the 4 pathogens examined. First, we conducted random forest and boosted tree analyses (*4*,*5*) to gauge the relative importance of the 6 characteristics in distinguishing between outbreak and sporadic cases. Random forest analysis is a data classification algorithm that seeks the best combination of factors to explain an outcome variable (i.e., outbreak or sporadic case). Boosted tree analysis pertains to the use of regression techniques (e.g., mean square errors) for measuring the fit of the trees to the data. We created random collections of classification trees and averaged those trees by a measure of how well each tree fit the data.

For each pathogen, we trained random forest models on ≈85% of the data; we used the remaining ≈15% of the data to validate the model's classifications of outbreak and sporadic cases. We used the G^2 statistic (a modified Wilk's statistic) to identify more and less important factors (*6*). In a stepwise fashion, we removed the least important factors to determine if model misclassification of outbreak status improved for the training dataset or the validation dataset. We stopped the model simplification whenever removal of a factor caused misclassification to worsen. Factors that were not eliminated were carried on to the next step.

The second step of the analysis was logistic regression modeling. We used stepwise model building routines (*7*) to examine all main effects and interactions among the factor levels (i.e., model parameters) explaining the fraction of cases that are outbreak-associated cases (i.e.,

$$\ln\left(\frac{p}{1-p}\right) = \alpha + \beta X$$

where p is the probability of a case being an outbreak case and X is a matrix of the data with the number of rows equal to the number of cases and the number of columns equal to the total levels of explanatory variables considered). As a model identification guide, we used forward selection procedures and minimum Bayesian information criterion scoring (BIC) (8). BIC is a preferred selection criterion because it penalizes the inclusion of additional parameters more strongly than alternative statistics (e.g., Akaike information criteria) (8,9).

We selected the logistic regression models with the lowest BIC scores as the best models. We used visual assessments of the residuals and interactions to assess the adequacy of the methods and models.

Results

During the study period (2004–2011), <1% of *Campylobacter* infections reported in FoodNet were outbreak cases, but ≈20% of *E. coli* O157 infections were outbreak cases. Outbreak cases represented ≈5% of *Listeria* and *Salmonella* infections (Table 1).

Seasonal quintiles were similar across pathogens except for *E. coli* O157; the first season was longer compared with the other pathogens, extending from January through the end of May (Figure 1). Age quintiles, however, differed substantially across pathogens. For example, to capture 20% of the data for *Listeria*, the first quintile was defined as cases in patients who were 0–38 years of age. In contrast, the first quintile for *Salmonella* only extended to patients 3 years of age. For *Listeria*, the relatively narrow quintile range for persons 60–80 years of age reflects the larger number of older persons among these cases. For the binary variables (sex and hospitalization), the frequency of male patients was ≈50% among all FoodNet cases for the 4 pathogens, and the percentages hospitalized for *Campylobacter*, *E. coli* O157, *Listeria,* and *Salmonella* infections were 16%, 44%, 93%, and 29%, respectively.

A descriptive treatment of the data shows that the frequency of outbreak cases among all FoodNet cases varied more for FoodNet site, year, patient age, and season than for sex and hospitalization status for each pathogen (Table 2). Compared with the other pathogens, *Listeria* exhibited substantial frequency ranges for some characteristics. For example, the percentage of *Listeria* cases that were outbreak versus sporadic cases per year varied from 0% versus 100% during 2007–2009 to 30.6% versus 69.4% in 2011. Variability was difficult to determine for *Campylobacter* because of the low frequency of outbreak-associated cases.

In general, FoodNet sites in Georgia and California had smaller percentages of outbreak cases, whereas Oregon and Colorado had larger percentages. California had small outbreak case percentages for *Campylobacter* (0.1%) and *E. coli* O157 (1.5%), whereas Georgia had the smallest

Table 1. Number of outbreak cases versus sporadic cases and outbreak fraction, FoodNet data, United States, 2004–2011*

Pathogen	Outbreak cases	Sporadic cases	Outbreak fraction, %
Campylobacter	195	42,744	0.5
Escherichia coli O157	730	3,117	19.0
Listeria	56	1,024	5.2
Salmonella	3,161	50,690	5.9

*Representing 101,717 reports with complete data for all study variables out of 110,157 total reports. FoodNet, Foodborne Diseases Active Surveillance Network.

percentage among all sites for *Listeria* (0.0%) and *Salmonella* (2.6%). Colorado had the largest outbreak case percentage among all sites for *Campylobacter* (1.0%) and *E. coli* O157 (38.9%), whereas Oregon and New Mexico had the largest percentages for *Salmonella* (20.5%) and *Listeria* (34.9%), respectively.

For each pathogen's random forest analysis, the G^2 statistic was smallest for the binary variables (sex and hospitalization). Furthermore, misclassification errors for the training and validation datasets were not substantively changed whether the analysis included all 6 factors or excluded sex and hospitalization status. Consequently, sex and hospitalization status were not important for classifying outbreak and sporadic cases for any of the pathogens, and these factors were excluded from the logistic modeling step.

Plots of the BIC statistic for increasingly complex models illustrate that its value decreases to a minimum and then increases for more complicated models (Figure 2).

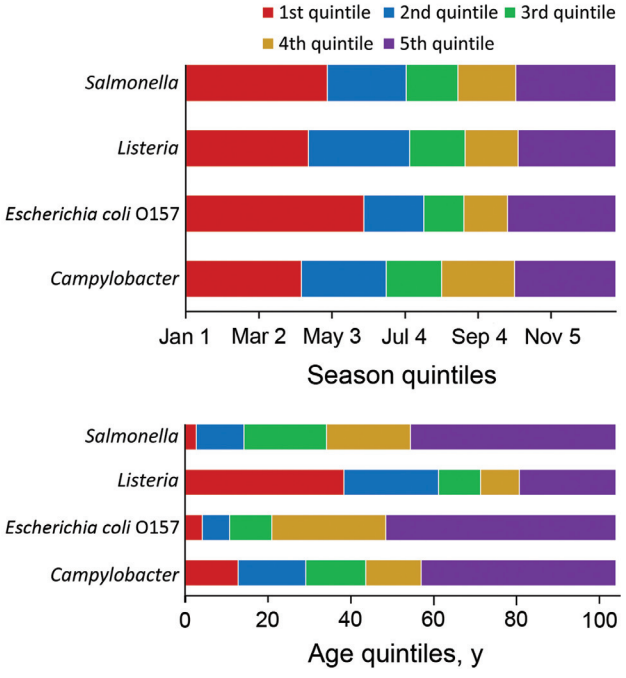

Figure 1. Quintile categorization of season and age for persons with foodborne illness included in the analysis of Foodborne Diseases Active Surveillance Network (FoodNet) data, United States, 2004–2011.

For *Campylobacter*, the minimum BIC corresponds to a model containing just the FoodNet site parameters. For *E. coli* O157 and *Listeria*, the minimum BIC corresponds to a model with 16 parameters (9 for FoodNet site and 7 for year, with 1 reference value for each factor included in the intercept term). For *Salmonella*, the minimum BIC corresponds to a model with 152 parameters that includes all 4 factors (24 parameters plus the reference intercept), the FoodNet site by year interactions (63 parameters), the year by season interactions (28 parameters), and the FoodNet site by season interactions (36 parameters). Residual plots of the best-fitting models demonstrate reasonable fit to the data (Figure 3). These plots illustrate that the studentized residuals ([observed frequency – predicted frequency of outbreak-associated cases]/SE of predicted frequency) generally cluster within 3 SD of the mean.

Interaction plots from the best-fitting *Salmonella* model (Figure 4) illustrate the complex relationships between some model factors. For example, interaction plots demonstrated that, for some FoodNet sites (e.g., Oregon, California, and Minnesota), the estimated proportion of outbreak-associated cases can change substantially across years. Moreover, the directions of changes are inconsistent across the sites. For example, the peaks and troughs of Oregon's proportions across years are nearly the opposite of Minnesota's pattern. Likewise, the *Salmonella* interaction plots demonstrated interactions between the seasonal quintile and both the surveillance year and the FoodNet site. In contrast, the patterns for the age quintiles are consistent across surveillance years. Nevertheless, the first age quintile (0–3 years of age) has a markedly lower proportion of outbreak-associated cases relative to the other age quintiles. This underrepresentation of outbreak-associated cases among the youngest age quintile drives the significance of the age parameter in the logistic regression model.

Discussion

If foodborne illness source attribution estimates are to be effectively used for food safety decision making and monitoring success of interventions, the data used to generate them must be collected in a systematic fashion over time. Foodborne outbreak surveillance data have been systematically collected since 1973 and provide direct links between human illnesses and food sources. Although other methods of source attribution (e.g.,

Table 2. Percentage of cases and total number of cases identified as outbreak-associated, by target pathogen and selected characteristics, FoodNet data, United States, 2004–2011*

Characteristic	% Outbreak cases (no. total observations)			
	Campylobacter	*Escherichia coli* O157	*Listeria*	*Salmonella*
FoodNet site				
California	0.1 (5,552)	1.5 (264)	1.7 (115)	3.0 (3,764)
Colorado	1.0 (3,391)	38.9 (319)	33.3 (72)	8.6 (2,491)
Connecticut	0.0 (3,689)	17.0 (277)	0.0 (148)	6.5 (3,335)
Georgia	0.2 (4,815)	8.4 (261)	0.0 (176)	2.6 (17,215)
Maryland	0.6 (2,920)	13.0 (200)	0.7 (140)	4.3 (6,020)
Minnesota	0.5 (7,308)	20.1 (1,078)	3.4 (58)	10.3 (5,379)
New Mexico	0.8 (2,640)	10.9 (92)	34.9 (43)	9.3 (2,497)
New York	0.4 (4,277)	22.9 (393)	3.7 (136)	8.2 (3,772)
Oregon	0.9 (5,147)	25.5 (545)	8.1 (86)	20.5 (3,067)
Tennessee	0.4 (3,200)	12.2 (418)	0.0 (106)	3.0 (6,311)
Year				
2004	0.2 (4,770)	9.0 (387)	0.8 (119)	6.0 (5,676)
2005	0.7 (5,009)	22.7 (467)	1.5 (136)	4.3 (5,982)
2006	0.7 (4,903)	15.9 (567)	4.4 (137)	7.6 (5,901)
2007	0.1 (5,377)	17.8 (546)	0.0 (122)	6.2 (6,540)
2008	0.6 (5,291)	25.8 (516)	0.0 (134)	7.9 (7,214)
2009	0.3 (5,546)	26.4 (458)	0.0 (157)	5.5 (6,844)
2010	0.4 (5,852)	21.1 (445)	2.3 (131)	5.2 (8,073)
2011	0.6 (6,191)	11.7 (461)	30.6 (144)	4.6 (7,621)
Age quintile				
1	0.7 (8,563)	20.6 (766)	2.3 (214)	2.2 (10,838)
2	0.7 (8,614)	18.1 (768)	4.6 (216)	4.4 (10,666)
3	0.3 (8,428)	19.3 (774)	5.1 (216)	9.2 (10,686)
4	0.3 (8,634)	19.6 (765)	5.5 (218)	7.7 (10,758)
5	0.3 (8,700)	17.3 (774)	8.3 (216)	6.0 (10,903)
Season quintile				
1	0.4 (8,552)	18.6 (774)	2.3 (218)	6.9 (10,962)
2	0.4 (8,761)	19.8 (773)	0.9 (215)	7.6 (10,804)
3	0.6 (8,545)	18.8 (775)	4.1 (218)	5.8 (10,773)
4	0.6 (8,666)	20.1 (770)	16.1 (217)	4.3 (10,671)
5	0.2 (8,415)	17.5 (755)	2.4 (212)	4.7 (10,641)
Sex				
F	0.4 (19,317)	19.4 (2,030)	6.4 (577)	6.1 (28,102)
M	0.4 (23,622)	18.4 (1,817)	3.8 (503)	5.4 (25,749)
Hospitalized				
No	0.5 (35,962)	20.1 (2,145)	4.1 (74)	6.3 (38,321)
Yes	0.3 (6,977)	17.5 (1,702)	5.3 (1,006)	4.8 (15,530)

*Age of persons with cases and season of specimen submission are classified by quintile of reported age and quintile of the day of year of the specimen submission date. FoodNet, Foodborne Diseases Active Surveillance Network.

case–control studies) can provide relevant estimates for different target populations, these estimates are potentially expensive, logistically complex, and not routinely conducted in the United States. Moreover, estimated attributable fractions are based on associations between illnesses and exposures, not proof of causality. The possibility that attribution estimates from outbreaks might not be reliably generalized to the bulk of estimated foodborne illnesses is recognized (*1*). Nevertheless, we cannot assess directly the validity of outbreak-based attribution estimates for application to the broader population of foodborne illnesses. Consequently, this study assessed similarities and differences between outbreak and sporadic cases across various case characteristics. If the examined characteristics of outbreak and sporadic cases are different for these data, then the assumption of similar exposure pathways is less plausible. FoodNet is particularly well-suited for this analysis, because it is the only US foodborne disease surveillance system that actively ascertains laboratory-confirmed human infections and distinguishes those cases that are associated with detected outbreaks.

In our analysis, the probability of a case being outbreak-associated varied significantly across the FoodNet surveillance sites for all 4 pathogens studied. Uncertainty exists for the causes of variability in the number of ascertained cases across FoodNet sites (*10*) and the number of outbreaks detected and reported across states (*2*,*11*,*12*). Previous research has demonstrated that differences in specimen collection and testing and outbreak surveillance and reporting practices, contribute to differences among states, and differences in funding or resource allocation have been hypothesized to be influential factors (*2*,*10*–*12*). We assume these sources of variability among sites are most influenced by differences in surveillance and do not suggest underlying differences in the sources of sporadic and outbreak illnesses.

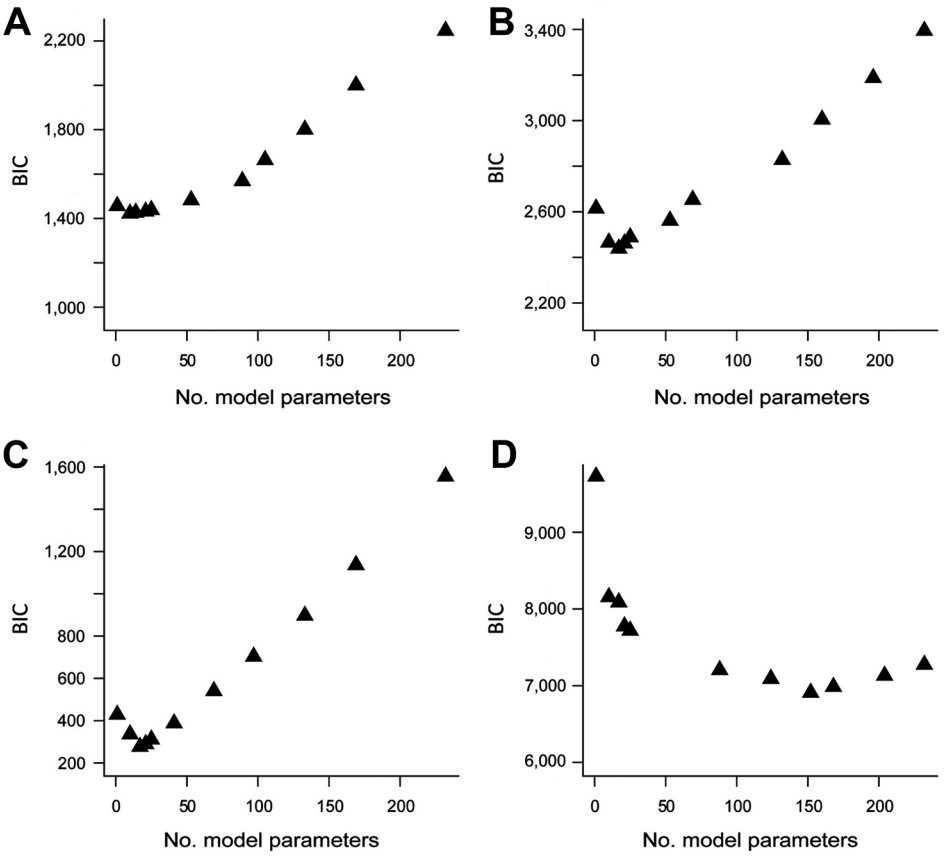

Figure 2. Patterns of the Bayesian information criterion (BIC) statistic as a function of the number of model parameters are shown for the four pathogens included in the analysis of Foodborne Diseases Active Surveillance Network (FoodNet) data, United States, 2004–2011. A) *Campylobacter*; B) *Escherichia coli* O157; C) *Listeria*; D) *Salmonella*. The BIC decreases to a minimum value and then increases as model complexity (as measured by the number of model parameters) increases.

The probability of a case being outbreak-associated also varied significantly with the surveillance year for *E. coli* O157, *Listeria,* and *Salmonella*. In addition, the season of specimen submission was a significant factor in the *Salmonella* model. In a study by Painter et al. (*1*), source attribution was estimated by aggregating multiple years of outbreak data and applying those to national annual burden of illness estimates (*13*). Gould et al. (*2*) similarly aggregated outbreak data for estimating source attribution. One justification for aggregating outbreak evidence across years (and seasons) is the need to capture more information than is available from a single year (or season). The significant association between the probability of an outbreak case and year (and state and season) suggests that aggregation of outbreak data across time and space might be appropriate to avoid biases introduced by significant local effects. Outbreak and sporadic cases might be dissimilar across periods of ≈1 year but more similar when multiple years are compared. For example, the fraction of outbreak-associated cases in the FoodNet *Salmonella* data are 5.7% for 2004–2007 and 5.8% for 2008–2011, despite year-to-year fluctuations ranging from 4.3% to 7.9% (Table 2).

Our analysis found no evidence that laboratory-confirmed outbreak and sporadic cases are dissimilar with respect to the sex or hospitalization status of patients. In particular, the data for *Salmonella* and *E. coli* O157 include substantial numbers of cases for comparisons of these factors. Therefore, the conclusion from the random forest analysis regarding these pathogens lends support to the same conclusion for the other 2 pathogens. Otherwise, the small number of outbreak-associated cases for *Campylobacter* and the generally small number of *Listeria* cases provides limited statistical power to detect real differences.

In the case of *Salmonella*, this analysis found that the percentage of outbreak-associated cases varied significantly by age cohort. In fact, the youngest age quintile (0–3 years of age) had the smallest proportion of outbreak-associated cases. Given this result, applying source attribution estimates derived from foodborne outbreak data to the youngest age strata of *Salmonella* sporadic cases might not be prudent. Because FoodNet epidemiologists cannot confirm the exposure pathway that resulted in FoodNet-captured illnesses, we cannot determine whether the lower frequency of outbreak-associated cases among the youngest cohorts of *Salmonella* patients reflects some fundamental difference in the distribution of exposure pathways, a difference in outbreak-associated case detection methods, or both.

The analytical methods we used rely on some assumptions. The initial random forest analysis was completed

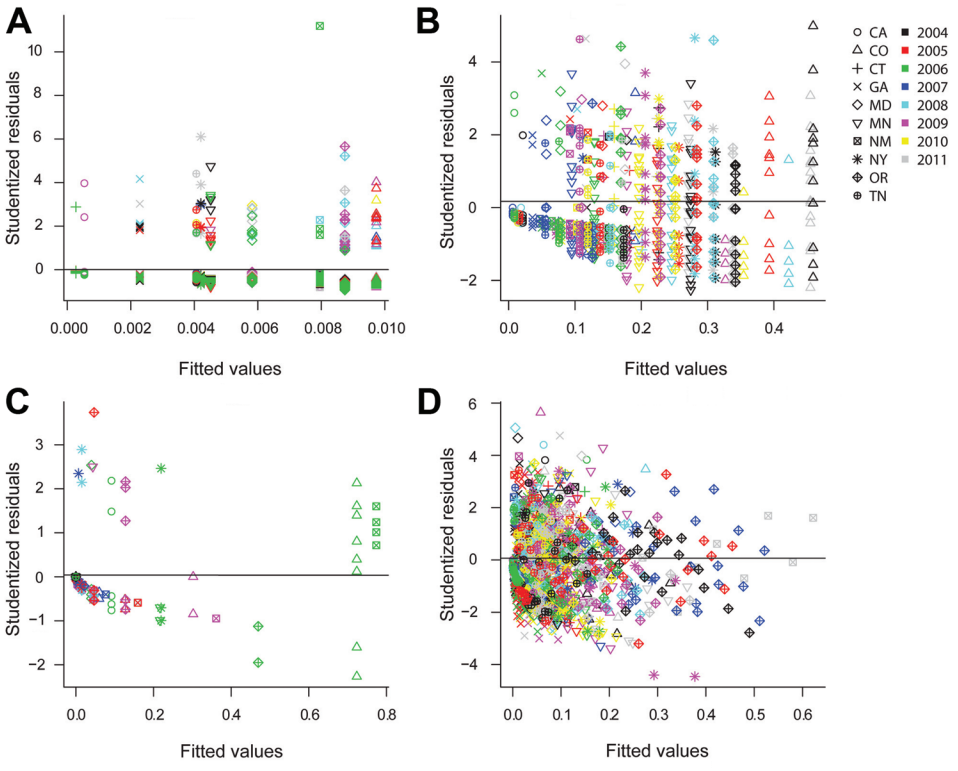

Figure 3. Residual plots relative to fitted estimates of outbreak-associated case frequency for the best-fitting models used in the analysis of Foodborne Diseases Active Surveillance Network (FoodNet) data, United States, 2004–2011. A) *Campylobacter*; B) *Escherichia coli* O157; C) *Listeria*; D) *Salmonella*. Generally, all 4 pathogen models demonstrate reasonable fit because the studentized residuals ([observed frequency – predicted frequency of outbreak-associated cases]/SE of predicted frequency) are mostly within 3 SD of the predicted mean frequency of outbreak-associated cases. The state variable is the only factor in the *Campylobacter* model, whereas year is included in the *E. coli* O157 and *Listeria* models. The *Salmonella* model includes state, year, season, age, and interaction terms.

because this technique demands few assumptions with respect to missing observations and factor interactions (*14*). Nevertheless, this technique was only used to eliminate those factors that had no evident association with outbreak status.

The logistic regression modeling we performed relies on a binomial process assumption for the frequency of outbreak cases among all FoodNet cases. Although this analysis assumes that all outbreak cases are unrelated to each other, detailed data about the specific outbreak for each outbreak case is not readily available and some outbreak cases might have stemmed from the same outbreak. Related outbreak cases might co-vary with respect to the factors we studied in violation of the binomial process assumption of independent trials. To address this possibility, we considered censoring outbreak cases in this analysis, but an unknown number of sporadic cases probably were also related to detected and undetected outbreaks.

This study also assumes that the probability of specimen collection and laboratory submission among ill persons is the same for outbreak and sporadic cases. Nevertheless, public awareness of an outbreak might increase healthcare-seeking behavior and submission of diagnostic samples by healthcare providers. In addition, during some outbreak investigations, investigators conduct active case-finding and collect additional laboratory specimens from persons reporting foodborne illness (*11*,*15*), resulting in laboratory-confirmed infections being identified in persons who had not sought healthcare. As a result, outbreak cases might be oversampled compared with sporadic infections.

Inherent dependencies among outbreak cases, combined with oversampling, might contribute to an increased strength of association between the proportion of outbreak-associated cases and the factors studied here. In addition to performing better than alternative criteria when the objective of modeling is to find the actual model, BIC penalizes the addition of parameters in models more harshly (*16*). We believe that this harsher assessment of factors reduces the likelihood of spurious associations.

Some of the persons with foodborne infections that were captured by FoodNet traveled internationally before their reported specimen collection date, and some of these persons probably became infected because of exposures that occurred outside the United States. The likelihood of their illness being associated with a disease outbreak might in turn be different from that of non-travelers. We were not able to exclude international travelers or adjust for this case characteristic because, except for cases of *E. coli* O157 infection, travel history information was missing for >20% of cases. Thus, our study population is not restricted to persons with infection caused by domestic exposures. Nevertheless, international travel was reported for <10% of cases for all pathogens except *Campylobacter*. Among *Campylobacter* infection cases in persons who reported a travel

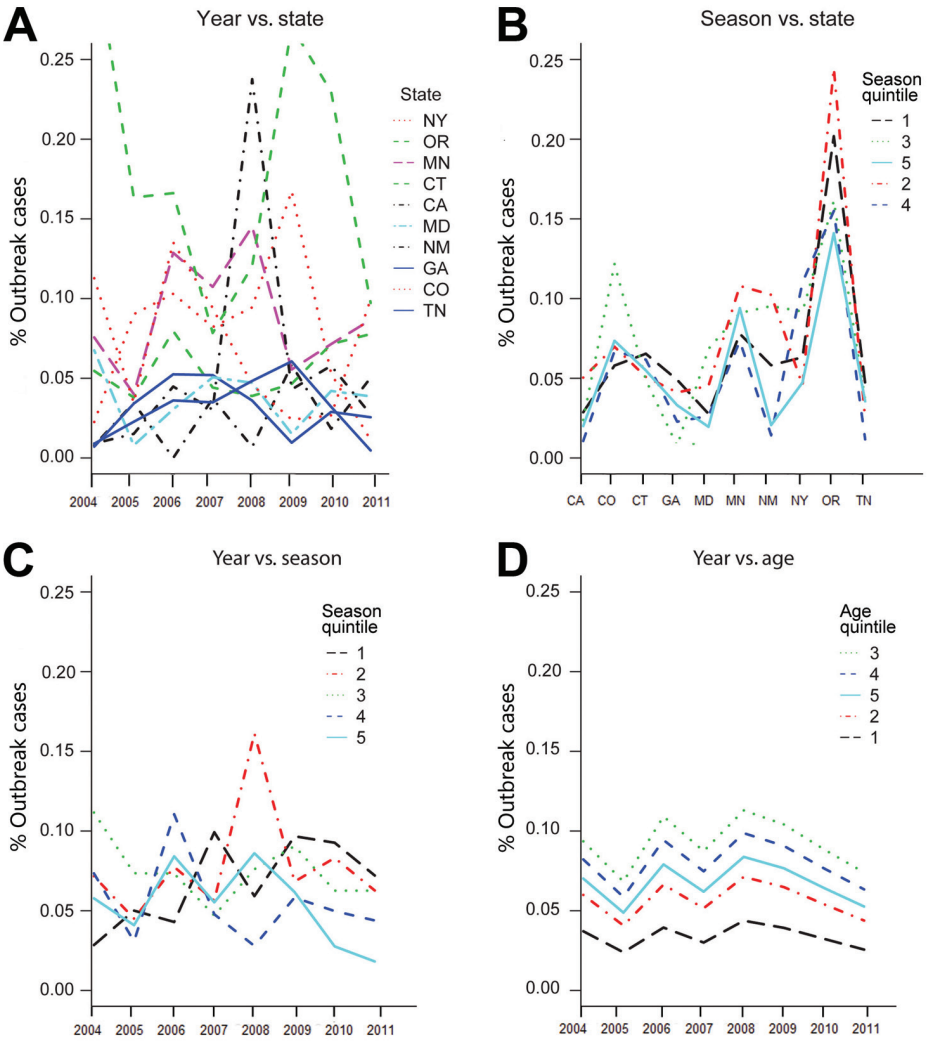

Figure 4. Interaction plots from the best-fitting *Salmonella* logistic regression model used in the analysis of Foodborne Diseases Active Surveillance Network (FoodNet) data, United States, 2004–2011. A) Year versus state; B) season versus state; C) year versus season; D) year versus age. The *y*-axis is the proportion of outbreak-associated cases. Crossing lines indicate interactions between 2 factors for the proportion of outbreak-associated case.

history, 18% involved international travel before illness onset; however, the small number of outbreak-associated cases is probably the primary limitation of the *Campylobacter* analyses.

We conclude that the characteristics of outbreak and sporadic cases captured by FoodNet vary for all 4 pathogens examined. Nevertheless, with the exception of season and age of patient for *Salmonella* cases, the differences between outbreak and sporadic cases pertain to factors that are probably associated with the inherent variability among complex surveillance systems. Our finding with respect to age differences for *Salmonella* outbreak and sporadic case-patients suggests that applying outbreak-based source attribution estimates to the youngest case-patients might be inappropriate. Otherwise, because our analysis generally finds that outbreak and sporadic illnesses have similar case characteristics, our impression is that this study does not refute the plausibility of outbreak-based source attribution methods demonstrated in Painter et al. (*1*).

Our study was limited to cases that were laboratory-confirmed. Consequently, our conclusions are based on the assumption that persons with foodborne illness who did not seek healthcare or did not have a specimen submitted for laboratory testing, are similar to those whose cases were included in our study. Nonetheless, source attribution methods will continue to evolve and will probably include data from multiple study populations. Recently, blending of outbreak-based and case-control source attribution estimates was evaluated (*15*). In the future, the type of analysis reported here could be used to examine more detailed case characteristics of illnesses transmitted commonly by food for similarities and differences between outbreak and sporadic cases. Currently, these types of data are not captured routinely in the US surveillance systems.

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References

1. Painter JA, Hoekstra RM, Ayers T, Tauxe RV, Braden CR, Angulo FJ, et al. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities, United States, 1998–2008. Emerg Infect Dis. 2013;19:407–15. http://dx.doi.org/10.3201/eid1903.111866
2. Gould LH, Walsh KA, Vieira AR, Herman K, Williams IT, Hall AJ, et al. Surveillance for foodborne disease outbreaks—United States, 1998–2008. MMWR Surveill Summ. 2013;62:1–34.
3. Scallan E, Mahon BE. Foodborne Diseases Active Surveillance Network (FoodNet) in 2012: a foundation for food safety in the United States. Clin Infect Dis. 2012;54(Suppl 5):S381–4. http://dx.doi.org/10.1093/cid/cis257
4. De'ath G. Boosted trees for ecological modeling and prediction. Ecology. 2007;88:243–51. http://dx.doi.org/10.1890/0012-9658(2007)88[243:BTFEMA]2.0.CO;2
5. Friedman J, Hastie T, Tibshirani R. Additive logistic regression: a statistical view of boosting (with discussion and a rejoinder by the authors). Ann Stat. 2000;28:337–407. http://dx.doi.org/10.1214/aos/1016218223
6. Hélie S. Understanding statistical power using noncentral probability distributions: chi-squared, G-squared, and ANOVA. Tutor Quant Methods Psychol. 2007;3:63–9.
7. R Development Core Team. R: a language and environment for statistical computing [cited 2015 Dec 12]. http://www.R-project.org
8. Schwarz G. Estimating the dimension of a model. Ann Stat. 1978;6:461–4. http://dx.doi.org/10.1214/aos/1176344136
9. Akaike H. Information theory and an extension of the maximum likelihood principle. In: Selected papers of Hirotugu Akaike. New York: Springer; 1998. p. 199–213.
10. Ailes E, Scallan E, Berkelman RL, Kleinbaum DG, Tauxe RV, Moe CL. Do differences in risk factors, medical care seeking, or medical practices explain the geographic variation in campylobacteriosis in Foodborne Diseases Active Surveillance Network (FoodNet) sites? Clin Infect Dis. 2012;54(Suppl 5):S464–71. http://dx.doi.org/10.1093/cid/cis050
11. Murphree R, Garman K, Phan Q, Everstine K, Gould LH, Jones TF. Characteristics of foodborne disease outbreak investigations conducted by Foodborne Diseases Active Surveillance Network (FoodNet) sites, 2003–2008. Clin Infect Dis. 2012;54(Suppl 5):S498–503. http://dx.doi.org/10.1093/cid/cis232
12. Jones TF, Rosenberg L, Kubota K, Ingram LA. Variability among states in investigating foodborne disease outbreaks. Foodborne Pathog Dis. 2013;10:69–73. http://dx.doi.org/10.1089/fpd.2012.1243
13. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, et al. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis. 2011;17:7–15. http://dx.doi.org/10.3201/eid1701.P11101
14. Breiman L. Random Forests. Mach Learn. 2001;45:5–32. http://dx.doi.org/10.1023/A:1010933404324
15. Cole D, Griffin PM, Fullerton KE, Ayers T, Smith K, Ingram LA, et al. Attributing sporadic and outbreak-associated infections to sources: blending epidemiological data. Epidemiol Infect. 2014;142:295–302. http://dx.doi.org/10.1017/S0950268813000915
16. Kuha J. AIC and BIC comparisons of assumptions and performance. Sociol Methods Res. 2004;33:188–229. http://dx.doi.org/10.1177/0049124103262065

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African Swine Fever Epidemic, Poland, 2014–2015

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In Poland, African swine fever (ASF) emerged in February 2014; by August 2015, the virus had been detected in >130 wild boar and in pigs in 3 backyard holdings. We evaluated ASF spread in Poland during these 18 months. Phylogenetic analysis indicated repeated incursions of genetically distinct ASF viruses of genotype II; the number of cases positively correlated with wild boar density; and disease spread was very slow. More cases were reported during summer than autumn. The 18-month prevalence of ASF in areas under various animal movement restrictions was 18.6% among wild boar found dead or killed by vehicles and only 0.2% in hunted wild boar. Repeated introductions of the virus into the country, the primary role of wild boar in virus maintenance, and the slow spread of the disease indicate a need for enhanced biosecurity at pig holdings and continuous and intensive surveillance for fast detection of ASF.

African swine fever (ASF) is an infectious and notifiable disease of domestic and wild animals of the family Suidae (*1,2*). First described in Kenya in 1921, ASF was territorially restricted to Africa only until 1957, when it spread from Angola to Lisbon. From then on, ASF has been repeatedly detected in many countries of Europe, Central America, and South America. In some countries (e.g., France, Belgium, the Netherlands), ASF outbreaks were rapidly contained, but in others (e.g., Portugal and Spain) ASF virus (ASFV) persisted for >30 years. Another longtime infected region in Europe is Sardinia (Italy), where ASFV has been circulating since 1978 and where the disease has been maintained as endemic (*3*). In 2007, the most recent epidemic started in Georgia and thereafter moved to Armenia, Azerbaijan, and the Russian Federation (*4,5*). In 2012 and 2013, ASF occurred in Ukraine and Belarus, respectively, and in 2014, it crossed into the European Union. According to the World Organisation for Animal Health, >550 ASF cases among wild boar and outbreaks among domestic pigs were detected through 2015 in Estonia, Latvia, Lithuania, and Poland (*5*).

In Poland, the first cases of ASF were detected in wild boar in February 2014 in the northeastern part of the country, very near (<1 km) the border with Belarus (*6*). As of August 31, 2015, a total of 76 cases in wild boar and 3 outbreaks among domestic pigs had been found in 3 counties (basic administrative regions of Poland).

Extensive surveillance revealed a unique pattern of disease spread that did not fit the commonly perceived concept of ASF epidemiology. Our study objective was to describe the spatiotemporal spread of ASF in Poland during the first 18 months after detection of the first cases.

Materials and Methods

Surveillance Design and Diagnostic Tests

After the first cases of ASF in Poland were confirmed, the affected area was differentiated into 3 levels of risk: area I (regions free from ASF but located near areas where ASF had been occurring in wild boar), area II (ASF detected in wild boar only), and area III (established after detection of ASF in pigs) (*7,8*). Despite differences with regard to animal movement restrictions, the surveillance strategy applied to areas I–III was the same: all wild boar found dead and those killed in road accidents (passive surveillance) and hunted wild boar (active surveillance) from all areas were submitted for testing. Samples collected from dead wild boar were whole blood, serum, marrow bones, kidneys, liver, spleen, lymph nodes, and lungs; samples from hunted wild boar were whole blood and serum. Homogenates (10% wt/vol) of individual tissues were prepared in phosphate-buffered saline. Clarified material was stored at −80°C or directly used for virus DNA extraction. Virus DNA was extracted directly from 200-mL aliquots of serum or tissue sample homogenates by using the commercial QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's recommended procedures. We used a PCR with the ASF diagnosis primers and a commercial probe (Universal ProbeLibrary no. 162; Roche Applied Science, Branford, CT, USA), which generates an amplicon of 74 bp within viral protein 72, to confirm the presence of ASFV DNA. Specific primers and probes were added to a LightCycler 480 Probes Master Kit (Roche Applied Science), and reactions were performed in a Stratagene Mx3005P real-time PCR thermocycler (Agilent Technologies, Santa Clara, CA,

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Table. African swine fever in wild boar in Poland during the first year after detection of the first cases in February 2014

	Active surveillance*				Passive surveillance†			
Season	No. positive	No. negative	Total	Prevalence, % (95% CI)	No. positive	No. negative	Total	Prevalence, % (95% CI)
Spring	0	446	446	0 (0–0.9)	4	45	49	8.2 (3.2–19.2)
Summer	0	988	988	0 (0–0.4)	26	81	107	24.3 (17.2–33.2)
Autumn	3	3,270	3,273	0.09 (0–0.3)	13	144	157	8.3 (4.913.7)
Winter	7	3,453	3,460	0.2 (0.1–0.4)	14	75	89	15.7 (9.6–24.7)
Total	10	8,157	8,167	0.12 (0.1–0.2)	57	345	402	14.2 (11.1–17.9)

*Hunted.
†Found dead.

USA) according to the protocol described by Fernández -Pinero et al. (*9*).

Altogether, from February 2014 through August 2015, samples from 609 dead/road accident wild boar and 12,253 hunted wild boar from areas I–III (*7,8*), as well as from ≈35,000 domestic pigs, were tested by real-time PCR; detailed results and difficulties encountered during the diagnostic process are described elsewhere (*10*). According to terminology adopted in Poland, outbreaks were defined as the detection of DNA of ASFV in pigs (irrespective of the number of pigs in a holding), and cases were defined as the presence of viral DNA in ≥1 wild boar found at the same time and in the same place. Thus, the number of infected animals outnumbered the number of cases or outbreaks. However, for the purpose of prevalence calculations, we took into account individual animals. To calculate the annual prevalence of ASF in wild boar during the first year of the epidemic and to analyze potential seasonal variations, we established prevalence rates (with 95% CIs) separately for wild boar tested within the scope of active and passive surveillance in quarterly intervals: spring (March–May 2014), summer (June–August 2014), autumn (September–November 2014), and winter (December 2014–February 2015). In addition, we calculated prevalence in monthly intervals to encompass the period from the beginning of the epidemic in February 2014 through August 2015. We mapped the locations of ASF outbreaks and cases by using sampling location coordinates in ArcGIS for Desktop software (Esri Inc., Redlands, CA, USA).

DNA Sequencing and Phylogenetic Analysis

We used the DNA of ASFV representing 64 cases and 3 outbreaks for phylogenetic analysis. So far we have failed to produce proper-length readable sequences for samples from case nos. 20, 24, 26–28, 32, 51, 56, 57, and 68. The primers specific to the MGF505-2R gene ASFV sequence were designed on the basis of the complete genome sequence of the BA71V strain (GenBank accession no. U18466.2) by using online Primer 3 Plus software (http://www.bioinformatics.nl/primer3plus/). The primers were also 100% complementary to the Georgia 2007/1 sequence strain. The expected product length was estimated to be 1,173 bp. The primer sequences used for amplification and sequencing of the MGF505–2R fragment were LVR13F: 5′-GCAGAGGTATGATGTCCTTA-3′ and LVR13F: 5′-TTCCTGTTGAACAAGTATCT-3′. The PCR products were separated in a 1.5% agarose gel (Invitrogen, Grand Island, NY, USA) and then purified according to the procedure for the QIAquick Gel Extraction Kit (QIAGEN). The amplicons were sequenced on a GS FLX/Titanium sequencer (Roche Applied Science) by Centrum Badań DNA Service (Poznań, Poland). Each product was sequenced in forward and backward directions and then assembled into a single contig by using Geneious R7 software (Biomatters Ltd., Auckland, New Zealand). The ClustalW alignment calculation parameters in MEGA6 (*11*) were as follows: gap opening penalty 15, gap extension penalty 6.66, transition weight 0.5, and delay divergent cutoff 30%. We plotted the phylogram by using the neighbor-joining algorithm in MEGA6 software and calculated the nucleotide similarity matrix providing the information about the sequence identity by using Geneious R7 software. The obtained nucleotide sequences of ASFV isolates were trimmed, assembled into contigs, and aligned by using Geneious R7 software. We also retrieved 2 sequences of ASFV representing genotype II (Georgia 2007/1 and Odintsovo/2014 Russia) from GenBank to use for comparison. The tree was rooted against ASFV strains Warmbaths South Africa and Malawi Lil 20/1, representing genotypes IV and VIII, respectively. We submitted the nucleotide sequences of ASFV successfully sequenced in Poland to GenBank under accession nos. KT366447–KT366459 and KT900042–KT900107.

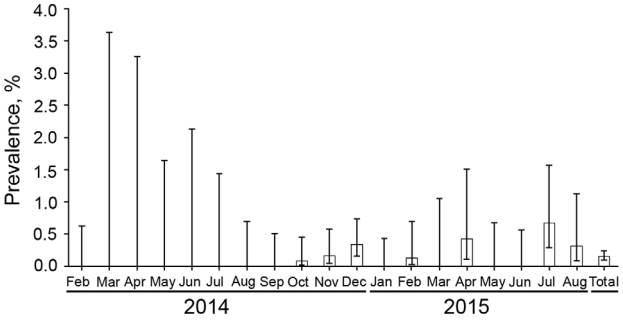

Figure 1. Monthly prevalence of African swine fever in hunted wild boar, Poland, February 2014–August 2015. Error bars indicate 95% CIs.

Statistical Analyses

To evaluate correlations between the number of ASFV-positive wild boar and wild boar density in the forestry units in which ASF detections were notified during the first year after the beginning of the epidemic, we performed a Pearson and Spearman correlation analysis (significance level 0.05). The follow-up analysis was performed 3 months after detection of ASF in new areas, which led to enlargement of the infected zone in August 2015. To assess statistical differences between seasonal prevalence of ASF, we used the Fisher exact test with a Bonferroni correction for each single comparison (significance level 0.05).

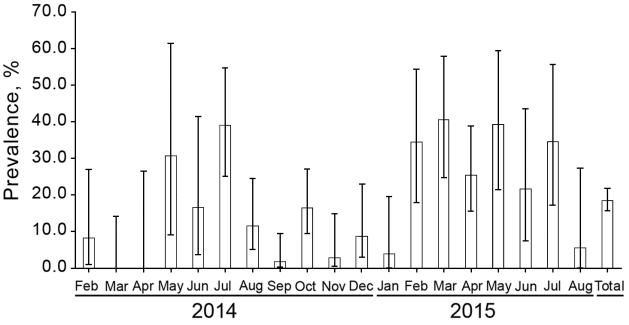

Figure 2. Monthly prevalence of African swine fever in dead (including road accident deaths) wild boar, Poland, February 2014–August 2015. Error bars indicate 95% CIs.

Results

The average annual prevalence of ASFV (based on positive PCRs) among hunted wild boar was 0.12% (95% CI 0.1%–0.2%) (Table). Prevalence did not differ significantly by season. With regard to detection of ASFV in dead wild boar, the annual prevalence was 14.2% (95% CI 11.1%–17.9%) and ranged from 8.2% in spring to 24.3% in summer. The only significant difference (after taking the Bonferroni correction into account) was between summer and autumn ($p<0.001$). The monthly prevalence ranged from 0 to 0.7% among hunted wild boar and from 0 to 40.5% among dead wild boar (Figures 1, 2). The overall 18-month prevalence in areas under animal movement restrictions was 18.6% (95% CI 15.7%–21.8%) according to passive surveillance and 0.2% (95% CI 0.1%–0.2%) according to active surveillance.

We found a correlation between the number of ASF notifications and the number of wild boar in the affected forestry units (Spearman rank correlation coefficient $R = 0.90$, $p<0.05$) in February 2015 (during the first year after detection of the first case). As of August 2015 (after detection of ASF in new areas in June 2015 and the enlargement of the infected zone), the correlation lost statistical significance (Figure 3).

The nucleotide and amino acid sequence identity of the MGF505-2R gene between ASFV isolates from Poland ranged from 99.47% to 100%. The largest cluster consisted of 42 sequences (41 from wild boar and 1 from pigs [outbreak 3]) exhibiting 100% homology between each other and indistinguishable from 2 references included for comparison: Georgia 2007/1 and Odintsovo 02/14 Russia (Figure 4). The second largest group containing 100% homologous sequences comprised 12 viruses (11 from wild boar and 1 from pigs [outbreak 2]) with 99.9% similarity to viruses of the previous group. The DNA fragment of the virus recovered from pigs identified as from the first outbreak differed slightly from those mentioned above, and the only identical sequence was from the virus from case no. 4. Sequences representing case nos. 15, 17, 41, 45, 55, and 72 formed a clearly separate and diverse cluster (within-group genetic diversity 99.5%–99.9%) (Figure 5).

Discussion

After the emergence of ASF in Poland, the preliminary forecasts had predicted that the virus would deplete the population of wild boar in the region or would spread quickly to new areas because it is inherently so highly contagious. These predicted events, however, did not occur. Nor did the concept that ASFV cannot be sustained among wild boar without spillover from domestic pigs (*12,13*) apply to the situation in Poland. So far, the number of cases in wild boar in Poland has greatly outnumbered outbreaks among domestic pigs. The virus has been found almost exclusively in wild boar, which seem to be the sole mediator for virus dissemination. The total area of the infected region is only ≈1,500 km^2. The slow spatial spread of ASF may be associated with the social behavior of wild boar, which has been studied quite extensively in Białowieża Primeval Forest, straddling the Poland–Belarus border (*14*). Wild boar show strong site fidelity, and most (≈70%) stay within 1–2 km of the center of their natal home ranges; only a relatively small percentage (5%–10%) of the population disperses from their natal range but not farther than 20–30 km. Spatial overlap of family groups is limited (*15*), which hampers transmission of the virus between groups by either direct contact between susceptible and sick animals or indirect contact with infected carcasses. In addition, the high virulence of the virus, which leads to the high case-fatality rate, prevents infected wild boar from long-distance movements. Therefore, long-distance dispersal of the virus by wild boar as carriers is assumed to be unlikely and mostly requires human involvement. However, specific socio-agricultural conditions in the affected region (i.e., low pig density, very few commercial farms, and small-scale national and international trade) create favorable barriers hitherto preventing the spread of the virus over long distances. It seems that the overall effect on the population was not significant and that,

despite a high lethality rate of genotype II for wild boar (no. deaths/no. infected animals) (*16,17*), the mortality rate (no. deaths/no. animals in the affected population) seems to not be very high. Therefore, the virus does not seem to be highly contagious, which can also be explained on one side by the inherent epidemiologic properties of ASF (no airborne transmission and required contact with blood or excretions of infected animals) and on the other side from the specific behavior of wild boar described above. The results obtained in our study provide grounds for redefining the role of wild boar, which after 18-months of observation can be considered as a reservoir host for ASFV.

The complete genetic identity between a large cluster of Poland ASFV isolates with Georgia 2007/1 isolates clearly shows that the examined region, although relatively variable, can remain highly conservative for a long

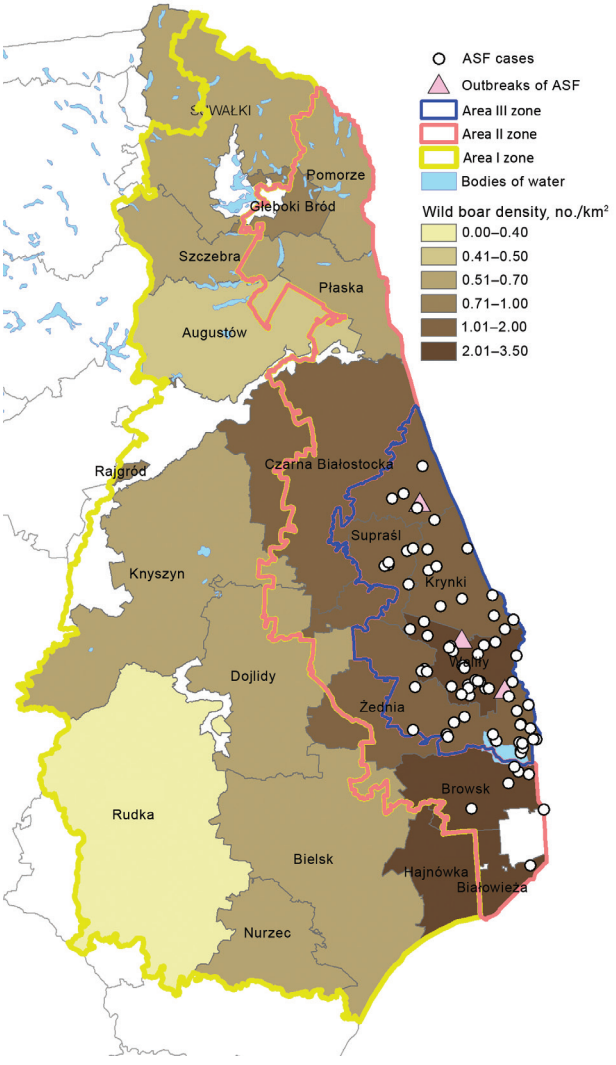

Figure 3. Locations of African swine fever (ASF) cases and outbreaks in Poland. Wild boar density data based on the National Forestry Service of Poland census.

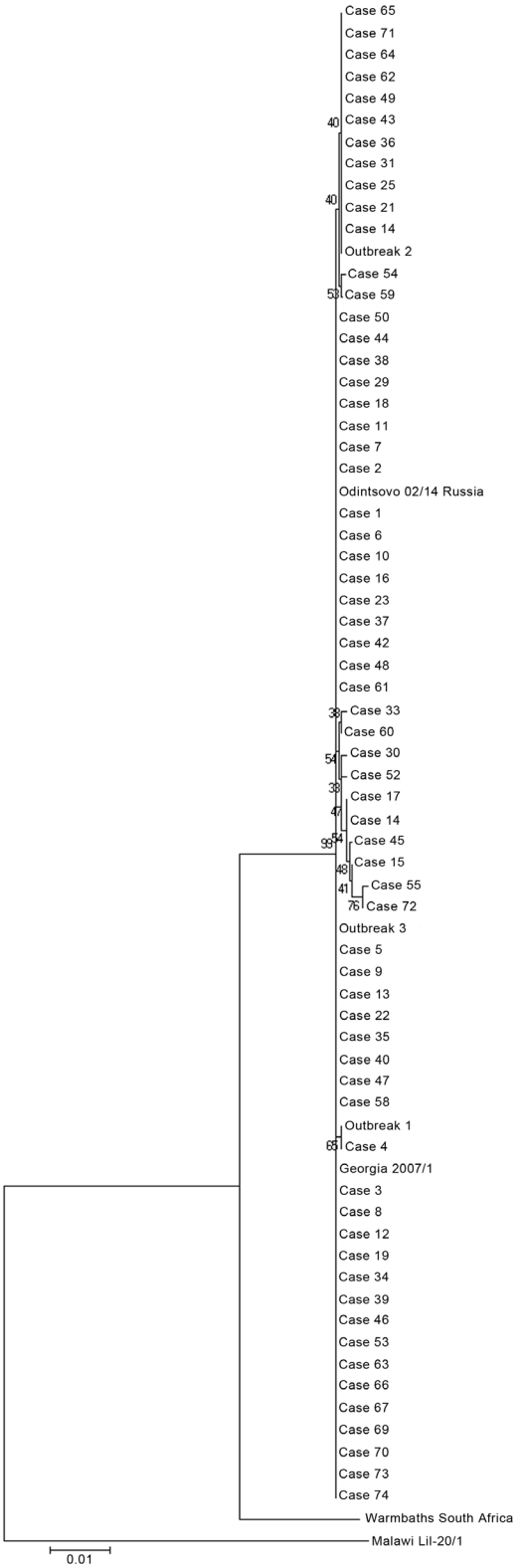

Figure 4. Phylogenetic analysis of African swine fever virus detected in pigs (outbreaks) and wild boar (cases) in Poland. Numbers on branches indicate bootstrap coefficient values. Scale bar indicates nucleotide substitutions per residue.

time (8 years). On the other hand, the genetic divergence of up to 0.5% in viruses from Poland highlighted by the presence of separate clusters on the phylogenetic tree clearly indicates that Poland has experienced a few incursions of genetically distinct ASFVs of genotype II. This finding is also supported by epidemiologic observations: 29 of 76 cases were located no farther than 5 km from the border with Belarus. With respect to outbreaks among pigs, the phylogenetic analysis clearly indicates no direct link between the 3 outbreaks. Epidemiologic investigations showed that wild boar were the most likely source of infection for domestic pigs (mainly poor biosecurity of pig holdings, enabling contact with wild animals). Overall, results of phylogenetic studies demonstrate the dynamic nature of the ASF epidemic in eastern Europe and raise serious concerns for control of ASF. We emphasize that without close and transparent collaboration between ASF-affected countries, eradication goals will be difficult to achieve.

The statistical relationship between wild boar density and the number of ASF cases was found after the 12 months after the beginning of the epidemic. The correlation was not statistically significant a few months after the virus spread to new forestry units with high wild boar density, apparently because of substantial changes in the population size in areas II and III as a result of introduced control measures (according to the most current census, the population in the aforementioned areas decreased by ≈25%). Moreover, the analysis of combined data for Poland and the Baltic States, conducted by a panel of European Food Safety Authority experts, found no correlation between wild boar density and ASF case notifications (*18*). This issue requires clarification, and the analysis will be continuously updated. However, during the first year, all cases in wild boar were detected in

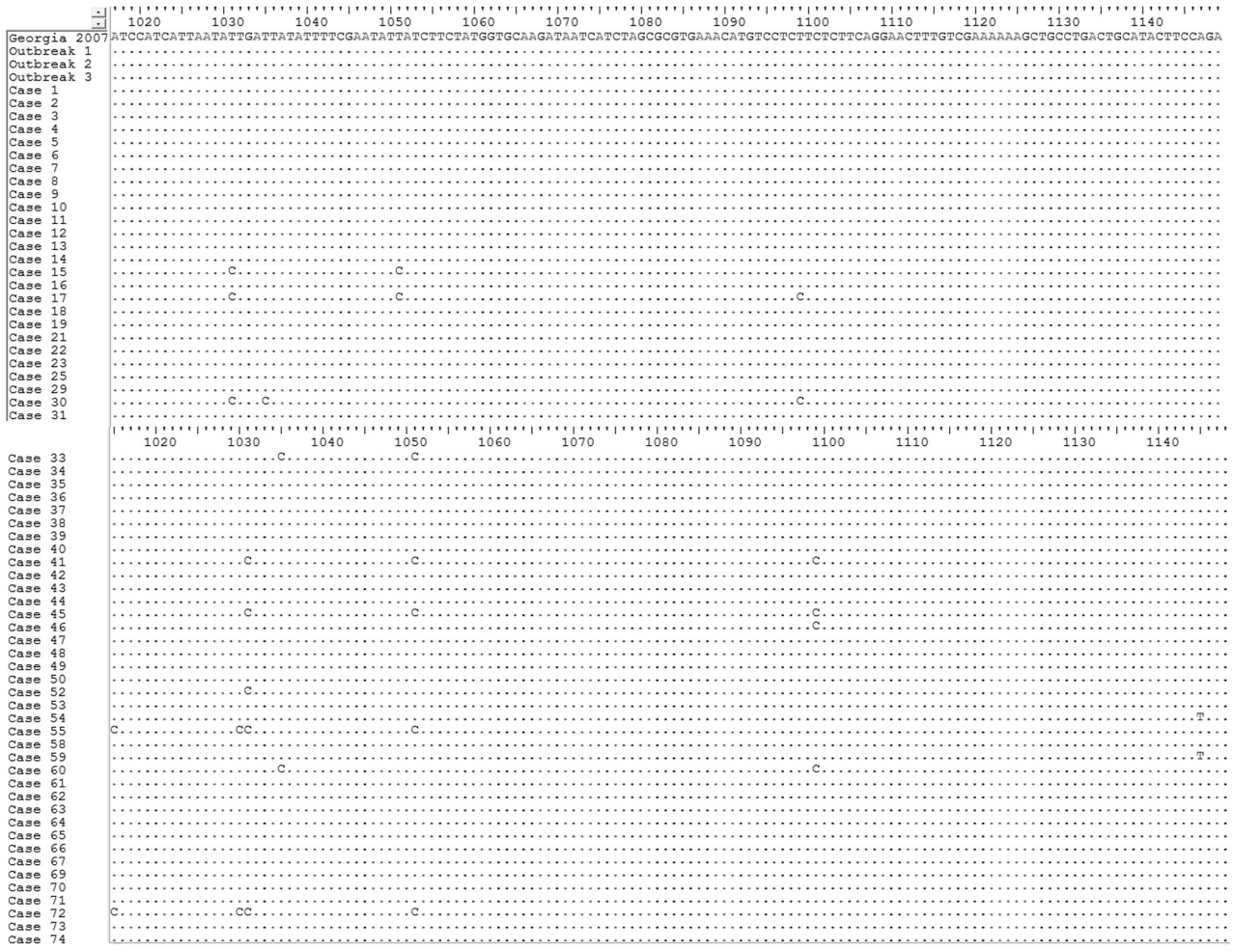

Figure 5. Nucleotide alignment of the MGF505–2R gene variable sequence fragment (residues from 1,015 to 1,149 nt) showing point mutations and differences between isolate Georgia 2007/1 isolate and African swine fever virus field isolates from Poland. The graph was generated by using Bioedit version 7.2.5 software (Ibis Biosciences, Carlsbad, CA, USA). The dots indicate identical nucleotide residues. The variable residues are visible as a nucleotide symbol.

areas with a wild boar density of ≥1 animal/km^2 (Figure 3); currently, in areas where substantial efforts have been undertaken to reduce wild boar populations, the number of ASF notifications has been reduced considerably. This finding raises potential implications for ASF control strategies (i.e., maintaining the wild boar population in the affected region at the level of ≈0.5–0.7 animals/km^2) and can be taken into account as a control option for reducing the number of cases among wild boar. Moreover, maintaining the population density in the surrounding regions at a low level may create a low-density barrier, preventing the virus from becoming established among wild boar in new areas, and infections, if they occur, can be expected to die out. As indicated in the most recent European Food Safety Authority report (*18*), the low density of wild boar can be achieved by female-targeted boar hunting and a feeding ban. However, this approach should be applied as a long-term control measure because intensive hunting is logistically demanding. It would also be desirable to significantly reduce the domestic pig population from backyard pig holdings, which do not fulfill biosecurity requirements. This process would thus create a chance to minimize the major risk for long-distance dispersal of the virus, which is attributed to human activity (e.g., transfer of contaminated pork, pig waste, or fomites to other, sometimes remote, regions). A new biosecurity regulation is being put in place in areas II and III, which, among other things, stipulates that holdings that do not fulfill the strict requirements will be closed.

In summary, during 18 months of ASF in Poland, we observed repeated introductions of ASFV into the country, slow spread of the disease in areas of dense wild boar populations, and a primary role of wild boar in virus maintenance. Enhancement of biosecurity practices at pig holdings is crucial for minimizing the risk for virus spillover virus from wild to domestic populations followed by long-distance spread of ASFV by human-related activities. Continuous and intensive surveillance enabling fast detection of ASF is needed, especially in previously disease-free areas.

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References

1. Costard S, Mur L, Lubroth J, Sánchez-Vizcaíno JM, Pfeiffer DU. Epidemiology of African swine fever virus. Virus Res. 2013;173:191–7. http://dx.doi.org/10.1016/j.virusres.2012.10.030
2. Sánchez-Vizcaíno JM, Mur L, Gomez-Villamandos JC, Carrasco L. An update on the epidemiology and pathology of African swine fever. J Comp Pathol. 2015;152:9–21. http://dx.doi.org/10.1016/j.jcpa.2014.09.003
3. Mur L, Atzeni M, Martínez-López B, Feliziani F, Rolesu S, Sanchez-Vizcaino JM. Thirty-five-year presence of African swine fever in Sardinia: history, evolution and risk factors for disease maintenance. Transbound Emerg Dis. 2016;63:e165–77. http://dx.doi.org/10.1111/tbed.12264
4. Oganesyan AS, Petrova ON, Korennoy FI, Bardina NS, Gogin AE, Dudnikov SA. African swine fever in the Russian Federation: spatio-temporal analysis and epidemiological overview. Virus Res. 2013;173:204–11. http://dx.doi.org/10.1016/j.virusres.2012.12.009
5. World Organisation for Animal Health. World Animal Health Information Database (WAHID) [cited 2016 Apr 23]. http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home
6. Pejsak Z, Truszczyński M, Niemczuk K, Kozak E, Markowska-Daniel I. Epidemiology of African swine fever in Poland since the detection of the first case. Pol J Vet Sci. 2014;17:665–72. http://dx.doi.org/10.2478/pjvs-2014-0097
7. Commission Implementing Decision of 9 October 2014 concerning animal health control measures relating to African swine fever in certain Member States and repealing Implementing Decision 2014/178/EU [cited 2016 Apr 23]. http://data.europe.eu/eli/dec-impl/2014/178/oj
8. Commission Implementing Decision (EU) 2015/1169 of 14 July 2015 amending the Annex to Implementing Decision 2014/709/EU concerning animal health control measures relating to African swine fever in certain Member States, as regards the entries for Estonia, Lithuania and Poland (notified under document C(2015) 4712). Official Journal of the European Union. L 188/45 [cited 2016 Apr 23]. http://data.europe.eu/eli/dec-impl/2015/1163/oj
9. Fernández-Pinero J, Gallardo C, Elizalde M, Robles A, Gómez C, Bishop R, et al. Molecular diagnosis of African swine fever by a new real-time PCR using universal probe library. Transbound Emerg Dis. 2013;60:48–58. http://dx.doi.org/10.1111/j.1865-1682.2012.01317.x
10. Woźniakowski G, Kozak E, Kowalczyk A, Łyjak M, Pomorska-Mól M, Niemczuk K, et al. Current status of African swine fever virus in a population of wild boar in eastern Poland (2014–2015). Arch Virol. 2016;161:189–95. http://dx.doi.org/10.1007/s00705-015-2650-5
11. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30:2725–9. http://dx.doi.org/10.1093/molbev/mst197
12. Laddomada A, Patta C, Oggiano A, Caccia A, Ruiu A, Cossu P, et al. Epidemiology of classical swine fever in Sardinia: a serological survey of wild boar and comparison with African swine fever. Vet Rec. 1994;134:183–7. http://dx.doi.org/10.1136/vr.134.8.183
13. Mur L, Boadella M, Martínez-López B, Gallardo C, Gortazar C, Sánchez-Vizcaíno JM. Monitoring of African swine fever in the wild boar population of the most recent endemic area of Spain. Transbound Emerg Dis. 2012;59:526–31. http://dx.doi.org/10.1111/j.1865-1682.2012.01308.x

14. Podgórski T, Scandura M, Jędrzejwska B. Next of kin next door—philopatry and socio-genetic population structure in wild boar. J Zool. 2014;294:190–7. http://dx.doi.org/10.1111/jzo.12167
15. Podgórski T, Lusseau D, Scandura M, Sönnichsen L, Jędrzejewska B. Long-lasting, kin-directed female interactions in a spatially structured wild boar social network. PLoS One. 2014;11;9:e99875. http://dx.doi.org/10.1371/journal.pone.0099875
16. Gallardo C, Soler A, Nieto R, Cano C, Pelayo V, Sánchez MA, et al. Experimental infection of domestic pigs with African swine fever virus Lithuania 2014 Genotype II field Isolate. Transbound Emerg Dis. 2015 Mar 22. Epub ahead of print. http://dx.doi.org/10.1111/tbed.12346
17. Pietschmann J, Guinat C, Beer M, Pronin V, Tauscher K, Petrov A, et al. Course and transmission characteristics of oral low-dose infection of domestic pigs and European wild boar with a Caucasian African swine fever virus isolate. Arch Virol. 2015;160:1657–67. http://dx.doi.org/10.1007/s00705-015-2430-2
18. EFSA AHAW Panel (EFSA Panel on Animal Health and Welfare. African swine fever. EFSA Journal. 2015;13:4163. http://dx.doi.org/10.2903/j.efsa.2015.416

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Restaurant Cooking Trends and Increased Risk for *Campylobacter* Infection

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In the United Kingdom, outbreaks of *Campylobacter* infection are increasingly attributed to undercooked chicken livers, yet many recipes, including those of top chefs, advocate short cooking times and serving livers pink. During 2015, we studied preferences of chefs and the public in the United Kingdom and investigated the link between liver rareness and survival of *Campylobacter*. We used photographs to assess chefs' ability to identify chicken livers meeting safe cooking guidelines. To investigate the microbiological safety of livers chefs preferred to serve, we modeled *Campylobacter* survival in infected chicken livers cooked to various temperatures. Most chefs correctly identified safely cooked livers but overestimated the public's preference for rareness and thus preferred to serve them more rare. We estimated that 19%–52% of livers served commercially in the United Kingdom fail to reach 70°C and that predicted *Campylobacter* survival rates are 48%–98%. These findings indicate that cooking trends are linked to increasing *Campylobacter* infections.

Foodborne illness is very costly, comprising medical expenses, loss of earnings, and reduced quality of life. In the United States, the annual healthcare cost is ≈$14 billion annually (*1*); in the United Kingdom, it is £1.8 billion (*2*). The foodborne illness most commonly responsible for these costs is campylobacteriosis (*3*–*5*). In the United States, cases increased by 13% between 2006–2008 and 2013 (*6*). In the United Kingdom, *Campylobacter* accounted for over half of the estimated 500,000 cases of foodborne disease during 2011–2012 (*3*,*7*); in the United States, it accounts for 9% of foodborne disease cases annually (*4*).

Foods implicated as *Campylobacter* vehicles include poultry, red meat, milk, and water (*7*–*11*). Studies of outbreaks and sporadic cases have identified the principal source of infection as undercooked chicken meat (*9*–*14*). In the United Kingdom, increasing numbers of outbreaks are attributed to undercooked chicken livers (*9*) despite the fact that the UK Food Standards Agency (FSA) has provided guidelines for safely cooking them. These increased infections seem to have coincided with a trend among leading chefs to advocate minimal cooking of chicken livers, despite recommendations to maintain liver cores at 70°C for 2–3 minutes to ensure they are *Campylobacter* free (*15*).

Although the association between consuming chicken livers and infection with *Campylobacter* is well known (*9*), the underlying reasons for the changing epidemiology of outbreaks associated with chicken liver consumption are unclear. We hypothesized that the trend toward including rarer, pinker meat in the recipes of leading chefs and by mass media representation of meat cooking may be contributing to changes in the way chicken livers are consumed.

We therefore conducted an interdisciplinary investigation by using a combination of methods from social and biological sciences. Participants were selected from the UK population, and the study was conducted during 2015. Our study objectives were 1) to investigate the ability of chefs and members of the public to identify cooked chicken livers that meet FSA guidelines for safe cooking, 2) to elicit the preferences of chefs and the public regarding the rareness of chicken livers, and 3) to model the survival of *Campylobacter* in chicken livers sautéed to various core temperatures.

Methods

Participants

We recruited a quota-based sample of 1,030 members of the UK public via an online market research panel (http://www.researchnow.com). Quotas were used to ensure representativeness in terms of age groups and social class. The quota permitted an unequal split by sex (up to 70% women) because in the United Kingdom, food preparation at home

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[1]These senior authors contributed equally to this article.

is more commonly performed by women than men. We also recruited 143 chefs through face-to-face convenience sampling at culinary shows and competitions and by online culinary forums.

All participants gave informed consent. Respondents were debriefed on the purpose of the survey after completion and given the opportunity to withdraw their data. Ethical approval was obtained from the College of Natural Science Ethics Committee at Bangor University (CNS/2014/AJ1).

Preparation of Visual Aids

To prepare cooked chicken liver dishes to serve as visual aids, we used methods similar to those used in studies of hamburgers (*16*) and beefsteaks (*17*). A chef cooked 7 batches of chicken livers for various times, recorded the maximum core temperature for each batch, and arranged each batch on a plate for photography by a professional photographer. The process was repeated (without the temperature being recorded) for 3 other meats (duck breasts, lamb racks, and beef burgers).

Surveys of Preference and Knowledge

To determine preferences and knowledge of safe cooking practices among chefs and members of the public, we used the images of cooked chicken livers as visual aids. The images were presented in surveys (online and print), arranged in order of cooking time/rareness (Figure 1). The surveys for chefs and the public were similar, except that the chefs were asked about serving preferences and the public was asked about eating preferences.

To avoid biases (such as social desirability bias) resulting from respondents perceiving the survey to be about food safety, we described the survey as being about food preferences. Respondents were first asked preference questions about 3 of the 4 meats (in random order) to obscure the focus on chicken livers and safety. Chefs were asked to indicate which chicken liver dish was cooked "the way you would like to serve it" and "the way you think most customers would like it." Members of the public were asked which dish they would prefer if "eating out" and "eating at home."

Respondents were subsequently asked which chicken liver dish (if any) was the first they thought would meet FSA safe cooking guidelines. Additional questions were asked about perceived trends and influences regarding cooking meat, dining habits, and demographic information such as class and age. Chefs provided additional information about their current position, such as their training and industry experience.

Campylobacter Survival

To prepare a suspension of *Campylobacter* for experimental inoculation, we streaked *Camplyobacter jejuni* M1 strain (sequence type 137, clonal complex 45) on Columbia agar base containing 5% defibrinated horse blood, incubated it at 37°C under microaerobic conditions for 48–72 h, and then inoculated it into *Camplyobacter* enrichment broth. After subculture for another 24 h, a bacterial suspension was prepared in maximum recovery diluent to an optical density of 600 nm (≈10^9 CFU/mL). The culture broth was diluted in *Camplyobacter* enrichment

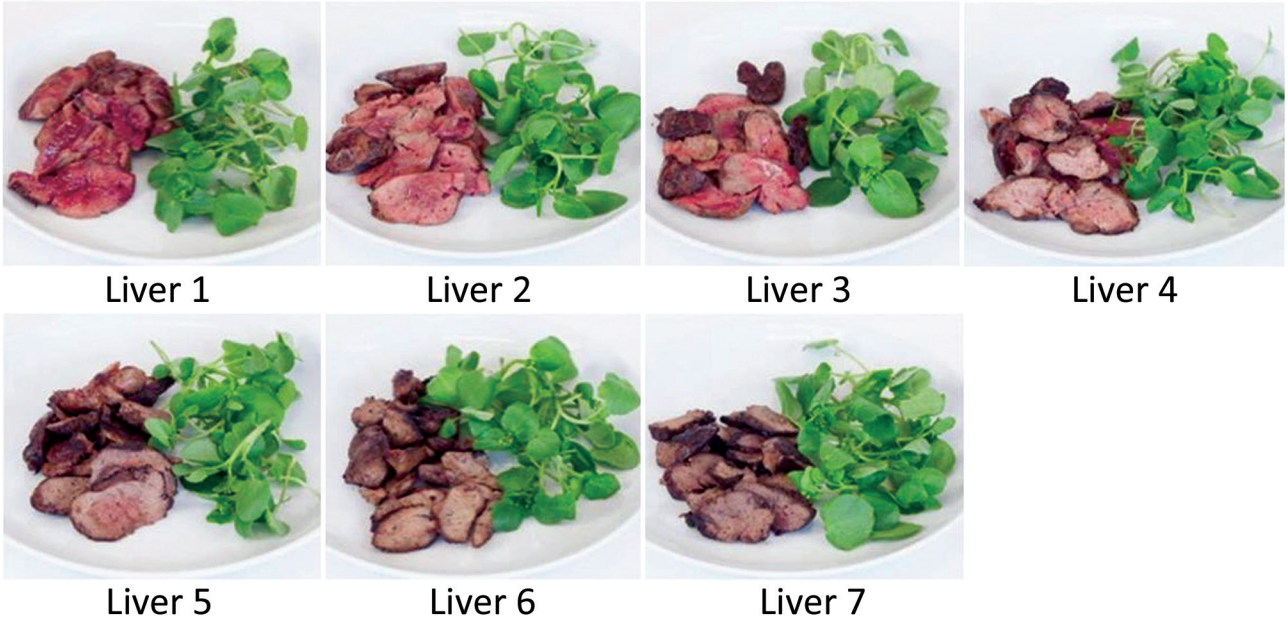

Figure 1. Chicken liver images, in order of cooking time/rareness, used in survey to determine preferences and knowledge of safe cooking practices among chefs and the public, United Kingdom.

broth to give a suspension of ≈10^5 CFU/mL for inoculation into fresh chicken livers.

The fresh chicken livers were purchased in packs from supermarkets and sorted into batches of 4 with similar weights. The connective tissue was cut between the 2 liver lobes, with the weight of the larger lobe recorded and assigned for inoculation with *Campylobacter* broth suspension; 4 livers were assigned to each cooking batch. A 1-cm^2 area of each liver was scored at its thickest point by using a sterile scalpel blade and injected with 100 μL (≈10^4 CFU) of culture broth, corresponding to the highest levels of *Campylobacter* reported to be found in naturally contaminated livers (*18*).

For each cooking time, 10 g butter was heated in a frying pan over moderate to high heat on an electric cooktop; when the butter had finished frothing, the 4 inoculated liver lobes in the batch were added. The maximum core temperature of the largest and smallest liver in each batch was recorded. To determine the survival of the inoculated M1 strain of *C. jejuni* within the cooked livers, we placed each liver in a sterile petri dish, and a 4–5-g portion around the scored inoculated region was removed and added to a Stomacher bag (Seward BA6040, Worthing, UK); 10 mL of Exeter broth was added to each bag before Stomaching (mechanical pounding of the outer surface of the bag to remove bacteria) for 1 min. The homogenized suspension was poured into a 20-mL universal container and incubated at 41°C under microaerobic conditions (Variable Atmosphere Incubator; Don Whitely Scientific, Shipley, UK) for 24 h, after which 1 loopful of broth was plated onto *Campylobacter* blood-free medium (modified charcoal cefoperazone deoxycholate agar, containing cefoperazone and amphotericin) at 41°C under microaerobic conditions for 48–72 h. We picked 1 typical *Campylobacter* colony from at least 1 plate in each batch and confirmed it as *C. jejuni* by PCR; for a cooked liver to be deemed positive, 1 isolate per batch was confirmed as *C. jejuni* positive (*19*).

Data Analyses

We modeled the probability of survival for the 60 livers for which temperature and *Campylobacter* presence/absence after cooking were recorded. We used logistic regression to model the relationship between the core temperature of the livers and the survival of C*ampylobacter*. The probability of *Campylobacter* survival as a function of core temperature was modeled via estimation of a logit model, which captured the nonlinear temperature-survival relationship (Figure 2). Parameter estimates were obtained by using logistic regression (Stata logit command; StataCorp LP, College Station, TX, USA) on the binary variable indicating *Camplyobacter* survival (1 = survival, 0 = nonsurvival) in a sample of 60 cooked chicken livers. Temperature was the maximum core temperature recorded for the batch from which the chicken liver was taken. This model was used to assign predicted survival rates for each photographed chicken liver dish.

We used the Kolmogorov Smirnov 2-sample test to compare differences in the distribution of knowledge and preferences between groups (chefs and the public). We investigated within-person differences by using the Wilcoxon signed-rank test for paired data. Ordered logit models (*20*) were estimated to determine the effects of observable characteristics on respondents' preferences for chicken liver rareness and their choices of FSA-compliant livers.

Results

Campylobacter Survival

We discuss the results of the *Campylobacter* survival experiment first because an understanding of those results is useful for interpreting the preferences and knowledge analyses. The relationship between core temperature and *Campylobacter* survival rate was inverse (Table; Figure 2). Of the 32 batches of 4 inoculated livers, the shortest cooking time was 1 minute, leading to a mean core temperature of 36°C and a 100% *Campylobacter* survival rate. At the maximum mean core temperature (72°C), *Campylobacter* survival rate was 8.3%.

The logistic model predicted a survival rate of 98% in liver with core temperature that reached 52°C (liver 1) and equivalent survival rates of 95% and 48% at core temperatures of 56°C and 66°C (livers 2 and 3). Liver 4 reached a maximum temperature of 70°C, but the temperature was not held for the recommended 2 minutes; predicted *Campylobacter* survival rate was 22%. Livers 6 and 7 met the FSA guidelines, and their predicted *Campylobacter* survival rate was <0.001%.

Preferences and Knowledge of the Public

Of the 1,030 members of the public surveyed, 43.0% ate chicken livers and hence were asked to select the chicken

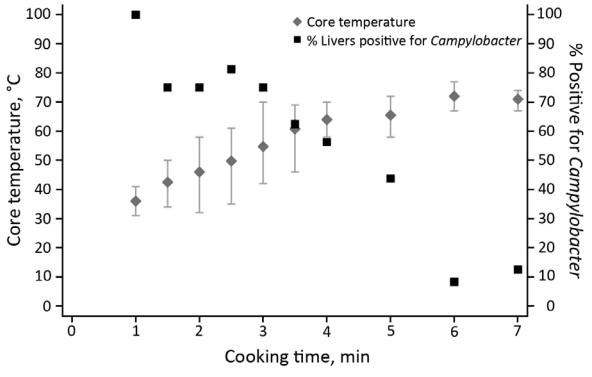

Figure 2. *Campylobacter* survival in cooked (pan-fried) chicken livers, by cooking time and temperature. Error bars represent minimum and maximum temperatures reached.

Table. *Campylobacter* survival in cooked chicken liver, by replicate*

Variable	Cooking time, min									
	1	1.5	2	2.5	3	3.5	4	5	6	7
Replicate 1										
No. positive	4.0	3.0	3.0	3.0	4.0	3.0	2.0	3.0	ND	ND
Mean weight, g	41.5	41.5	43.8	41.5	41.5	41.5	40.3	40.8	ND	ND
Mean core temp, °C	36.0	46.0	44.0	41.0	47.5	55.5	60.5	61.5	ND	ND
Replicate 2										
No. positive	ND	3.0	3.0	4.0	3.0	3.0	3.0	3.0	0	ND
Mean weight, g	ND	34.0	34.0	34.0	34.3	34.0	34.3	34.5	34.3	ND
Mean core temp, °C	ND	39.0	42.5	44.0	50.5	59.0	65.5	65.0	72.0	ND
Replicate 3										
No. positive	ND	ND	2.0	4.0	4.0	4.0	3.0	3.0	1.0	1.0
Mean weight, g	ND	ND	40.0	40.3	39.3	40.5	40.5	40.3	39.5	39.0
Mean core temp, °C	ND	ND	41.5	55.5	57.5	61.0	69.0	64.0	69.0	72.5
Replicate 4										
No. positive	ND	ND	4.0	2.0	1.0	0.0	1.0	1.0	0	0
Mean weight, g	ND	ND	25.8	26.3	28.0	26.5	27.3	24.8	27.8	29.5
Mean core temp, °C	ND	ND	56.0	58.5	63.5	67.5	61.0	71.5	75.0	69.5
No. livers	4	8	16	16	16	16	16	16	12	8
No. positive	4	6	12	13	12	10	9	10	1	1
Mean no. positive per batch of 4	4.0	3.0	3.0	3.3	3.0	2.5	2.3	2.5	0.3	0.5
Overall mean % of positives	100	75.0	75.0	81.3	75.0	62.5	56.3	62.5	8.3	12.5
Overall mean liver weigh, g	41.5	37.8	35.9	35.5	35.8	35.6	35.6	35.1	33.8	34.3
Overall mean core temperature, °C	36.0	42.5	46.0	49.8	54.8	60.8	64.0	65.5	72.0	71.0

*ND, not detected.

liver dishes they preferred and which they thought met FSA guidelines. Half (49.3%) of all male respondents and 38.4% of all female respondents ate chicken livers. Rates of chicken liver consumption varied by age group: 18–34 years, 34.7%; 35–44 years, 44.7%; 45–54 years, 49.0%, 55–64 years: 51.5%; and ≥65: 42.9%. Chicken livers were eaten by half (51.0%) of respondents belonging to UK socioeconomic grouping ABC1 (upper, middle, and lower middle class) and 32.3% of those belonging to C2DE (working class and those at the lowest level of subsistence).

Members of the public poorly identified whether a chicken liver met FSA guidelines for safe cooking (Figure 3). Thirty percent identified livers 1–3 as being safe to eat; the predicted rates of *Campylobacter* survival in these livers were 48%–98%. Another 22% thought that liver 4 (*Campylobacter* survival rate 22%) was safe to eat.

No significant difference was found between the public's choices of FSA-compliant livers and their preferences when dining out ($p = 0.776$, Wilcoxon signed-rank test; $n = 386$) (Figure 4); respondents were consistent between what they wanted to eat and what they thought was safe. Respondents showed a significant preference for pinker livers when eating out rather than at home ($p = 0.007$, Wilcoxon signed-rank test; $n = 446$). Paradoxically, respondents reported being more concerned about food safety when eating out than at home ($p<0.001$, Wilcoxon signed-rank test; $n = 999$).

Ordered logit results (not reported) identified no systematic differences in rareness preferences by respondent sex, age, or class. Livers that were more pink were preferred by respondents who described themselves as adventurous ($p<0.030$, $n = 444$) and who were less concerned about restaurant food safety ($p<0.001$, $n = 444$).

Perceptions and Knowledge of Chefs

Among the 143 chefs, of those who indicated their sex, 134 (88%) were male. Among the 141 who indicated their type of work, 31.9% worked in fine dining, 17% in contract catering, 11.3% in casual restaurants, 5.7% in pubs, and 19.1% in multiple kitchen types. The most commonly held position among 131 chefs who responded was head chef (54.0%), followed by chef trainer (11.5%), chef de partie (10.7%), commis chef (6.9%), and sous chef (6.1%).

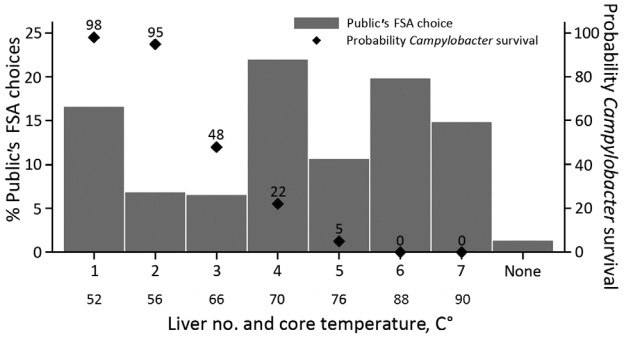

Figure 3. Rarest chicken livers visually identified by members of the public as complying with FSA cooking guidelines and associated core temperatures and probabilities of *Campylobacter* survival in survey to determine preferences and knowledge of safe cooking practices among chefs and the public, United Kingdom. Liver image numbers correspond to those shown in Figure 1. FSA, Food Standards Agency.

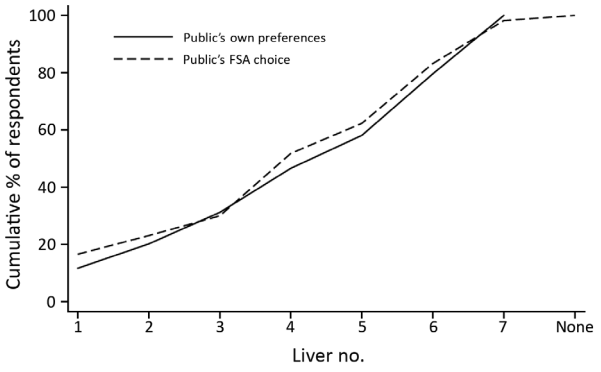

Figure 4. Proportion of public identifying which chicken liver dishes they preferred and which they believed complied with FSA cooking guidelines in survey to determine preferences and knowledge of safe cooking practices among chefs and the public, United Kingdom. Liver image numbers correspond to those shown in Figure 1. FSA, Food Standards Agency.

Chefs were much better than members of the public at identifying whether a chicken liver met FSA guidelines; only 9.8% of chefs (vs. 30% of the public) selected livers 1–3 as being FSA compliant (Figure 5), and another 19.8% thought that liver 4 met FSA guidelines. Although they outperformed the public, 30% of the chefs identified livers with *Campylobacter* survival rates of 22%–98% as being FSA compliant.

Chefs preferred to serve livers more pink than they thought would meet FSA guidelines ($p<0.001$, Wilcoxon signed-rank test; $n = 143$) (Figure 5). Chefs also preferred to serve livers substantially more pink than the public preferred when eating out ($p<0.001$, Kolmogorov-Smirnov 2-sample test). Chefs' perceptions of customers' preferences for rareness differed significantly from customer's true preferences: not only did chefs prefer to serve livers more rare than customers wanted them served, they also thought that customers wanted chicken livers more rare than the customers themselves indicated ($p = 0.008$, Kolmogorov-Smirnov 2-sample test).

As with the members of the public, in the ordered logit model to explain serving preferences, chef preference for pinkness of served livers did not vary according to chef characteristics such as age, sex, and class. The only significant results indicated that chefs holding senior positions preferred to serve liver more pink than did their less experienced colleagues holding junior kitchen positions ($p = 0.002$).

Culinary Trends

Almost half (47.8%) of the members of the public sampled agreed that "cooking programmes on TV and/or recipes in magazines have influenced the way the general public cook meat, people now serve it pinker in the middle." Among chefs, >45% agreed that they had noticed a trend of rarer and pinker chicken livers on television, in recipes, and among other chefs.

Discussion

Members of the public poorly identified whether chicken livers had been cooked to a safe microbiological state. Their preferences for chicken livers were consistent with their (often inaccurate) perceptions of safely cooked livers. Among chefs, these variables differed; chefs outperformed the public at identifying whether chicken livers had been cooked to FSA guidelines. We found that chef preferences for serving chicken livers were inconsistent with their perceptions of safe cooking—they preferred to serve livers more rare than is microbiologically safe and believed that their customers also prefer them more rare than is safe. Chefs systematically overpredicted their customers' preferences for pinkness of livers served. This finding probably means that an estimated 19%–52% of livers being served in commercial UK food establishments fail to reach a core temperature of 70°C and could have *Campylobacter* survival rates of 48%–98%.

Chefs preferred rarer livers than the FSA guidelines would recommend. Chefs (correctly) thought that customers preferred livers less rare than their own preferences ($p<0.001$, Wilcoxon signed-rank test), but they still overestimated customers' preference for pinkness. Chefs' preferences, rather than their ignorance of FSA microbiological guidelines, seem to be leading them to serve undercooked livers. This finding resonates with previous findings that knowledge is not necessarily a driver of behavior (*21–23*). We contend that the explanation for the discrepancy between cooking practices and recommended guidelines is a cultural one, resulting in preferences for taste and texture overriding the desire to avoid foodborne illness (*24–26*). In extremis, this preference ultimately led chef Raymond

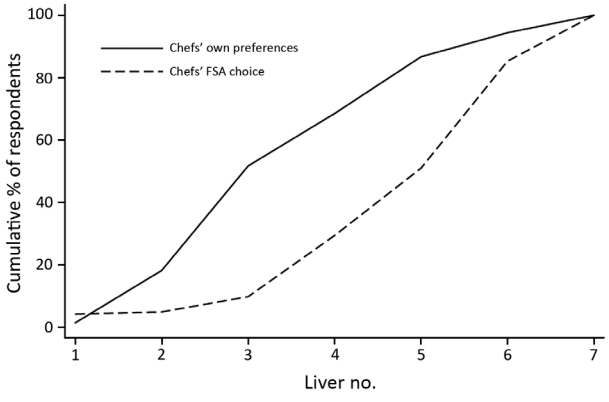

Figure 5. Proportion of chefs identifying which chicken liver dishes they preferred and which they believed complied with FSA cooking guidelines in survey to determine preferences and knowledge of safe cooking practices among chefs and the public, United Kingdom. Liver image numbers correspond to those shown in Figure 1. FSA, Food Standards Agency.

Blanc to remove liver dishes from the menu rather than increase cooking times/temperatures after cases of campylobacteriosis were attributed to diners having eaten liver in his restaurant (*27*).

The public health implications of the contrast between chef preferences and safe practices depend largely on what chefs provide for customers. Given that chefs prefer livers more pink than they believe customers do, we take the chef perception of customer preference as the lower bound and chefs' own preference as the upper bound on the rareness of chicken livers served. This finding implies that 18.9%–51.7% of livers being served in commercial UK food establishments are failing to reach a core temperature of 70°C and have *Campylobacter* survival rates of 48%–98% (Figure 6). Extending the range of livers considered unsafe to liver 4 from our testing implies that 38.5%–68.5% of chicken livers being served commercially may have *Campylobacter* survival rates of 22%–98%.

This preference for rare chicken livers is part of a broader shift in contemporary cooking culture toward rarer meats, a trend that is reflected in the mass media (*28*,*29*) but not yet in the peer-reviewed literature. Periodically, the preference among chefs for serving rarer meat results in conflicts with recommendations of public health officials (*30*–*32*). The trend toward serving meat more pink has now extended from meats such as beefsteaks to meats such as chicken livers, for which the microbiological risks associated with rareness are far greater.

Our interdisciplinary approach, using relatively large samples of chefs and members of the general public, provides a unique insight into the possible public health implications of a divergence between preferences and safe cooking. A limitation of our approach is basing selection of preferred dishes on visual inspection alone. However, an experimental design that enabled respondents to physically assess cooked dishes would have severely limited study size. Another limitation is use of a laboratory-cultured inoculum, which might be less heat resistant than naturally occurring bacteria. Therefore, the projected death rates might be overestimated, and undercooked livers might pose even more of a risk than this study suggests. Our results relate to the *C. jejuni* M1 strain only; other *Campylobacter* strains may exhibit different survival characteristics. *Campylobacter* survival is reported here in terms of presence or absence, not as colony counts. Results indicate public risk for exposure to *Campylobacter*, not risk for infection or subsequent illness. The low doses required for infection and illness (*33*,*34*) are part of a stochastic process that can happen at any dose, suggesting that the presence of any *Campylobacter* in cooked livers poses a public health threat.

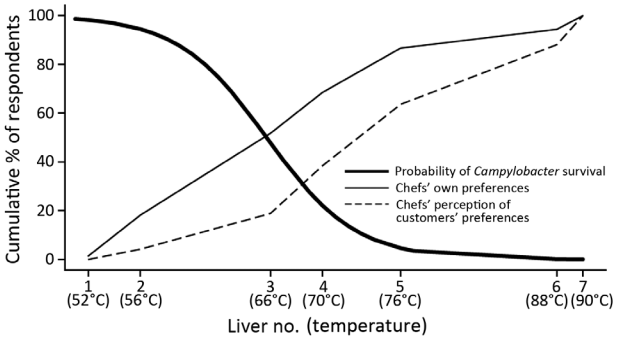

Figure 6. Proportion of chefs identifying which chicken liver dishes they preferred and which they believed their customers would prefer and associated probabilities of *Campylobacter* survival in survey to determine preferences and knowledge of safe cooking practices among chefs and the public, United Kingdom. Liver image numbers correspond to those shown in Figure 1.

Because all experimental livers were inoculated with *Campylobacter*, our results have been framed in terms of probability of *Campylobacter* survival rather than exposure. Hence, our reported rates at which chefs serve *Camplyobacter*-positive livers may be slightly overestimated.

The temperature–survival results presented here, supported by those of Whyte et al. (*15*), suggest that the chicken liver cooking techniques practiced by many chefs, and promoted in the culinary and mass media, are leading to increased exposure to *Campylobacter*. The role of celebrity chefs and the mass media in pushing the trend toward serving pink meat were evident in our results. Recipes by top chefs frequently recommend serving chicken livers pink in the middle in warm salads, pâtés, and parfaits (*35*,*36*). This trend toward pink resonates with our estimate, based on our survey and experimental results, that 19%–52% of livers served in UK food outlets do not reach a core temperature of 70°C and our predicted *Campylobacter* survival rates of 48%–98%. Given *Campylobacter* prevalence rates among UK retail chicken livers (81%–100% externally, 90% internally [*15*,*37*]), our results suggest that contemporary cooking trends are leading to the "gourmet-fication" of foodborne disease.

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References

1. Hoffmann S, Batz MB, Morris JJ. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. J Food Prot. 2012;75:1292–302. http://dx.doi.org/10.4315/0362-028X.JFP-11-417
2. Food Standards Agency. Annual report of the chief scientist 2012/13. Safer food for the nation. London: The Agency; 2013.
3. Tam CC, Rodrigues LC, Viviani L, Dodds JP, Evans MR, Hunter PR, et al. Longitudinal study of infectious intestinal disease in the United Kingdom (IID2 study): incidence in the community and presenting to general practice. Gut. 2012;61:69–77. http://dx.doi.org/10.1136/gut.2011.238386
4. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis. 2011;17:7–15. http://dx.doi.org/10.3201/eid1701.P11101
5. Kirk MD, Pires SM, Black RE, Caipo M, Crump JA, Devleesschauwer B, et al. World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. PLoS Med. 2015;12:e1001921. http://dx.doi.org/10.1371/journal.pmed.1001921
6. Crim SM, Iwamoto M, Huang JY, Griffin PM, Gilliss D, Cronquist AB, et al. Incidence and trends of infection with pathogens transmitted commonly through food—Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006–2013. MMWR Morb Mortal Wkly Rep. 2014;63:328–32.
7. Tam C, Larose T, O'Brien S. Costed extension to the second study of infectious intestinal disease in the community: identifying the proportion of foodborne disease in the United Kingdom and attributing foodborne disease by food commodity. Report no.: Project B18021 (FS231043). London: Food Standards Agency; 2014.
8. Frost JA, Gillespie IA, O'Brien SJ. Public health implications of *Campylobacter* outbreaks in England and Wales, 1995–9: epidemiological and microbiological investigations. Epidemiol Infect. 2002;128:111–8. http://dx.doi.org/10.1017/S0950268802006799
9. Little CL, Gormley FJ, Rawal N, Richardson JF. A recipe for disaster: outbreaks of campylobacteriosis associated with poultry liver pâté in England and Wales. Epidemiol Infect. 2010;138:1691–4. http://dx.doi.org/10.1017/S0950268810001974
10. Humphrey T, O'Brien S, Madsen M. Campylobacters as zoonotic pathogens: a food production perspective. Int J Food Microbiol. 2007;117:237–57. http://dx.doi.org/10.1016/j.ijfoodmicro.2007.01.006
11. Adak GK, Meakins SM, Yip H, Lopman BA, O'Brien SJ. Disease risks from foods, England and Wales, 1996–2000. Emerg Infect Dis. 2005;11:365–72. http://dx.doi.org/10.3201/eid1103.040191
12. Butzler JP. *Campylobacter*, from obscurity to celebrity. Clin Microbiol Infect. 2004;10:868–76. http://dx.doi.org/10.1111/j.1469-0691.2004.00983.x
13. Sheppard SK, Dallas JF, Strachan NJC, MacRae M, McCarthy ND, Wilson DJ, et al. *Campylobacter* genotyping to determine the source of human infection. Clin Infect Dis. 2009;48:1072–8. http://dx.doi.org/10.1086/597402
14. Domingues AR, Pires SM, Halasa T, Hald T. Source attribution of human campylobacteriosis using a meta-analysis of case–control studies of sporadic infections. Epidemiol Infect. 2012;140:970–81. http://dx.doi.org/10.1017/S0950268811002676
15. Whyte R, Hudson JA, Graham C. *Campylobacter* in chicken livers and their destruction by pan frying. Lett Appl Microbiol. 2006;43:591–5. http://dx.doi.org/10.1111/j.1472-765X.2006.02020.x
16. Røssvoll EH, Lavik R, Ueland Ø, Jacobsen E, Hagtvedt T, Langsrud S. Food safety practices among Norwegian consumers. J Food Prot. 2013;76:1939–47.
17. López Osornio MM, Hough G, Salvador A, Chambers E IV, McGraw S, Fiszman S. Beef's optimum internal cooking temperature as seen by consumers from different countries using survival analysis statistics. Food Qual Prefer. 2008;19:12–20. http://dx.doi.org/10.1016/j.foodqual.2007.06.004
18. Baumgartner A, Felleisen R. Market surveillance for contamination with thermotolerant campylobacters on various categories of chicken meat in Switzerland. J Food Prot. 2011;74:2048–54. http://dx.doi.org/10.4315/0362-028X.JFP-11-228
19. Klena JD, Parker CT, Knibb K, Ibbitt JC, Devane PM, Horn ST, et al. Differentiation of *Campylobacter coli, Campylobacter jejuni, Campylobacter lari*, and *Campylobacter upsaliensis* by a multiplex PCR developed from the nucleotide sequence of the lipid A gene *lpxA*. J Clin Microbiol. 2004;42:5549–57. http://dx.doi.org/10.1128/JCM.42.12.5549-5557.2004
20. Greene WH. Econometric analysis. 7th ed. Harlow (UK): Pearson Education Limited; 2011. p. 926.
21. Levy AS, Choinière CJ, Fein SB. Practice-specific risk perceptions and self-reported food safety practices. Risk Anal. 2008;28:749–61. http://dx.doi.org/10.1111/j.1539-6924.2008.01051.x
22. Olsen NV, Røssvoll E, Langsrud S, Scholderer J. Hamburger hazards and emotions. Appetite. 2014;78:95–101. http://dx.doi.org/10.1016/j.appet.2014.03.007
23. Green Brown L, Khargonekar S, Bushnell L; The Environmental Health Specialists Network Working Group. Frequency of inadequate chicken cross-contamination prevention and cooking practices in restaurants. J Food Prot. 2013;76:2141–5. http://dx.doi.org/10.4315/0362-028X.JFP-13-129
24. McIntosh WA, Christensen LB, Acuff GR. Perceptions of risks of eating undercooked meat and willingness to change cooking practices. Appetite. 1994;22:83–96. http://dx.doi.org/10.1006/appe.1994.1007
25. Ralston K, Brent CP, Starke Y, Riggins T, Jordan Lin CT. Consumer food safety behavior: a case study in hamburger cooking and ordering. Agricultural Economic Report no. 804. Washington (DC): US Department of Agriculture; 2001. p. 11-2.
26. O'Leary MC, Harding O, Fisher L, Cowden J. A continuous common-source outbreak of campylobacteriosis associated with changes to the preparation of chicken liver pâte. Epidemiol Infect. 2009;137:383–8. http://dx.doi.org/10.1017/S0950268808001003
27. Davies C. Raymond Blanc ordered to take lamb's liver off menu after food poisoning [cited 2015 May 6]. http://www.theguardian.com/lifeandstyle/2012/nov/13/raymond-blanc-lambs-liver-food-poisoning
28. Morris S. Bloody marvellous: how did we fall in love with rare meat? [cited 2015 May 6]. http://www.independent.co.uk/life-style/food-and-drink/features/bloody-marvellous-how-did-we-fall-in-love-with-rare-meat-6294303.html
29. Winter G. Celebrity chefs are trying to make undercooked pork fashionable. But could it kill you? [cited 2015 May 6]. http://www.dailymail.co.uk/health/article-1202601/Celebrity-chefs-trying-make-undercooked-pork-fashionable-But-kill-you.html
30. Copping J, Malnick E. Chefs defy health inspectors over rare meat [cited 2015 May 6]. http://www.telegraph.co.uk/foodanddrink/10249764/Chefs-defy-health-inspectors-over-rare-meat.html.
31. Doward J. Chefs fight for the right to serve their pork pink [cited 2015 May 6]. http://www.theguardian.com/lifeandstyle/2013/sep/21/chefs-pork-pink
32. Fagge N. How health police are making rare steaks rarer: Prue Leith says officials are enforcing rules designed for factories to ensure meat is cooked through [cited 2015 May 6]. http://www.dailymail.co.uk/news/article-2396605/Prue-Leith-warns-rare-steak-rarer-restaurants-councils-demands.html
33. Black RE, Levine MM, Clements ML, Hughes TP, Blaser MJ. Experimental *Campylobacter jejuni* infection in humans. J Infect Dis. 1988;157:472–9. http://dx.doi.org/10.1093/infdis/157.3.472

34. Teunis P, Van Den Brandhof W, Nauta M, Wagenaar J, Van Den Kerkhof H, Van Pelt W. A reconsideration of the *Campylobacter* dose–response relation. Epidemiol Infect. 2005;133:583–92. http://dx.doi.org/10.1017/S0950268805003912
35. Kerridge T. Tom Kerridge's proper pub food. Bath (UK): Absolute Press; 2013. p. 49.
36. Oliver J. Jamie's kitchen. London: Penguin Books Limited; 2010. p. 204–5.
37. Strachan NJC, MacRae M, Thomson A, Rotariu O, Ogden ID, Forbes KJ. Source attribution, prevalence and enumeration of *Campylobacter* spp. from retail liver. Int J Food Microbiol. 2012; 153:234–6. http://dx.doi.org/10.1016/j.ijfoodmicro.2011.10.033

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Heat Wave–Associated Vibriosis, Sweden and Finland, 2014

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During summer 2014, a total of 89 *Vibrio* infections were reported in Sweden and Finland, substantially more yearly infections than previously have been reported in northern Europe. Infections were spread across most coastal counties of Sweden and Finland, but unusually, numerous infections were reported in subarctic regions; cases were reported as far north as 65°N, ≈100 miles (160 km) from the Arctic Circle. Most infections were caused by non-O1/O139 *V. cholerae* (70 cases, corresponding to 77% of the total, all strains were negative for the cholera toxin gene). An extreme heat wave in northern Scandinavia during summer 2014 led to unprecedented high sea surface temperatures, which appear to have been responsible for the emergence of *Vibrio* bacteria at these latitudes. The emergence of vibriosis in high-latitude regions requires improved diagnostic detection and clinical awareness of these emerging pathogens.

Vibrio species are among the most common gram-negative bacteria that inhabit surface waters throughout the world and are responsible for several severe infections in humans and animals (*1*). Infection usually begins after exposure to seawater or ingestion of raw or undercooked seafood (*2*,*3*). Several reports recently showed that human *Vibrio* illnesses are increasing worldwide; these illnesses include fatal acute diarrheal diseases, such as cholera, gastroenteritis, wound infections, and septicemia (*1*,*4*). Fatalities associated with *Vibrio* infections are more common in persons who are immunocompromised or who have underlying diseases or syndromes, such as immune disorders, diabetes, and HIV/AIDS, than in persons without these conditions. Critically, *Vibrio* bacteria grow preferentially in warm (>15°C), low salinity (<25 parts per thousand NaCl) seawater (*4*,*5*). Warming of low-salinity marine environments is likely to support larger numbers of *Vibrio* populations and consequently increase the risk for vibriosis. In this regard, during the past 2 decades, reported infections have increased that have spread poleward and in areas not usually associated with these bacteria, including temperate and cold regions, such as the US Pacific Northwest (*6*,*9*), South America (*7*,*8*), and northern Europe (*4*,*5*). We describe a highly unusual instance of a large number of *Vibrio* infections reported in high-latitude coastal counties in northern Europe during summer 2014.

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Materials and Methods

During winter 2014 and into the early spring 2015, we became aware of an unusual number of reported *Vibrio* infections in northern Europe. Colleagues at the European Centre for Disease Control relayed the initial information to the Centre for Environment, Fisheries and Aquaculture Science (Weymouth, UK) and the University of Bath (Bath, UK). The information suggested that an unprecedented number of *Vibrio* infections had been observed in Sweden and Finland during summer 2014 and that many cases were reported in high-latitude coastal counties.

To scrutinize cases of infection, we took several approaches. We initially contacted the Public Health Agency of Sweden (Stockholm, Sweden) and the National Institute for Health and Welfare (Helsinki, Finland), as well as other northern Europe reference laboratories, in December 2014. Although vibriosis is not regionally notifiable in Europe, Finland and Sweden maintain national databases of *Vibrio* infections. In Finland, *V. cholerae* is a notifiable infection, and isolates from persons with suspected infections are submitted to the reference laboratory for confirmation, serotyping, and PCR testing for the cholera toxin gene (*ctx*). Also, other *Vibrio* species (e.g., *V. vulnificus*, *V. parahaemolyticus*) may be sent to the reference laboratory for subsequent species-level confirmation. In Sweden, diarrhea with CTX-producing *V. cholera* O1 or O139 is a notifiable disease, as is infection with other *Vibrio* species, including *V. cholerae* not producing CTX that causes wound infections, septicemia, enteritis, and otitis. Isolates of *V. cholerae* are sent to the Public Health Agency of Sweden for serotyping and

confirmation of virulence factors, such as *ctx*, using appropriate molecular methods, such as PCR.

For cases identified in 2014, the geographic location of each reported infection was established (e.g., town or city where the patient was treated). Where possible, information relevant to disease transmission, such as possible water-associated activities, also was gathered; however, for many cases, this information was not available. Basic epidemiologic data on each case, including patient sex and age, was subsequently collated, as was the site of bacterial isolation (e.g., wound, ear, blood). The date the case was reported to regional authorities was determined, and for a subset of cases, data on the onset of reported symptoms also were established. To assess recent trends regarding infections, we collated *Vibrio* cases identified in Finland and Sweden from 2005 onward and omitted from analysis cases we suspected of being foreign-acquired.

To assess the possible role of extreme weather events on the emergence and dynamics of *Vibrio* disease in Finland and Sweden, we analyzed the epidemiologic data alongside long-term sea surface temperature (SST) records (HadISST [Hadley Centre Sea Ice and Sea Surface Temperature dataset] and ERSST [Extended Reconstructed Sea Surface Temperature dataset, v3b from the US National Oceanic and Atmospheric Administration (NOAA)] [*4*]). We used satellite-derived data to scrutinize temperature conditions and changes in the Baltic Sea area using NOAA's Optimum Interpolation v2 Daily SST Analysis dataset that integrates satellite SST data retrievals. NOAA data (baseline period of 30 years [1971–2000]) was used to determine anomalies from this dataset. We also scrutinized daily SST and SST anomaly retrieval data from 6 fixed positions in the Baltic Sea area, which included the transitional waters between southern Sweden and Denmark, the southeastern and mideastern Baltic coasts of Sweden, and Bay of Bothnia (northern Baltic) and southern coast of Finland. To assess the significance of climatologic data from summer 2014, we also used long-term oceanographic datasets to analyze SST. In situ SST was provided by the Finnish Meteorological Institute and was downloaded on November 14, 2014. We also used instrumental measurements of SST in coastal areas in the Baltic Sea area. We removed short-term fluctuations from the buoy data by applying a 1-hour wide median filter to the original dataset.

Statistical tests used to infer the relationship between maximum SST and annual *Vibrio* case occurrence were investigated by using a generalized linear model that assumed a quasi-Poisson error distribution (log link function) in R version 3.1.3 (http://www.R-project.org). We analyzed daily long-term SST and anomaly data (1981–2015) using a Welch *t* test (which enables analysis of the unbalanced size of the 2 datasets).

Results

A total of 89 *Vibrio* infections were reported in Sweden and Finland during the summer and autumn 2014, the largest yearly total number of cases, to our knowledge, identified in these countries. Infections were apparent across most Baltic coastal counties of Sweden and Finland. Numerous cases were reported at extreme subarctic regions, and as far north as >65°N, <100 miles (160 km) from the Arctic Circle. Reported infections began in July 2014 and peaked in August, before decreasing significantly in September (Figure 1). Infections were spread across persons of widely varying ages (range 3–93 years; median 36.2 years). In general, those infected were more commonly male (61 [67%] cases). One known fatality was noted: a *V. cholerae* non–O1/O139 infection reported in August 2014 from southern Sweden. Data on possible transmission was largely absent from the dataset from Finland; however, most cases in Sweden during 2014 occurred among persons who reported recreational exposure to seawater (e.g., the Baltic Sea) or lake water before infection (33 [78%] cases). Most (70 [77%]) infections were attributed to *V. cholerae* non–O1/O139; in 1 case, a *ctx*-negative O1 strain was reported. Other species reported were *V. alginolyticus* (3 cases), *V. parahaemolyticus* (4 cases), *V. vulnificus* (2 cases), *V. mimicus* (1 case), and unspecified *Vibrio* species (8 cases) (Table). Thirty-three (37%) infections were associated with ear or ear secretion isolations; however, for 17 (19%) of the 89 reported cases, *Vibrio* organisms were isolated directly from blood, suggesting more serious systemic disease progression.

The temporal and spatial distribution of reported cases corresponded closely with a highly anomalous heat wave in northern Finland and Sweden during July and August 2014, where SSTs in the northern Baltic exceeded all known long-term climatic and oceanographic records. A persistent and long-lasting period of high pressure occurred in northern Finland and Sweden beginning in May 2014, and this weather pattern persisted until mid-August. Concomitantly, SST in the Baltic Sea area was highly anomalous

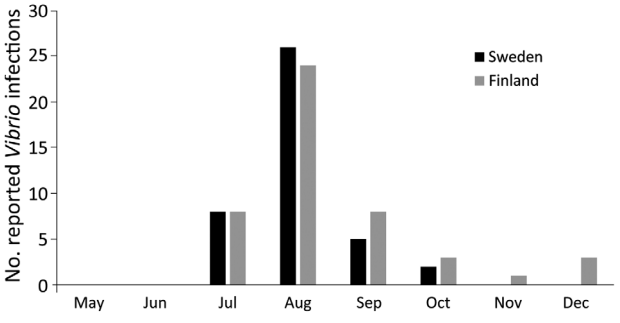

Figure 1. Monthly reported *Vibrio* infections in Sweden and Finland, May–December 2014. Beginning in July and increasing in August, reported infections spiked, corresponding with the heat wave in Scandinavia during that time.

Table. Relevant microbiologic data about *Vibrio* pathogens identified from reported cases, Finland and Sweden, 2014*

Species	Country of isolation (no. cases)
V. cholerae non-O1/O139	Finland (45), Sweden (25)
V. cholerae, O1, Inaba, El Tor, *ctx*–	Finland (1)
V. alginolyticus	Sweden (3)
V. parahaemolyticus	Sweden (4)
V. vulnificus	Sweden (2)
V. mimicus	Finland (1)
Vibrio spp.	Sweden (8)

**ctx*–, negative for the cholera toxin gene.

during July and August 2014; temperatures peaked toward the end of July. In some coastal regions, SSTs were ≈10°C higher than the long-term average, indicating the extreme severity of this anomaly (Figure 2). Across the northern Baltic Sea area, SSTs were several degrees Celsius warmer than had been reported since the early 1980s. SSTs across large swathes of the Baltic and the Gulf of Bothnia area, in particular, had SSTs >18°C for several weeks beginning in mid-July and ceasing in mid-August (Figure 3). SST's reported in the Gulf of Bothnia at the end of July were the most extreme reported during 1981–2016, exceeding 21.7°C on July 29, 2014, and with several days of temperatures >20°C. The observed SST anomaly during this period was also the largest ever seen in this dataset, encompassing almost 13,000 data points, with an anomaly of 9.79°C on July 29, 2014.

A statistical analysis between maximum SST and annual *Vibrio* cases using a generalized linear model showed that maximum SST explained a significant amount of the variability in cases (as determined by a significant reduction in the residual deviance from 120.55 to 42.16). The model predicted that, as the maximum SST increases, the number of annual number of cases also will increase significantly (β = 0.33002, SE = 0.08045, t = 4.102, p = 0.00343).

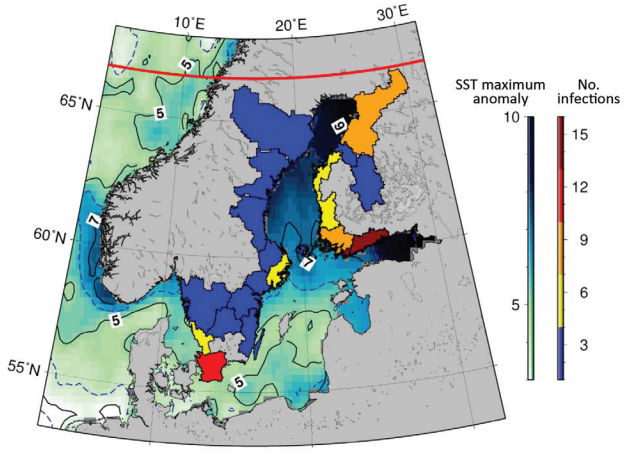

Figure 2. Location of reported *Vibrio* infections in coastal areas, Sweden and Finland, 2014. The number of infections coupled with the extreme SST anomaly, particularly in northern latitude areas, is particularly noteworthy. SST, sea surface temperature. Red line indicates the location of the Arctic Circle.

Discussion

Domestically acquired *Vibrio* infections are rare in northern Europe, and the spike in recorded cases of vibriosis reported in this region is particularly noteworthy. The cases in 2014 are the largest yearly total of reported *Vibrio* infections in Sweden and Finland, more than double the number of reported cases than in other recent years (Figure 4). In Sweden, 2014 was the warmest year on record since recordkeeping began in 1860; in Finland, 2014 was the second-warmest year on record (*10*,*11*). Across Finland, 50 days of hot summer weather (temperatures >25°C) were recorded during May–August, which is 14 days more than the long-term average (*10*). The large number of reported infections corresponded closely with an intense and northerly SST anomaly, suggesting that these unusual oceanographic and climatic conditions drove this episode of waterborne disease. A subsequent quantitative and statistical analysis of SST data from this region revealed 3 further observations: 1) the peak SSTs in late July 2014 were the most intense observed in the Bay of Bothnia; 2) the anomaly is the most intense in almost 35 years of climate data (1981–2015); and 3) the likelihood of such an event occurring based on recent climate data (1981–2015) is highly unlikely—the 2014 maximum observed temperature was significantly higher than the maximum expected based on the data for other years, and based on the distribution of maximum temperatures observed, a temperature this much higher than the mean would be expected only in 0.78% of years (once every 128 years).

Vibrio species such as *V. cholerae* grow preferentially in low-salinity warm water, and recreational exposure to water, which appears to have been responsible for a sizeable proportion of these reported infections, also increases substantially during heat waves. That 2014 followed several other recent heat wave years (e.g., 1994, 1997, 2003, 2006, and 2010), during which recorded domestically acquired *Vibrio* cases spiked in northern Europe (*4*,*5*), is particularly noteworthy. Previous epidemiologic analysis regarding the emergence of *Vibrio* infections in the region (*5*) indicated that sustained SSTs >18°C were a notable risk factor, significantly increasing reported cases. The relation between maximum SST and annual *Vibrio* case occurrence analyzed by using generalized linear model–based methods demonstrated similarly to previous studies in the region (*4*) that maximum temperature correlates highly with risk, and cooler years (e.g., 2005, 2007, and 2012) indicate lower levels of reported infections than heat wave years (e.g., 2006, 2010, and 2014). In our study, the observation that a sizeable proportion of described cases were reported in subarctic latitudes (>65°N) and within 100 miles (160 km)

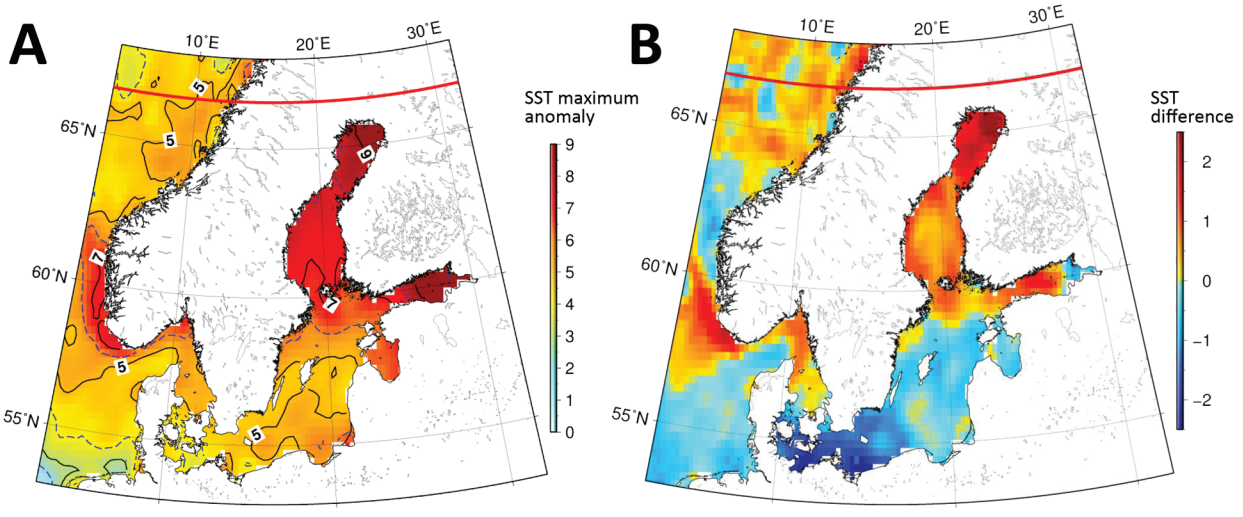

Figure 3. SST anomaly data for coastal areas of Sweden and Finland. A) Maximum SST anomalies during July and August 2014. The anomalies were substantially high throughout the region but especially in the northern Baltic Sea area. B) Differences between the maximum temperatures during 2014 and those during 1982–2013. SST, sea surface temperature.

of the Arctic Circle is striking. Ten *V. cholerae* infections were reported above 63°N, of which 6 cases were identified in the Oulo area (≈65°N). The cases recorded here are, to our knowledge, the most northerly reported instances of vibriosis documented, exceeding previous studies where cases have been reported at high latitudes, such as Alaska (*9*) and previously in northern Europe (*5*).

Disease data, such as those reported here, often are sporadic and usually grossly underreported. Likewise, a major limitation of our investigation was the lack of detailed trace-back epidemiologic data, which limits the assessment of exposure and subsequent risk. For many reported cases, data about prior exposure (e.g., specific information about the timing and location of recreational exposure to water) and subsequent routes of transmission were absent. However, almost without exception, cases from Finland and Sweden were reported in coastal rather than inland medical centers. Second, when prior transmission information was available from confirmed cases, most patients reported exposure to seawater in the days before symptom onset. These 2 factors, coupled with the striking climatic and oceanographic conditions during summer 2014, suggest that exposure to seawater was largely responsible for these episodes of disease emergence. The limitations underscore the need for a centralized system of surveillance and reporting. In the United States, the Centers for Disease Control and Prevention's COVIS (Cholera and Other Vibrio Illness Surveillance) maintains a national database of vibriosis that contained detailed epidemiologic and transmission route information (*12*). A similar centralized reporting, monitoring, and surveillance system would greatly enhance risk assessment and risk management of vibriosis in Europe. Across the region, and with the exception of toxigenic *V. cholerae* infection, vibriosis is not a notifiable disease (*5*). Given that these rare waterborne infections appear to have emerged and increased in northern Europe recently (*13*) (e.g., 1994, 2006, 2014), this event underlies the need for clinicians to identify possible exposure to seawater. This event is particularly relevant for patients who have a history of conditions where progression of vibriosis to systemic infection is more likely, including diabetes, immune disorders, and liver dysfunction.

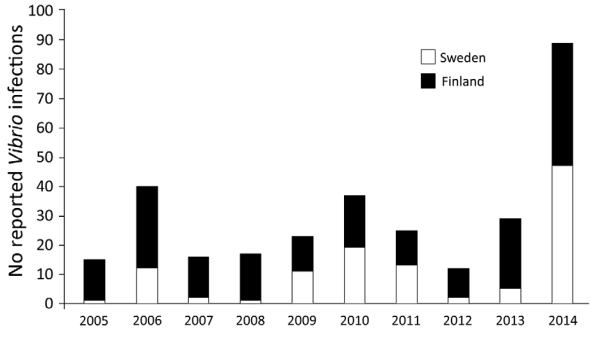

Figure 4. Total reported *Vibrio* infections in Finland and Sweden, 2005–2014. Foreign-acquired infections (where known) were omitted from the analyses. Epidemiologic data were gathered from public health agencies in Sweden and Finland (see Materials and Methods).

Climatic anomalies, such as the heat wave conditions during summer 2014 in northern Europe, appear to be responsible for restructuring the geographic distribution of waterborne infectious diseases and resulted in major and far reaching consequences for the identification, treatment, and management of these pathogens. The greater

number and intensity of large heat wave events in northern Europe during the past 20 years or so (1994, 1997, 2003, 2006, 2010, 2014) further highlights the need for improved epidemiology and reporting, coupled with enhanced diagnostic capability in clinical settings to manage and ameliorate risk.

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References

1. Vezzulli L, Colwell RR, Pruzzo C. Ocean warming and spread of pathogenic vibrios in the aquatic environment. Microb Ecol. 2013;65:817–25. http://dx.doi.org/10.1007/s00248-012-0163-2
2. Altekruse SF, Bishop RD, Baldy LM, Thompson SG, Wilson SA, Ray BJ, et al. *Vibrio* gastroenteritis in the US Gulf of Mexico region: the role of raw oysters. Epidemiol Infect. 2000;124:489–95. http://dx.doi.org/10.1017/S0950268899003714
3. Dechet AM, Yu PA, Koram N, Painter J. Nonfoodborne *Vibrio* infections: an important cause of morbidity and mortality in the United States, 1997-2006. Clin Infect Dis. 2008;46:970–6. http://dx.doi.org/10.1086/529148
4. Baker-Austin CL, Trinanes JA, Taylor NGH, Hartnell R, Siitonen A, Martinez-Urtaza J. Emerging *Vibrio* risk at high latitudes in response to ocean warming. Nat Clim Chang. 2012; 3:73–7. http://dx.doi.org/10.1038/nclimate1628.
5. Baker-Austin C, Stockley L, Rangdale R, Martinez-Urtaza J. Environmental occurrence and clinical impact of *Vibrio vulnificus* and *Vibrio parahaemolyticus*: a European perspective. Environ Microbiol Rep. 2010;2:7–18. http://dx.doi.org/10.1111/j.1758-2229.2009.00096.x
6. Centers for Disease Control and Prevention. Outbreak of *Vibrio parahaemolyticus* infections associated with eating raw oysters—Pacific Northwest, 1997. MMWR Morb Mortal Wkly Rep. 2008;47:457–62.
7. González-Escalona N, Cachicas V, Acevedo C, Rioseco ML, Vergara JA, Cabello F, et al. *Vibrio parahaemolyticus* diarrhea, Chile, 1998 and 2004. Emerg Infect Dis. 2005;11:129–31. http://dx.doi.org/10.3201/eid1101.040762
8. Martinez-Urtaza J, Huapaya B, Gavilan RG, Blanco-Abad V, Ansede-Bermejo J, Cadarso-Suarez C, et al. Emergence of Asiatic *Vibrio* diseases in South America in phase with El Niño. Epidemiology. 2008;19:829–37. http://dx.doi.org/10.1097/EDE.0b013e3181883d43
9. McLaughlin JB, DePaola A, Bopp CA, Martinek KA, Napolilli NP, Allison CG, et al. Outbreak of *Vibrio parahaemolyticus* gastroenteritis associated with Alaskan oysters. N Engl J Med. 2005;353:1463–70. http://dx.doi.org/10.1056/NEJMoa051594
10. Finnish Meteorological Institute. 2014 was Finland's second warmest year on record [press release] [cited 2015 Mar 17]. http://en.ilmatieteenlaitos.fi/press-release/42503751
11. Swedish Meteorological and Hydrological Institute. Swedish weather summary for the year 2014 [cited 2015 Mar 17]. http://www.smhi.se/en/news-archive/swedish-weather-summary-for-the-year-2014-1.83987
12. Newton A, Kendall M, Vugia DJ, Henao OL, Mahon BE. Increasing rates of vibriosis in the United States, 1996-2010: review of surveillance data from 2 systems. Clin Infect Dis. 2012;54(Suppl 5):S391–5. http://dx.doi.org/10.1093/cid/cis243
13. Vezzulli L, Brettar I, Pezzati E, Reid PC, Colwell RR, Höfle MG, et al. Long-term effects of ocean warming on the prokaryotic community: evidence from the vibrios. ISME J. 2012;6:21–30. http://dx.doi.org/10.1038/ismej.2011.89

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High Incidence of Chikungunya Virus and Frequency of Viremic Blood Donations during Epidemic, Puerto Rico, USA, 2014

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Chikungunya virus (CHIKV) caused large epidemics throughout the Caribbean in 2014. We conducted nucleic acid amplification testing (NAAT) for CHIKV RNA (n = 29,695) and serologic testing for IgG against CHIKV (n = 1,232) in archived blood donor samples collected during and after an epidemic in Puerto Rico in 2014. NAAT yields peaked in October with 2.1% of donations positive for CHIKV RNA. A total of 14% of NAAT-reactive donations posed a high risk for virus transmission by transfusion because of high virus RNA copy numbers (10^4–10^9 RNA copies/mL) and a lack of specific IgM and IgG responses. Testing of minipools of 16 donations would not have detected 62.5% of RNA-positive donations detectable by individual donor testing, including individual donations without IgM and IgG. Serosurveys before and after the epidemic demonstrated that nearly 25% of blood donors in Puerto Rico acquired CHIKV infections and seroconverted during the epidemic.

Chikungunya virus (CHIKV), a mosquitoborne, positive-sense RNA virus of the family *Togaviridae*, causes an acute febrile illness and severe polyarthralgia that can persist for months or years in some patients (*1*–*3*). Serious outcomes and deaths are rarely observed. However, newborns and other vulnerable populations are at risk for severe complications (*4*).

In late 2013, cases of CHIKV infection were reported in the French Collectivity of Saint Martin, which is part of the French Antilles (*5*), constituting the first instance of autochthonous transmissions of CHIKV in the Americas in the past century (*6*). In an immunologically naive population, CHIKV spread rapidly throughout the Caribbean region and beyond to most countries in the Western Hemisphere (*7*), including 11 autochthonous cases reported in Florida, USA, in September 2014 (*8*).

CHIKV has yet to be demonstrated to be transmissible by blood transfusion (*9*). However, this finding might result from difficulties in discriminating transfusion transmission from locally acquired mosquitoborne infection. Transfusion transmission is probable, given previous instances of laboratory-acquired infections and infection of healthcare workers by blood exposures (*10*). Asymptomatically infected persons can have viral loads >10^5 PFU/mL (*11*,*12*) and are a substantial risk for transfusion transmission.

Estimates of asymptomatic CHIKV infection vary widely. A recent study in Puerto Rico (*13*) confirmed previous estimates that 10%–25% of total infections are subclinical (*14*–*16*). However, other studies with the Asian genotype suggest that a greater proportion of cases might be asymptomatic or have only mild and transient symptoms (*17*,*18*). CHIKV infection can result in viral loads >10^8 PFU/mL (*19*). Thus, relatively high viral loads likely present in some presymptomatic donors might be a threat for transfusion transmission. Recently, a case of transfusion transmission of the related alphavirus Ross River virus, has been reported (*20*), stemming from transfusion of the erythrocyte component from a blood donor who reported symptoms of Ross River virus infection 2 days after donating blood.

To mitigate the theoretical risk for transmission, some blood collection organizations in regions with large CHIKV epidemics have suspended local blood collection, implemented nucleic acid amplification testing (NAAT) of erythrocyte and plasma donations for CHIKV RNA, and introduced pathogen-reduction technology for platelet components (*21*,*22*). To directly assess the threat that CHIKV poses to the blood supply, and given the absence of

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licensed NAAT for donor screening, we conducted NAAT surveys of blood donors in Puerto Rico during the 2014 epidemic and complementary serosurveys before and after the epidemic.

Materials and Methods

Human Subjects Research Approval

We performed retrospective testing of anonymous blood donor samples and minipools. The study was approved by the University of California, San Francisco Committee for Human Research.

Specimens

Creative Testing Solutions (Tempe, AZ, USA) retained, aliquoted, and archived at −70°C residual plasma from EDTA-anticoagulated blood collected in Puerto Rico and supplied for routine blood donor screening during the second half of 2014 and for a brief period during March 2015. Current molecular testing procedures at Creative Testing Solutions require that plasma samples be pooled into a minipool of 16 donor samples. Minipools prepared from blood donations in Puerto Rico were frozen during June 20–December 31, 2014. The sample set consisted of 1,667 minipools representing 26,672 individual donation samples from donors in Puerto Rico. Minipools were irreversibly stripped of their original labels and given a unique bar code that was linked only to month of collection.

In addition, 3,007 individual donor samples (IDS) were collected during the epidemic (September–November 2014), and ≈1,000 samples were saved per month. IDS were irreversibly stripped of all identifying information and given a unique bar code. Only basic demographic data (donor's age, race, sex, county of residence, and week of collection) were retained in a secure database. Anonymous minipools and individual donor samples were retained, aliquoted, frozen, and stored at −70°C.

Finally, we retained 1,031 individual donation samples obtained during March 1–9, 2015, for a postepidemic serosurvey. Demographic data, including the donor's age, sex, and zip code of residence, but not individual donor identifiers, were retained for these samples to enable analysis of serologic test results by using demographic strata.

Viral RNA Testing

We performed viral RNA testing by using a prototype real-time CHIKV/dengue virus (DENV) target-capture, transcription-mediated amplification (TC-TMA) assay (*12*) (Hologic, Inc., San Diego, CA, USA). Plasma samples (0.5 mL) were tested by using the fully automated Panther System (Hologic, Inc.), which performs target capture, amplification, and real-time detection in the presence of an internal control. We achieved detection by using single-stranded, fluorescent-labeled nucleic acid probes that were present during amplification of the target. The time for the fluorescent signal to reach a specified threshold was proportional to the starting CHIKV and DENV RNA concentrations. Target capture oligonucleotides, TMA primers, and detection probes hybridize with highly conserved regions of CHIKV or DENV RNA genomes and were designed to detect all 3 major CHIKV lineages and all 4 DENV types. We set the cutoff value for reactive specimens at 1,000 relative fluorescent units.

Estimated viral loads for CHIKV were calculated relative to the emergence time of the emitted fluorescence of a calibration curve generated by testing logarithmic dilutions of a CHIKV in vitro–synthesized transcript. ID-NAAT–reactive specimens were diluted 1:16 in defibrinated, delipidated, pooled plasma (SeraCare, Gaithersburg, MD, USA) to mimic minipool testing and tested by TC-TMA assay to assess whether donation samples detected by ID-NAAT would have been detectable by minipool NAAT (MP-NAAT).

We determined limits of detection (LODs) by using an in vitro transcript corresponding to each analyte and calculation by using Enterprise Guide 5.1 Probit analysis and the Normal model (SAS Institute, Cary, NC, USA). For DENV-1–4, the 50% LOD was 1.7–2.1 copies/mL, and the 95% LOD was 7.1–13.0 copies/mL in the IDS format. For CHIKV, the 50% LOD was 4.6 copies/mL, and the 95% LOD was 19.7 copies/mL in the IDS format. In 16-member minipools for DENV-1–4, the 50% LOD was from 27.2–33.6 copies/mL, and the 95% LOD was 116.8–208.0 copies/mL. For CHIKV, the 50% LOD was 73.6 copies/mL, and the 95% LOD was 315.2 copies/mL in the MP format.

Serologic Analysis

Plasma samples were tested for CHIKV IgM or IgG by using 2 ELISAs (Euroimmun US, LLC, Morris Plains, NJ, USA). These CHIKV ELISAs had specificities of 82% and 95% and sensitivities of 85% and 88% for IgM and IgG, respectively, when compared with those for 2 established in-house assays (*23*). Samples were diluted 1:100 and tested in duplicate according to the manufacturer's instructions. Sample-to-calibrator ratios were calculated. In validating the assay, we found that preepidemic samples (n = 201) yielded no strongly positive samples when the manufacturer's cutoff value >1.1 sample-to-calibrator ratio was used. However, 5 samples showed borderline reactivity (sample-to-calibrator ratios 1.13–1.37).

These 5 samples did not show positive results by reflex IgM testing, plaque-reduction neutralization testing (PRNT), or Western blot analysis when cell culture–propagated virus (strain 99659) was used as antigen. Testing of randomly chosen highly and moderately IgG-reactive samples from March 2015 by PRNT showed strong neutralization in all instances. Thus, the assay

does not appear to yield strongly reactive false-positive results, but might yield a small frequency (5/201, 2.5%) of low-level reactive false-positive results. Therefore, a new cutoff value was established by using mean sample-to-calibrator ratios of preepidemic samples plus 5 SDs (1.42). Testing of multiple IgG-negative samples from both sample sets by IgM ELISA (20 samples), PRNT (20 samples), and Western blot analysis (10 samples) did not yield any suspected false-negative results, which suggested that false-negative results were also not common.

Estimation of Detection Periods for MP-NAAT and IDS-NAAT

On the basis of the estimate for incidence of infection during the 2014 epidemic derived from serosurveys and MP-NAAT–positive results for the study period, we derived an estimate for duration of viremia detectable by the CHIKV TMA NAAT applied to minipools by using the approach of Busch et al. (*24*). We estimated the number of NAAT-positive donations in each minipool from minipool-testing results by using a program developed at the Centers for Disease Control and Prevention (Atlanta, GA, USA) (*25*). If T_i is the proportion of NAAT-positive donations in month i and P is seroprevalence of CHIKV at the end of the epidemic, then the TMA detection interval of CHIKV virus RNA (W) is estimated as

$$W = \frac{30 \sum_i T_i}{P}$$

Confidence limits for W were estimated by using a delta method estimate of the variance of W. Estimates for length of the individual donor sample-positive detection periods preceding and following the MP-NAAT–detectable period were derived from results of screening 3,007 individual donor samples by using ratios of samples detectable only by ID-NAAT that lacked IgG or contained IgG relative to the number of samples detectable at a dilution of 1:16. Confidence limits for these detection periods were derived by bootstrapping the assay results ratios (2/21) and (33/21) to obtain their variances, and then combining those with the variance associated with the estimate for the minipool detection period to obtain the variance of each of the 2 window estimates.

Results

Of 1,668 minipools tested, 1 was positive for DENV RNA, and 161 (9.7%) were positive for CHIKV RNA (Table 1). This finding indicates a minimum MP-NAAT–detectable infection rate of 0.6% (161 positive donations of 26,688 total donations), assuming only 1 of the 16 donations in each positive minipool was viremic. However, because the reactive minipool proportion peaked at 19.5% in September 2014 (Table 1), some pools would probably contain >1 viremic donation.

Individual donations comprising reactive minipools were not archived for further testing. Thus, we could not directly determine numbers of reactive IDS per reactive minipool. Therefore, we used a published algorithm (*25*) to estimate the proportion of donations that would contain CHIKV RNA at levels detectable by MP-NAAT (Table 1). This modification yielded an estimate for MP-NAAT detectable viremia of 0.65% for the overall season and an upper limit of 0.93%. The highest estimated proportion of MP-NAAT–detectable CHIKV RNA-positive donations was during September and October (1.34% and 1.31% of donations reactive for CHIKV RNA by MP-NAAT, respectively) (Table 1). This estimation represented a slightly delayed peak when compared with suspected and confirmed clinical cases reported in Puerto Rico (Figure 1).

Although not optimized to be quantitative, the TC-TMA assay provided approximate viral RNA copy numbers (Figure 2, panel A). Several minipools, particularly from early in the epidemic, had $>10^7$ copies/mL, although they were tested as a minipool, and thus effectively diluted 1:16. Of 161 reactive minipools, 125 had quantifiable viral loads. Remaining minipools had viral loads less than an estimated value of 0.5 log copies/mL (according to the calibration curve). The median viral load of 161 reactive minipools was 550 copies/mL (range <3.16 copies/mL–2.3×10^7 copies/mL). Donations from November and December had lower viral loads than donations from preceding months.

We also performed testing of archived IDS for CHIKV RNA for 3,007 donations collected in Puerto Rico during September–November 2014. We identified 56 confirmed positive donations, and ID-NAAT yields were 1.7%–2.1% for the 3 months tested (Table 2). When samples were

Table 1. Nucleic acid amplification testing for chikungunya virus in minipools of blood donations during a chikungunya epidemic, Puerto Rico, USA, 2014

Month	No. reactive minipools/no. tested (%)	Infection rate* (upper limit), %
June	0/106 (0.0)	0.0 (0.00)
July	8/193 (4.1)	0.26 (0.50)
August	26/293 (8.9)	0.58 (0.83)
September	51/262 (19.5)	1.34 (1.75)
October	57/299 (19.1)	1.31 (1.69)
November	12/243 (4.9)	0.32 (0.54)
December	7/272 (2.6)	0.16 (0.32)
Total	161/1,668 (9.7)	0.65 (0.93)

*In individual donors on the basis of minipools of 16 samples.

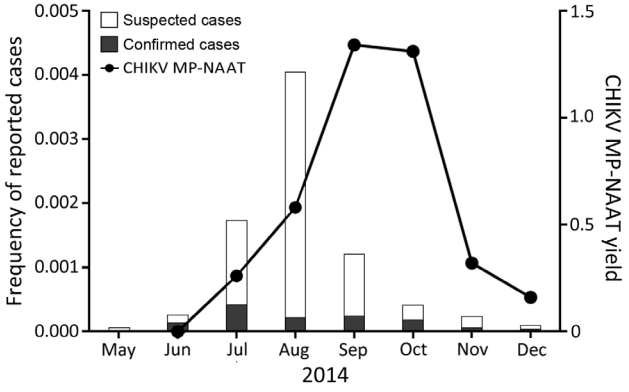

Figure 1. Estimated percentage of blood donations positive for chikungunya virus (CHIKV) RNA during a chikungunya epidemic, Puerto Rico, USA, 2014. CHIKV RNA-positive minipools of 16 donors were used to estimate the percentage of positive donations for the last 7 months of 2014. Estimates were made by using an algorithm for calculating infection rates from pooled data. Data from the Puerto Rico Department of Health for reported (suspected) and confirmed chikungunya case reports was used to transform data into estimated frequency of reported cases in a population in Puerto Rico of ≈3,548,400. MP-NAAT, minipool nucleic acid amplification testing.

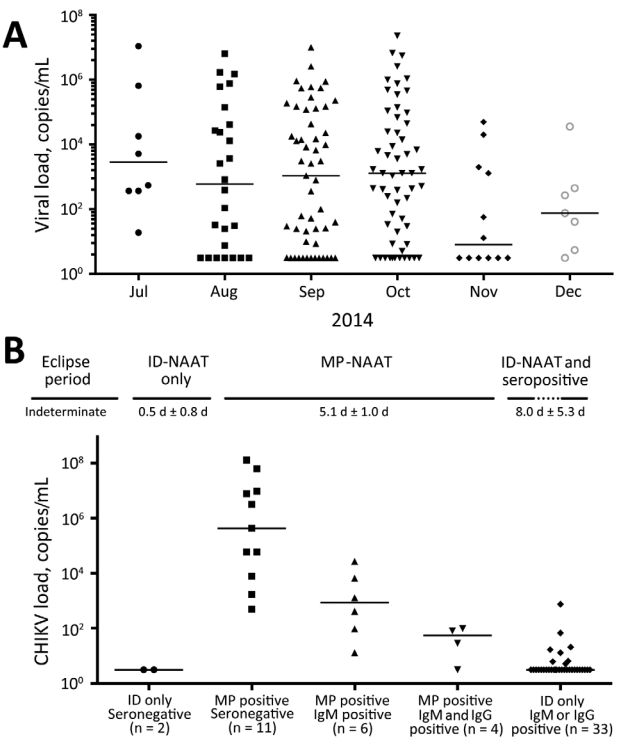

Figure 2. Viral loads for chikungunya virus (CHIKV) in blood donations during a chikungunya epidemic, Puerto Rico, USA, 2014. A) Positive minipool (MP) viral loads. Estimated viral loads (RNA copies/mL) were calculated for each reactive MP identified by using target capture transcription-mediated amplification (TC-TMA) during the epidemic. June 2014 (n = 106) is not plotted because of a lack of positive samples. Positive samples with unquantifiable viral loads are plotted as being at the limit of quantification (3.16 copies/mL) and were included in calculation of medians (horizontal bars). B) Individual donor (ID) viral loads for CHIKV. Estimated viral loads were calculated for each positive specimen identified by using TC-TMA during the 3 peak months of the epidemic. Positive samples with unquantifiable viral loads are plotted as being at the limit of quantification (3.16 copies/mL) and were included in calculation of medians (horizontal bars). Samples are arranged in order of projected time postinfection on the basis of predicted time course of acute infection (shown as estimated mean ±SD time intervals in days). ID only, samples positive by nucleic acid amplification testing (NAAT) but not positive for a 1:16 dilution mimicking minipooling. MP positive, samples positive by ID-NAAT and at a 1:16 dilution. Dynamics of acute infection with CHIKV (*26*) from the eclipse period (negative for virus RNA and IgM and IgG against CHIKV) to the end of infection (positive or negative for virus RNA and positive for IgM and IgG against CHIKV) is based on similar staging of dynamics of acute infection for other arboviruses (*27*) and approximate detection periods as described in the text.

diluted 1:16 to mimic minipools, proportions of RNA-positive samples detectable by MP-NAAT for September–November decreased to 0.4%–0.9%. Only 21 (37.5%) of 56 ID-NAAT–reactive specimens were reactive when tested for CHIKV RNA at a dilution of 1:16. Thus, 35 (62.5%) of 56 specimens would probably have been missed by routine MP-NAAT (Table 2). As expected, viral loads were low in donations reactive only by ID-NAAT. Only 8 of the ID-NAAT only–reactive samples had quantifiable viral loads (range 5.2–760 copies/mL) (Figure 2, panel B).

We performed assays to detect IgM and IgG in the 56 ID-NAAT–reactive specimens to characterize the relationship between development of IgG and IgM, viral load, and the ability of minipool testing to detect viremic donations (Table 2). Thirteen (23.2%) of 56 samples were seronegative; 2 were detectable only by ID-NAAT. These 2 samples are presumed to represent donors detected in the earliest stages of acute infection. The remaining 11 seronegative viremic donations had detectable viral loads (range 5 $\times 10^2$–1.3×10^8 copies/mL) (Figure 2, panel B), including 8 (14.3%) of 56 with viral loads $>10^4$ copies/mL. These samples were probably from donors who were near the peak of viremia, but still collected before seroconversion occurred.

Most CHIKV RNA-reactive samples were IgM positive (75%) and IgG positive (64%); 1 sample was IgM negative and IgG positive. Development of IgG titers is an inverse correlate of CHIKV RNA detection (*28*); of the IgG-reactive samples, only 4 (11.1%) of 36 were detectable by the less sensitive MP-NAAT. Viral loads of samples sorted on the basis of NAAT results (ID only vs MP-NAAT detectable) and serologic data demonstrate a typical profile of acute viral infection (Figure 2, panel B). The 43 viremic IgM-positive or IgG-positive donations had significantly lower viral loads (median <3.16 copies/mL) than 13 viremic seronegative donations (60,000 copies/mL; p<0.0001 by 2-tailed Mann-Whitney test). Although

similar proportions of ID-NAAT–positive samples were detected in November (1.7%) and September (1.8%), only 2 (11.8%) of 17 were seronegative in November compared with 6 (33.3%) of 18 in September, which suggested waning of the epidemic and a higher proportion of donations at the end of acute infection.

To estimate the incidence of CHIKV infection during the 2014 epidemic, we performed IgG serologic studies on blood donor specimens collected at the beginning of the epidemic (June 2014; preepidemic) and after the epidemic had subsided (March 2015; postepidemic). Collection was delayed until March to maximize detection of IgG seroconversion and to enable the maximum period for potential donors to recover from symptomatic infection, which would result in self-deferral, or deferral by the blood collection organization.

On the basis of IgG testing, we found that there were no unequivocally seroreactive samples in preepidemic samples (n = 201). In contrast, 241 (n = 1,031) postepidemic samples were strongly reactive (sample-to-calibrator ratio >2.5) (Figure 3). An additional indeterminate sample was positive by confirmatory testing with IgM ELISA, PRNT, and Western blot analysis. Thus, 242 (23.5%) of 1,031 samples were conservatively characterized as reactive (Figure 3).

Before we relabeled samples so that CHIKV testing was anonymous, basic demographic data were extracted for many of the specimens from March 2015 tested for seroreactivity (Table 3). No differences were observed in seropositivity rates between men and women. Persons 16–19 years of age had the highest rate of CHIKV recent infection; 40 (43.0%) of 93 of these persons were seropositive. In contrast, only 30 (18.3%) of 164 persons 40–49 years of age were seropositive.

We combined results from MP-NAAT and ID-NAAT screening and the serosurvey to estimate lengths of time that CHIKV RNA is detectable in serial stages of viremia in asymptomatic donors by MP-NAAT and ID-NAAT used in this study (Figure 2, panel B). We estimated that the length of the MP-NAAT–detectable phase for acute CHIKV infection in asymptomatic persons who donated blood was 5.1 days (confidence limit 4.1–6.0 days). By applying the ratios of seronegative ID-NAAT–only donations (2/56), MP-NAAT–detectable donations (21/56), and ID-NAAT–only seropositive donations (33/56), we estimated that there is a transient stage of low viral load infection preceding viremia detectable by MP-NAAT (0.5 days; confidence limit 0–1.3 days), whereas there is a relatively long stage of persistent viremia after seroconversion (8 days; confidence limit 2.7–13.3 days).

Discussion

Large epidemics of CHIKV infection occurred in the Caribbean Islands and in Central and South America over the past 2 years. Although >1.5 million confirmed and suspected cases have been reported (*29*), continued monitoring of CHIKV in these immunologically naive populations is needed for understanding population immunity and predicting dynamics of future epidemics. Using MP-NAAT, we estimated that 0.58% of individual blood donations were positive for CHIKV RNA during August 2014, a finding that is consistent with reported rates for Puerto Rico (*12*) and other Caribbean Islands (*22*).

As the 2014 epidemic in Puerto Rico continued, proportions of CHIKV viremia peaked in blood donors during September and October; >2% of donors were viremic, as indicated by individual donor NAAT results. During September and October, 1,440 chikungunya cases confirmed by real-time reverse transcription PCR were reported to the Puerto Rico Department of Health, which indicated sustained levels of CHIKV in the general population. However, reports of suspected chikungunya cases by month of illness onset received by the Puerto Rico Department of Health through passive surveillance peaked in August 2014 (Figure 1), which resulted in ≈14,000 suspected chikungunya cases in August, including 741 chikungunya cases confirmed by real-time reverse transcription PCR (Puerto Rico Department of Health, 2015, unpub. data).

Several factors probably affect the relative frequency of viremia and seroincidence of CHIKV in blood donors compared with clinical cases documented in the general population, including the focal nature of the epidemic in Puerto Rico during 2014 in relation to blood donor center

Table 2. Individual blood donations tested for chikungunya virus by nucleic acid amplification testing and serologic analysis during a chikungunya epidemic, Puerto Rico, USA, 2014*

Month	No. samples	No. ID-NAAT reactive samples	ID-NAAT yield, %	No. reactive at 1:16 dilution (MP-NAAT)	IgM reactive		IgG reactive	
					Total	IgM+/ID-only reactive	Total	IgG+/ID-only reactive
September	987	18	1.8	8	11	7†	8	7‡
October	1,010	21	2.1	9	15	10	14	10
November	1,010	17	1.7	4	16	12	14	12
Total	3,007	56	1.9	21	42	32†	36	32‡

*ID, individual donor; NAAT, nucleic acid amplification testing; MP, minipool.
†Includes 1 IgM-positive/IgG-negative ID-only positive specimen.
‡Includes one IgM-negative/IgG-positive ID-only positive specimen.

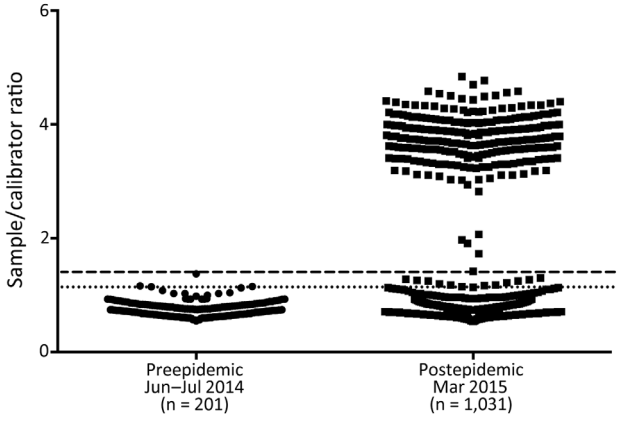

Figure 3. Serosurvey for chikungunya virus IgG in blood donations during a chikungunya epidemic, Puerto Rico, USA, 2014. Preepidemic samples collected in June and July 2014 were tested by using an IgG ELISA. A stringent cutoff value of mean + 5 SD (dashed line) was calculated from preepidemic samples. A less stringent cutoff value of mean + 3 SD (dotted line) was also calculated. These cutoff values were then applied to postepidemic samples collected in March 2015.

locations. It is also likely that many cases went unreported and that as the epidemic progressed many infected persons might not have sought medical care (*13*). Our finding that ≈25% of blood donors had serologic evidence of CHIKV infection after the 2014 epidemic supports these suggestions. Given a population of >3.5 million, and assuming that blood donors are representative of the total population of Puerto Rico with respect to risk for arbovirus transmission, a seroincidence of 23.5% would suggest that >800,000 persons were infected in Puerto Rico during the 2014 epidemic.

Blood safety protocols in place during the study included a Puerto Rico Departmen t of Health requirement for questioning of donors concerning symptoms in the month preceding donation and passive reporting of postdonation febrile illness. Thus, in the absence of specific NAAT screening, asymptomatic donors are likely to result in most viremic donations (*30*).

It is not clear whether asymptomatic infection is correlated with lower viremia levels, and thus would decrease the likelihood of transfusion transmission. However, similar to previous findings (*12*), many presumably asymptomatic donors in our study had viral loads comparable with those for symptomatic patients (*11*,*19*), including some viral loads >10^8 copies/mL. Most donations with low viral loads were IgM positive, which indicates recent acute infections. The proportion of these viremic specimens increased as the epidemic waned, and the percentage of ID-NAAT–only samples increased from 56% in September to 77% in November. Lower average viral copy numbers were also observed in November and December by testing of minipools. Furthermore, all RNA-positive donors in November were seropositive compared with only 78% of NAAT-reactive donors in September.

We estimate that the RNA-detectable window for MP-NAAT was 5.1 days. This value matches viremic periods observed for experimentally infected nonhuman primates (*31*) but is somewhat shorter than estimates for symptomatic patients of 1–2 days before disease onset and 8 days postonset (*9*,*11*,*32*,*33*). This finding is probably caused by a loss in the ability to detect viremia at the 1:16 dilution inherent in creating minipools, but might be a reflection that this study was limited to asymptomatic persons who donated blood. In addition, we calculated a relatively short ramp-up period before MP-NAAT–detectable viremia (0.5 days) and a longer low-level (MP-NAAT negative) viremia at the end of acute infection after seroconversion (8 days). Nevertheless, the 5-day MP-NAAT–detectable period for high-titer viremia is probably the most infectious period in terms of transfusion transmission and transmission to mosquitoes.

The overall threat CHIKV poses to the blood supply remains an open question that requires urgent attention, including in the continental United States, given the risk for travel-acquired and autochthonous transmission. In the absence of routine NAAT for CHIKV, and in regions where pathogen-reduction technology is not implemented, the largest threat is probably from donors with high viral

Table 3. Demographic characteristics of blood donors tested for chikungunya virus during a chikungunya epidemic, Puerto Rico, USA, 2014

Characteristic	No. (%) nonreactive for IgG, n = 786*	No. (%) reactive for IgG, n = 242*	Total, n = 1,031*	Odds ratio (95% CI)
Sex				
F	235 (75.81)	75 (24.19)	310	1.00
M	348 (74.95)	117 (25.05)	567	1.05 (0.75–1.47)
Age, y				
16–19	53 (56.99)	40 (43.01)	93	1.00
20–29	139 (81.29)	32 (18.71)	171	0.31 (0.17–0.55)
30–39	119 (79.33)	31 (20.67)	150	0.35 (0.19–0.62)
40–49	134 (81.71)	30 (18.29)	164	0.30 (0.16–0.54)
50–59	90 (70.54))	38 (29.46)	129	0.55 (0.31–0.97)
60–78	49 (70.00)	21 (30.00)	69	0.57 (0.29–1.10)

*Some specimens did not have complete demographic data.

loads who have not fully seroconverted because it can be assumed that donors with neutralizing IgG responses have a lower probability of transmitting an infectious dose to a recipient.

Although convalescent-phase serum is protective in animal studies (*34*), the ability of IgM and IgG in viremic donors to mitigate CHIKV transfusion transmission requires further study. Likewise, if viral RNA screening is introduced, studies will be needed to evaluate the relative usefulness of ID versus MP-NAAT. In screening of 3,007 individual donations, we identified 7 viremic donors with only IgM responses. However, only 1 of these donors had viremia detected only by ID-NAAT. We also identified 2 seronegative donors who showed reactivity by ID-NAAT, but not minipool testing. Whether blood components from these donations, together with specimens in the so-called eclipse phase between acquisition of infection and detectable ID-NAAT reactivity, are infectious remains unanswered.

In summary, our results indicated a sizable proportion of blood donors had detectable CHIKV RNA during the chikungunya epidemic in Puerto Rico in 2014. Several donations with high viremias were negative for IgM and IgG, which suggested that donors were in the peak phase of acute infection and highlights the risk for transfusion transmission. However, most viremic donations had low levels of viral RNA and were seropositive, which suggests recent subclinical infection and low risk for infectivity. However, these donors were healthy enough to donate blood. Finally, serosurveys before and after peak epidemic months showed that ≈25% of blood donors in Puerto Rico acquired CHIKV during the 2014 epidemic. On the basis of findings of this study, we are now conducting further investigations to determine the risk for transfusion transmission of CHIKV by virus RNA–positive transfusions and outcomes of infection in recipients.

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References

1. Charrel RN, de Lamballerie X, Raoult D. Chikungunya outbreaks: the globalization of vectorborne diseases. N Engl J Med. 2007;356:769–71. http://dx.doi.org/10.1056/NEJMp078013
2. Pialoux G, Gauzere BA, Jaureguiberry S, Strobel M. Chikungunya, an epidemic arbovirosis. Lancet Infect Dis. 2007;7:319–27. http://dx.doi.org/10.1016/S1473-3099(07)70107-X
3. Powers AM, Logue CH. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. J Gen Virol. 2007;88:2363–77. http://dx.doi.org/10.1099/vir.0.82858-0
4. Rampal, Sharda M, Meena H. Neurological complications in chikungunya fever. J Assoc Physicians India. 2007;55:765–9.
5. Cassadou S, Boucau S, Petit-Sinturel M, Huc P, Leparc-Goffart I, Ledrans M. Emergence of chikungunya fever on the French side of Saint Martin Island, October to December 2013. Euro Surveill. 2014;19:pii: 20752. http://dx.doi.org/10.2807/1560-7917.ES2014.19.13.20752
6. Halstead SB. Reappearance of chikungunya, formerly called dengue, in the Americas. Emerg Infect Dis. 2015;21:557–61. http://dx.doi.org/10.3201/eid2104.141723
7. Rolph MS, Foo SS, Mahalingam S. Emergent chikungunya virus and arthritis in the Americas. Lancet Infect Dis. 2015;15:1007–8. http://dx.doi.org/10.1016/S1473-3099(15)00231-5
8. Kendrick K, Stanek D, Blackmore C.; Centers for Disease Control and Prevention. Notes from the field: transmission of chikungunya virus in the continental United States—Florida, 2014. MMWR Morb Mortal Wkly Rep. 2014;63:1137.
9. Petersen LR, Epstein JS. Chikungunya virus: new risk to transfusion safety in the Americas. Transfusion. 2014;54:1911–5. http://dx.doi.org/10.1111/trf.12790
10. Cordel H, Quatresous I, Paquet C, Couturier E. Imported cases of chikungunya in metropolitan France, April 2005–February 2006. Euro Surveill. 2006;11:E060420.3.
11. Appassakij H, Khuntikij P, Kemapunmanus M, Wutthanarungsan R, Silpapojakul K. Viremic profiles in asymptomatic and symptomatic chikungunya fever: a blood transfusion threat? Transfusion. 2013;53:2567–74. http://dx.doi.org/10.1111/j.1537-2995.2012.03960.x
12. Chiu CY, Bres V, Yu G, Krysztof D, Naccache SN, Lee D, et al. Genomic assays for identification of chikungunya virus in blood donors, Puerto Rico, 2014. Emerg Infect Dis. 2015;21:1409–13. http://dx.doi.org/10.3201/eid2108.150458
13. Sharp TM, Roth NM, Torres J, Ryff KR, Perez Rodriguez NM, Mercado C, et al. Chikungunya cases identified through passive surveillance and household investigations—Puerto Rico, May 5–August 12, 2014. MMWR Morb Mortal Wkly Rep. 2014;63:1121–8.
14. Sissoko D, Moendandze A, Malvy D, Giry C, Ezzedine K, Solet JL, et al. Seroprevalence and risk factors of chikungunya virus infection in Mayotte, Indian Ocean, 2005–2006: a population-based survey. PLoS ONE. 2008;3:e3066. http://dx.doi.org/10.1371/journal.pone.0003066
15. Moro ML, Gagliotti C, Silvi G, Angelini R, Sambri V, Rezza G, et al. Chikungunya virus in North-Eastern Italy: a seroprevalence survey. Am J Trop Med Hyg. 2010;82:508–11. http://dx.doi.org/10.4269/ajtmh.2010.09-0322
16. Queyriaux B, Simon F, Grandadam M, Michel R, Tolou H, Boutin JP. Clinical burden of chikungunya virus infection. Lancet Infect Dis. 2008;8:2–3. http://dx.doi.org/10.1016/S1473-3099(07)70294-3
17. Nakkhara P, Chongsuvivatwong V, Thammapalo S. Risk factors for symptomatic and asymptomatic chikungunya infection. Trans R Soc Trop Med Hyg. 2013;107:789–96. http://dx.doi.org/10.1093/trstmh/trt083
18. Yoon IK, Alera MT, Lago CB, Tac-An IA, Villa D, Fernandez S, et al. High rate of subclinical chikungunya virus infection and association of neutralizing antibody with protection in a prospective cohort in the Philippines. PLoS Negl Trop Dis. 2015;9:e0003764. http://dx.doi.org/10.1371/journal.pntd.0003764
19. Chow A, Her Z, Ong EK, Chen JM, Dimatatac F, Kwek DJ, et al. Persistent arthralgia induced by chikungunya virus infection is

associated with interleukin-6 and granulocyte macrophage colony-stimulating factor. J Infect Dis. 2011;203:149–57. http://dx.doi.org/10.1093/infdis/jiq042
20. Hoad VC, Speers DJ, Keller AJ, Dowse GK, Seed CR, Lindsay MD, et al. First reported case of transfusion-transmitted Ross River virus infection. Med J Aust. 2015;202:267–70. http://dx.doi.org/10.5694/mja14.01522
21. Rasonglès P, Angelini-Tibert MF, Simon P, Currie C, Isola H, Kientz D, et al. Transfusion of platelet components prepared with photochemical pathogen inactivation treatment during a chikungunya virus epidemic in Ile de La Reunion. Transfusion. 2009;49:1083–91. http://dx.doi.org/10.1111/j.1537-2995.2009.02111.x
22. Gallian P, de Lamballerie X, Salez N, Piorkowski G, Richard P, Paturel L, et al. Prospective detection of chikungunya virus in blood donors, Caribbean 2014. Blood. 2014;123:3679–81. http://dx.doi.org/10.1182/blood-2014-03-564880
23. Prat CM, Flusin O, Panella A, Tenebray B, Lanciotti R, Leparc-Goffart I. Evaluation of commercially available serologic diagnostic tests for chikungunya virus. Emerg Infect Dis. 2014;20:2129–32. http://dx.doi.org/10.3201/eid2012.141269
24. Busch MP, Wright DJ, Custer B, Tobler LH, Stramer SL, Kleinman SH, et al. West Nile virus infections projected from blood donor screening data, United States, 2003. Emerg Infect Dis. 2006;12:395–402. http://dx.doi.org/10.3201/eid1205.051287
25. Biggerstaff BJ. Confidence intervals for the difference of two proportions estimated from pooled samples. Journal of Agricultural, Biological, and Environmental Statistics. 2008;13:478–96. http://dx.doi.org/10.1198/108571108X379055
26. Schwartz O, Albert ML. Biology and pathogenesis of chikungunya virus. Nat Rev Microbiol. 2010;8:491–500. http://dx.doi.org/10.1038/nrmicro2368
27. Busch MP, Kleinman SH, Tobler LH, Kamel HT, Norris PJ, Walsh I, et al. Virus and antibody dynamics in acute West Nile virus infection. J Infect Dis. 2008;198:984–93. http://dx.doi.org/10.1086/591467
28. Prince HE, Seaton BL, Matud JL, Batterman HJ. Chikungunya virus RNA and antibody testing at a national reference laboratory since the emergence of chikungunya virus in the Americas. Clin Vaccine Immunol. 2015;22:291–7. http://dx.doi.org/10.1128/CVI.00720-14
29. Pan American Health Organization. Number of reported cases of chikungunya fever in the Americas—epidemiological week 45, 2015 [cited 2016 Apr 4]. http://www.paho.org/hq/index.php?option=com_docman&task=doc_download&Itemid=270&gid=32212&lang=en.
30. Appassakij H, Promwong C, Rujirojindakul P, Wutthanarungsan R, Silpapojakul K. The risk of blood transfusion-associated chikungunya fever during the 2009 epidemic in Songkhla Province, Thailand. Transfusion. 2014;54:1945–52. http://dx.doi.org/10.1111/trf.12575
31. Messaoudi I, Vomaske J, Totonchy T, Kreklywich CN, Haberthur K, Springgay L, et al. Chikungunya virus infection results in higher and persistent viral replication in aged rhesus macaques due to defects in anti-viral immunity. PLoS Negl Trop Dis. 2013;7:e2343. http://dx.doi.org/10.1371/journal.pntd.0002343
32. Chusri S, Siripaitoon P, Silpapojakul K, Hortiwakul T, Charernmak B, Chinnawirotpisan P, et al. Kinetics of chikungunya infections during an outbreak in Southern Thailand, 2008–2009. Am J Trop Med Hyg. 2014;90:410–7. http://dx.doi.org/10.4269/ajtmh.12-0681
33. Lanciotti RS, Kosoy OL, Laven JJ, Panella AJ, Velez JO, Lambert AJ, et al. Chikungunya virus in US travelers returning from India, 2006. Emerg Infect Dis. 2007;13:764–7. http://dx.doi.org/10.3201/eid1305.070015
34. Couderc T, Khandoudi N, Grandadam M, Visse C, Gangneux N, Bagot S, et al. Prophylaxis and therapy for chikungunya virus infection. J Infect Dis. 2009;200:516–23. http://dx.doi.org/10.1086/600381

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Tropheryma whipplei as a Cause of Epidemic Fever, Senegal, 2010–2012

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The bacterium *Tropheryma whipplei*, which causes Whipple disease in humans, is commonly detected in the feces of persons in Africa. It is also associated with acute infections. We investigated the role of *T. whipplei* in febrile patients from 2 rural villages in Senegal. During June 2010–March 2012, we collected whole-blood finger-prick samples from 786 febrile and 385 healthy villagers. *T. whipplei* was detected in blood specimens from 36 (4.6%) of the 786 febrile patients and in 1 (0.25%) of the 385 apparently healthy persons. Of the 37 *T. whipplei* cases, 26 (70.2%) were detected in August 2010. Familial cases and a potential new genotype were observed. The patients' symptoms were mainly headache (68.9%) and cough (36.1%). Our findings suggest that *T. whipplei* is a cause of epidemic fever in Senegal.

Determining the etiologic causes of febrile illness in tropical settings provides public health and local community benefits. In the context of a decline in malaria cases in many parts of sub-Saharan Africa, the few studies that have been conducted in recent years to analyze the burden of bacterial infections used traditional blood cultures and identified typhoid fever and *Streptococcus pneumoniae* as the leading documented causes of nonmalarial bloodstream infections (*1–3*). However, this method does not enable the identification of intracellular organisms, and most causes of fever remain unknown. In 2008, we initiated a study of the etiologies of fevers of unknown origin in Africa, particularly in Senegal. Our preliminary studies showed the presence of previously known pathogenic microorganisms, such as *Borrelia crocidurae*, *Rickettsia felis*, *R. conorii*, and *Coxiella burnetii*, and the unexpected presence of *Tropheryma whipplei* (*4–9*).

T. whipplei was first considered to be an uncommon bacterium that causes Whipple disease, a rare chronic disease (*10*). However, *T. whipplei* is in fact a common bacterium associated with various conditions, such as acute infections (pneumonia and gastroenteritis) and chronic infections (classic Whipple disease and other infections without digestive involvement, including endocarditis and encephalitis) (*10–19*). *T. whipplei* can also be carried in human feces and, less commonly, in the saliva (*20–23*); carriage prevalence varies by the age and exposure of the population and by geographic area (*21–30*).

T. whipplei is highly prevalent in rural Senegal, where carriage rates reach 75% among children <2 years of age, and overall seroprevalence is 72% (*21–26*). In our preliminary study in Senegal, which was conducted in 2 villages (Dielmo and Ndiop) during December 2008–July 2009, we detected *T. whipplei* bacteremia in 6.4% of the analyzed specimens (*8*). Bacteremia was significantly associated with cough, but no link to feces carriage was observed (*8*). However, our study had several limitations, such as a small number of febrile patients, no local control group of afebrile persons, and a short study period. In this same area, we recently showed that humans comprise the only source of *T. whipplei* among the populations in whom the bacterium is highly prevalent. Moreover, our findings showed that limited access to toilets and exposure to human feces was associated with the high prevalence of *T. whipplei*, suggesting that these conditions may facilitate fecal–oral transmission of the bacterium (*31*). To better characterize *T. whipplei* bacteremia, we extended our analysis, beginning in 2010, in this same area of rural Senegal to include the collection of >1,000 blood samples from healthy persons and ambulatory patients with acute fever.

Materials and Methods

We conducted the study during June 2010–March 2012 in Senegal's rural Sine-Saloum area, a dry sahelian ecosystem with 2 typical seasons: dry (November–May) and rainy (June–October). We obtained written consent for every person included in the study. The National Ethics Committee of Senegal approved the study.

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Participants

Study participants included 786 febrile patients at the healthcare center for the villages of Dielmo and Ndiop; 78% of the patients were <15 years of age, and the sex ratio was 1:1. For all patients with fever (defined as axillary temperature of >37.5°C), we conducted a medical examination, completed a questionnaire, and collected a whole-blood finger-prick sample (200-μL [4 drops]) (*8*). In parallel, we collected blood samples from a control group of 385 healthy, afebrile villagers; 62.5% of these study participants were <15 years of age, and the sex ratio was 1:1.

Molecular Analyses

DNA Extraction

For DNA extraction, we used a BioRobot EZ1 Workstation (QIAGEN, Courtaboeuf, France) according to the manufacturer's instructions. Extraction was performed in Senegal, and specific quantitative real-time PCR (qPCR) was performed in France.

Specific qPCR

We used a 7900HT-thermocycler (Applied Biosystems, Foster City, CA, USA) with the QuantiTect-Probe PCR Kit (QIAGEN) to perform qPCR. First, we analyzed specimens for *T. whipplei* by using the primer pair Twhi3F (5′-TTG TGT ATT TGG TAT TAG ATG AAA CAG-3′)/Twhi3R (5′-CCC TAC AAT ATG AAA CAG CCT TTG-3′) and the specific Twhi3 probe (6-FAM-GGG ATA GAG CAG GAG GTG TCT GTC TGG-TAMRA). For specimens with positive results, we ran a second, confirmatory qPCR with the Twhi2F (5′-TGA GGA TGT ATC TGT GTA TGG GAC A-3′)/Twhi2R (5′-TCC TGT TAC AAG CAG TAC AAA ACA AA-3′) primer pair and the specific Twhi2 probe (6-FAM-GAG AGA TGG GGT GCA GGA CAG GG-TAMRA) (*8*,*21*). To validate the assays, we included positive (*T. whipplei*) and negative (PCR mix) controls in each run, as previously reported (*8*,*21*).

We considered samples to be *T. whipplei*–positive if qPCR results for the 2 specific genes were positive at a log-based fluorescence cycle threshold (C_t) of <38. We used qPCR for the β-actin housekeeping gene, as previously described (*7*), to check the quality of DNA handling and blood specimen extraction; only positive samples were considered reliable.

Genotyping

We performed genotyping of *T. whipplei* as previously described (*32*). We attempted to amplify and sequence each of 4 multispacer sequences (TW133, ProS, SecA, and Pro184) from positive specimens. When sequences were obtained, we compared them with those available in the GenBank database and our internal laboratory database to determine their corresponding genotype.

Statistical Analyses

We performed statistical analyses by using Epi Info 6 software (http://www.cdc.gov/epiinfo/index.html); results with $p<0.05$ were considered statistically significant. The corrected χ^2 test or the Fisher exact test was used where indicated.

Results

Prevalence of *T. whipplei* Bacteremia

A total of 786 febrile patients and 385 healthy controls were included in the study, among whom 36 (4.6%) and 1 (0.25%), respectively, were positive for *T. whipplei* DNA ($p<0.00007$). The positive control participant was a 13-year-old boy who had low concentrations of *T. whipplei* DNA (C_t of 36.85 and 37.99). The C_t for febrile patients ranged from 26.10 to 36.41 (mean ± SD 33.40 ± 2.53).

Age Distribution

The prevalence of *T. whipplei* bacteremia was 4% (3/75) for febrile patients <12 months of age, 4.8% (12/250) for those 1–3 years of age, 4.2% (5/119) for those 4–6 years of age, 5.4% (9/167) for those 7–15 years of age, 2.7% (2/75) for those 16–29 years of age, and 5.2% (5/97) for those ≥30 years of age. Age data were not available for 3 patients. No significant differences in age distribution were observed.

Clinical Manifestations

Clinical data were available for 786 febrile patients (Table 1). The main symptoms in the 36 *T. whipplei*–positive febrile patients were headache (23 [68.9%]), cough (13 [36.1%]), rhinorrhea (8 [22.2%]), nausea (5 [13.9%]), vomiting (4 [11.1%]), and diarrhea (3 [8.3%]). No significant clinical differences were observed by C_t level.

Seasonality

All 36 *T. whipplei* cases detected among the 786 febrile patients were in the 466 patients tested during the June–October rainy season; no cases were detected among the 320 febrile patients sampled during the November–May dry season ($p = 0.0000001$). Moreover, 33 (92%) of these 36 cases were diagnosed during the 2010 rainy season, and the other 3 were diagnosed during August 2011 (2 cases) and October 2011 (1 case) (Figure). The highest prevalence of *T. whipplei* bacteremia cases was detected during August, when 28 (30%) of 93 febrile patients were found to be positive (19 [28%] of 73 patients in Dielmo and 9 [45%] of 20 patients in Ndiop). In fact, the data were affected by the high prevalence of cases observed in August 2010, which seemed to be indicative of an outbreak.

Table 1. Clinical manifestations observed in 786 febrile *Tropheryma whipplei*–positive or –negative patients in 2 villages, Dielmo and Ndiop in the Sine-Saloum area of Senegal, June 2010–March 2012.

Clinical manifestation	*T. whipplei*–positive patients, no. (%), n = 36	*T. whipplei*–negative patients, no. (%), n = 750	p value by χ^2 test
Headache	23 (68.9)	439 (58.5)	0.52
Arthralgia	0	19 (2.5)	0.46
Myalgia	0	53 (7.0)	0.07
Diarrhea	3 (8.3)	39 (5.2)	0.3
Vomiting	4 (11.1)	94 (12.5)	0.56
Nausea	5 (13.9)	100 (13.3)	0.53
Abdominal pain	1 (2.8)	21 (2.8)	0.68
Cough	13 (36.1)	274 (36.5)	0.95
Expectoration	2 (5.6)	42 (5.6)	0.67
Otalgia	1 (2.8)	28 (3.7)	0.61
Otorrhea	0	2 (0.3)	0.91
Rhinorrhea	8 (22.2)	229 (30.5)	0.28
Burning urination	1 (2.8)	33 (4.4)	0.53
Rash	0	10 (1.3)	0.62
Meningeal signs	2 (5.5)	25 (3.3)	0.35

In July 2010, *T. whipplei* infection was detected in 2 febrile patients, an 18-year-old boy in Dielmo (case detected July 24) and a 15-year-old girl in Ndiop (case detected July 27). In August 2010, a total of 29 febrile patients from Dielmo were tested; 17 (58.5%) of the 29 patients had samples (18 total samples) positive for *T. whipplei* bacteremia. During the same month in Ndiop, 9 (69%) of 13 febrile patients had positive samples. In September 2010, 2 patients were positive in Dielmo and 1 in Ndiop, and in October, 2 patients were positive in Dielmo and none in Ndiop. For almost 1 year, all specimens from febrile patients were negative for *T. whipplei*. Then, in August 2011, only 2 patients were positive in Dielmo, and in October 2011, only 1 patient was positive in Ndiop.

Treatment and Follow-Up

Data about antimicrobial drug therapy was available for 33 patients, 23 of whom benefited from treatment with amoxicillin (18 patients), metronidazole (3 patients), or cotrimoxazole (2 patients). In Dielmo, 24 specimens from 23 patients were positive for *T. whipplei*; 1 patient was sampled twice 15 days apart, and both specimens were positive. For 17 patients, blood specimens were also sampled during other febrile episodes. Nine specimens from 5 patients were sampled from 15 days to 13 months before the positive sample was detected, and 43 specimens from 17 patients were sampled from 3 weeks to 16 months after the positive sample was detected; all of these samples were negative. Moreover, our previously published data (*8*) included test results for a 4-year-old boy who was diagnosed with *T. whipplei* bacteremia in January 2009 (19 months before August 2010). Four other blood specimens from this patient were tested 1 month before (1 sample) or 4, 11, and 15 months after (3 samples) the positive specimen was detected, and all were negative for *T. whipplei*.

In Ndiop, 12 specimens from 12 patients were positive. For 8 of these patients, blood specimens were sampled during other febrile episodes. The specimen for 1 patient was sampled 1 month before the positive sample, and 9 specimens from 6 patients were sampled from 7 weeks to 18 months after the positive samples; all of these specimens were negative. No data were available for these patients about antibody response against *T. whipplei*.

Genotyping

Because of the lack of specimens available for genotyping and the low sensitivity of genotyping, we could obtain multispacer sequences for only 8 patients at the time of the 2010 peak in *T. whipplei* bacteremia cases (Table 2). The *T. whipplei* genotype corresponds to the concatenation of the 4 spacers (TW133-ProS-SecA-Pro184); however, TW133 sequencing was not successful, so the corresponding spacer was not available (NA) for any of the patients. ProS sequence was obtained for 5 patients, SecA for 6 patients, and Pro184 for all patients. For 4 patients, 3 spacers were available, enabling the detection of the same multispacer sequence combination (NA-7-2-1) for the 4 patients. For another 4 patients, 2 spacers were available, enabling the detection of the NA-7-NA-1 combination for

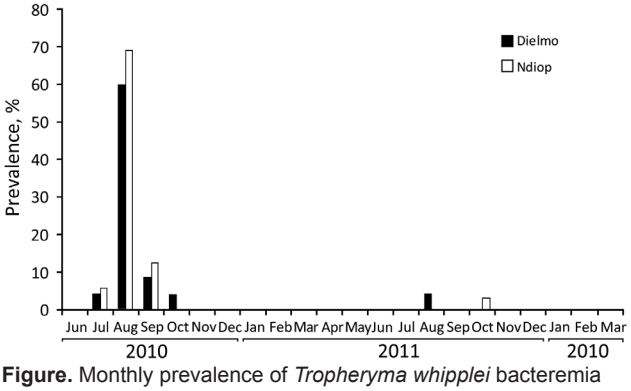

Figure. Monthly prevalence of *Tropheryma whipplei* bacteremia in Dielmo and Ndiop, Senegal, June 2010–March 2012. These 2 rural villages are located in the Sine-Saloum area, a dry sahelian ecosystem.

Table 2. *Tropheryma whipplei* multispacer typing results for 8 patients in the Sine-Saloum area of Senegal, 2010*

Patient no.	Age, y/sex	Sampling date	Village	Household no.	Spacers			
					TW133	ProS	SecA	Pro184
1	1/M	2010 Aug 4	Dielmo	14	NA	NA	2	1
2	1/M	2010 Aug 10	Dielmo	39	NA	7	2	1
3	5/M	2010 Aug 16	Dielmo	19	NA	NA	2	1
4	1/F	2010 Aug 22	Dielmo	6	NA	7	NA	1
5	4/M	2010 Aug 24	Dielmo	39	NA	7	2	1
6	13/F	2010 Jul 27	Ndiop	2	NA	7	2	1
7	2/F	2010 Aug 6	Ndiop	38	NA	7	2	1
8	2/M	2010 Aug 13	Ndiop	10	NA	7	NA	1

*NA, not available.

2 of the patients and the NA-NA-2-1 combination for the other 2 patients. None of the potential combinations has previously been sequenced in Senegal. Moreover, the NA-7-2-1 combination has also not previously been detected in any other area of the world and is thus a new genotype. Overall, our data suggest that the same genotype was detected in Dielmo and Ndiop during the summer of 2010. However, *T. whipplei* genotyping was performed (sometimes only partially) for only 8 of 36 patients, so we can only suspect, but not confirm, that an epidemic clone was present and that an outbreak was ongoing at that time.

Affected Households

In Dielmo during the peak of the August 2010 outbreak, multiple persons in several households were positive for *T. whipplei* bacteremia: 4 of 6 persons in household no. 19, 3 of 4 persons in household no. 39, 2 of 2 persons in household no. 9, and 2 of 3 persons in household no. 14. In Ndiop, 2 of 2 persons in household no. 3 and 2 of 3 persons in household no. 8 were positive for *T. whipplei* bacteremia. Of note, during this time, the family in household no. 39 had a furnace in which they baked bread that they marketed locally. In December 2010, most of the family left the village and the furnace was shut down; no additional *T. whipplei* bacteremia cases were subsequently observed.

Discussion

We report the detection of *T. whipplei* DNA in the blood of patients in Dielmo and Ndiop, Senegal. The validity of our data is based on strict experimental procedures and controls, including rigorous positive and negative controls, used to validate test results. In addition, we confirmed each positive PCR result by the successful amplification of an additional specific DNA sequence, and we performed *T. whipplei* genotyping on several specimens. We also showed that the presence of *T. whipplei* in blood is significantly linked to the presence of fever; *T. whipplei* DNA was detected (at a low level) in the blood of only 1 afebrile person in the study area. Moreover, we included a control group of afebrile persons from the same area, thereby reinforcing the validity of our data. Indeed, several well-known pathogens have been detected in recently analyzed specimens from healthy persons. For example, *Plasmodium falciparum* has been detected in 32% of blood specimens from healthy, afebrile persons in Senegal (*33*); respiratory viruses, including influenza virus, have been detected in 12% of nasopharyngeal samples from symptom-free Hajj pilgrims (*34*); and *S. pneumoniae* has been detected in 6.3% of blood specimens from afebrile children in Tanzania (*35*). Thus, because of the significantly higher prevalence of *T. whipplei* among febrile patients compared with healthy controls, we suspect that this microorganism is a pathogenic agent.

The overall prevalence of *T. whipplei* bacteremia is 4.6%. However, in August 2010, we observed a peak in *T. whipplei* bacteremia cases in Dielmo and Ndiop, where *T. whipplei* was involved in more than half of the observed cases of fever. This peak corresponds to a short outbreak of *T. whipplei* bacteremia with 1 potential genotype. A similar new genotype was observed for the patients from Dielmo and Ndiop for whom genotyping was available at the time of the outbreak. To date, 35 different *T. whipplei* genotypes have been detected in Senegal, but only 1 common genotype has been detected in Dielmo and Ndiop, even though the villages are 5 km apart (*25*). All of the other genotypes detected in the Sine-Saloum area were specific to each village, including the 2 that were more prevalent: genotype 52 was detected in 54% of feces samples in Dielmo, and genotype 49 was detected in 28% of feces samples from Ndiop (*25*).

Several familial cases also occurred during this outbreak. The family in household no. 39 in Dielmo was 1 of the most affected families: 3 of 4 persons living in the home had fever and *T. whipplei* bacteremia. Genotyping was available for 2 of these patients, both of whom exhibited the same potential genotype. The family in household no. 39 was involved in the management of a traditional oven for preparing bread, which was thoroughly cooked and sold directly to other residents. Since the departure of the baker and his family, no other outbreaks have been observed, and the prevalence of *T. whipplei* bacteremia has dramatically decreased. Thus, this family may have contributed to spread of the outbreak on a daily basis in Dielmo and possibly on a weekly basis at traditional markets, which served as the main contact between villagers from Dielmo

and Ndiop. Also of note, no toilet facilities were present in household no. 39, and a link between a lack of toilet facilities and the high detection of *T. whipplei*, mainly in feces, has previously been reported (*31*). Thus, we hypothesize that *T. whipplei* was transmitted to customers who bought bread contaminated with infectious feces (*31*). Overall, all of our data confirm human-to-human transmission of the bacterium (*22*,*23*,*26*,*31*).

One of the main symptoms among febrile patients with *T. whipplei* bacteremia is cough (36.1%). In our preliminary study of *T. whipplei* bacteremia, cough was also the main manifestation observed (*36*). Thus, *T. whipplei* could be involved in respiratory infections (*13*,*14*,*36*,*37*). However, the presence of cough in ≈36% of febrile patients who were either *T. whipplei*–positive or –negative may also suggest that this symptom was poorly specific.

Of note, a 4-year-old patient had 2 febrile episodes associated with *T. whipplei* bacteremia 18 months apart (*8*); however, it was not possible to make a distinction between relapse and reinfection because genotyping was not available (*38*). Blood specimens from this patient that we tested for *T. whipplei* before and after the last febrile episode were negative, confirming that the infection was acute. Thus, these data suggest that some patients may have several febrile episodes linked to *T. whipplei*.

T. whipplei bacteremia cannot be diagnosed in tropical regions that lack the proper laboratory facilities or in industrialized countries that lack or do not routinely perform molecular biology–based diagnostics due to the specific training, expensive reagents, and excessive time required to perform such tests. Moreover, even recent studies that have looked for causes of nonmalarial fevers, including by performing molecular detection in blood for intracellular bacteria, such as *R. felis*, have not included the molecular detection of *T. whipplei* (*39*). Thus, it is currently difficult to estimate the prevalence of *T. whipplei* bacteremia. In conclusion, the results of our large-scale study clearly confirm the role of *T. whipplei* in febrile episodes as well as its contagiousness and epidemic character.

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References

1. Brent AJ, Ahmed I, Ndiritu M, Lewa P, Ngetsa C, Lowe B, et al. Incidence of clinically significant bacteraemia in children who present to hospital in Kenya: community-based observational study. Lancet. 2006;367:482–8. http://dx.doi.org/10.1016/S0140-6736(06)68180-4
2. Hotez PJ, Kamath A. Neglected tropical diseases in sub-Saharan Africa: review of their prevalence, distribution, and disease burden. PLoS Negl Trop Dis. 2009;3:e412. http://dx.doi.org/10.1371/journal.pntd.0000412
3. Reddy EA, Shaw AV, Crump JA. Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. Lancet Infect Dis. 2010;10:417–32. http://dx.doi.org/10.1016/S1473-3099(10)70072-4
4. Parola P, Diatta G, Socolovschi C, Mediannikov O, Tall A, Bassene H, et al. Tick-borne relapsing fever borreliosis, rural Senegal. Emerg Infect Dis. 2011;17:883–5. http://dx.doi.org/10.3201/eid1705.100573
5. Socolovschi C, Mediannikov O, Sokhna C, Tall A, Diatta G, Bassene H, et al. *Rickettsia felis*–associated uneruptive fever, Senegal. Emerg Infect Dis. 2010;16:1140–2. http://dx.doi.org/10.3201/eid1607.100070
6. Mediannikov O, Diatta G, Fenollar F, Sokhna C, Trape JF, Raoult D. Tick-borne rickettsioses, neglected emerging diseases in rural Senegal. PLoS Negl Trop Dis. 2010;4:e821. http://dx.doi.org/10.1371/journal.pntd.0000821
7. Mediannikov O, Fenollar F, Socolovschi C, Diatta G, Bassene H, Molez JF, et al. *Coxiella burnetii* in humans and ticks in rural Senegal. PLoS Negl Trop Dis. 2010;4:e654. http://dx.doi.org/10.1371/journal.pntd.0000654
8. Fenollar F, Mediannikov O, Socolovschi C, Bassene H, Diatta G, Richet H, et al. *Tropheryma whipplei* bacteremia during fever in rural West Africa. Clin Infect Dis. 2010;51:515–21. http://dx.doi.org/10.1086/655677
9. Mediannikov O, Socolovschi C, Edouard S, Fenollar F, Mouffok N, Bassene H, et al. Common epidemiology of *Rickettsia felis* infection and malaria, Africa. Emerg Infect Dis. 2013;19:1775–83. http://dx.doi.org/10.3201/eid1911.130361
10. Fenollar F, Lagier JC, Raoult D. *Tropheryma whipplei* and Whipple's disease. J Infect. 2014;69:103–12. http://dx.doi.org/10.1016/j.jinf.2014.05.008
11. Moos V, Schneider T. Changing paradigms in Whipple's disease and infection with *Tropheryma whipplei.* Eur J Clin Microbiol Infect Dis. 2011;30:1151–8. http://dx.doi.org/10.1007/s10096-011-1209-y
12. Harris JK, De Groote MA, Sagel SD, Zemanick ET, Kapsner R, Penvari C, et al. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. Proc Natl Acad Sci U S A. 2007;104:20529–33. http://dx.doi.org/10.1073/pnas.0709804104
13. Bousbia S, Papazian L, Auffray JP, Fenollar F, Martin C, Li W,et al. *Tropheryma whipplei* in patients with pneumonia. Emerg Infect Dis. 2010;16:258–63. http://dx.doi.org/10.3201/eid1602.090610
14. Fenollar F, Ponge T, La Scola B, Lagier JC, Lefebvre M, Raoult D. First isolation of *Tropheryma whipplei* from bronchoalveolar fluid and clinical implications. J Infect. 2012;65:275–8. http://dx.doi.org/10.1016/j.jinf.2011.11.026
15. Raoult D, Fenollar F, Rolain JM, Minodier P, Bosdure E, Li W, et al. *Tropheryma whipplei* in children with gastroenteritis. Emerg Infect Dis. 2010;16:776–82. http://dx.doi.org/10.3201/eid1605.091801
16. Lagier JC, Lepidi H, Raoult D, Fenollar F. Systemic *Tropheryma whipplei*: clinical presentation of 142 patients with infections diagnosed or confirmed in a reference center. Medicine (Baltimore). 2010;89:337–45. http://dx.doi.org/10.1097/MD.0b013e3181f204a8
17. Gubler JG, Kuster M, Dutly F, Bannwart F, Krause M, Vögelin HP, et al. Whipple endocarditis without overt gastrointestinal disease:

report of four cases. Ann Intern Med. 1999;131:112–6. http://dx.doi.org/10.7326/0003-4819-131-2-199907200-00007
18. Goldenberger D, Künzli A, Vogt P, Zbinden R, Altwegg M. Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. J Clin Microbiol. 1997;35:2733–9.
19. Rickman LS, Freeman WR, Green WR, Feldman ST, Sullivan J, Russack V, et al. Brief report: uveitis caused by *Tropheryma whippelii* (Whipple's bacillus). N Engl J Med. 1995;332:363–6. http://dx.doi.org/10.1056/NEJM199502093320604
20. Fenollar F, Trani M, Davoust B, Salle B, Birg ML, Rolain JM, et al. Prevalence of asymptomatic *Tropheryma whipplei* carriage among humans and nonhuman primates. J Infect Dis. 2008;197:880–7. http://dx.doi.org/10.1086/528693
21. Fenollar F, Laouira S, Lepidi H, Rolain JM, Raoult D. Value of *Tropheryma whipplei* quantitative polymerase chain reaction assay for the diagnosis of Whipple's disease: usefulness of saliva and stool specimens for first-line screening. Clin Infect Dis. 2008;47:659–67. http://dx.doi.org/10.1086/590559
22. Fenollar F, Keita AK, Buffet S, Raoult D. Intrafamilial circulation of *Tropheryma whipplei*, France. Emerg Infect Dis. 2012;18:949–55. http://dx.doi.org/10.3201/eid1806.111038
23. Keita AK, Brouqui P, Badiaga S, Benkouiten S, Ratmanov P, Raoult D, et al. *Tropheryma whipplei* prevalence strongly suggests human transmission in homeless shelters. Int J Infect Dis. 2013;17:e67–8. http://dx.doi.org/10.1016/j.ijid.2012.05.1033
24. Fenollar F, Trape JF, Bassene H, Sokhna C, Raoult D. *Tropheryma whipplei* in fecal samples from children, Senegal. Emerg Infect Dis. 2009;15:922–4. http://dx.doi.org/10.3201/eid1506.090182
25. Keita AK, Bassene H, Tall A, Sokhna C, Ratmanov P, Trape JF, et al. *Tropheryma whipplei*: a common bacterium in rural Senegal. PLoS Negl Trop Dis. 2011;5:e1403. http://dx.doi.org/10.1371/journal.pntd.0001403
26. Keita AK, Raoult D, Fenollar F. *Tropheryma whipplei* as a commensal bacterium. Future Microbiol. 2013;8:57–71. http://dx.doi.org/10.2217/fmb.12.124
27. Dutly F, Altwegg M. Whipple's disease and "*Tropheryma whippelii*." Clin Microbiol Rev. 2001;14:561–83. http://dx.doi.org/10.1128/CMR.14.3.561-583.2001
28. Amsler L, Bauernfeind P, Nigg C, Maibach RC, Steffen R, Altwegg M. Prevalence of *Tropheryma whipplei* DNA in patients with various gastrointestinal diseases and in healthy controls. Infection. 2003;31:81–5. http://dx.doi.org/10.1007/s15010-002-3083-0
29. Ramharter M, Harrison N, Bühler T, Herold B, Lagler H, Lötsch F, et al. Prevalence and risk factor assessment of *Tropheryma whipplei* in a rural community in Gabon: a community-based cross-sectional study. Clin Microbiol Infect. 2014;20:1189–94. http://dx.doi.org/10.1111/1469-0691.12724
30. Keita AK, Dubot-Pérès A, Phommasone K, Sibounheuang B, Vongsouvath M, Mayxay M, et al. High prevalence of *Tropheryma whipplei* in Lao kindergarten children. PLoS Negl Trop Dis. 2015;9:e0003538. http://dx.doi.org/10.1371/journal.pntd.0003538
31. Keita AK, Mediannikov O, Ratmanov P, Diatta G, Bassene H, Roucher C, et al. Looking for *Tropheryma whipplei* source and reservoir in rural Senegal. Am J Trop Med Hyg. 2013;88:339–43. http://dx.doi.org/10.4269/ajtmh.2012.12-0614
32. Li W, Fenollar F, Rolain JM, Fournier PE, Feurle GE, Müller C, et al. Genotyping reveals a wide heterogeneity of *Tropheryma whipplei*. Microbiology. 2008;154:521–7. http://dx.doi.org/10.1099/mic.0.2007/011668-0
33. Males S, Gaye O, Garcia A. Long-term asymptomatic carriage of *Plasmodium falciparum* protects from malaria attacks: a prospective study among Senegalese children. Clin Infect Dis. 2008;46:516–22. http://dx.doi.org/10.1086/526529
34. Gautret P, Charrel R, Benkouiten S, Belhouchat K, Nougairede A, Drali T, et al. Lack of MERS coronavirus but prevalence of influenza virus in French pilgrims after 2013 Hajj. Emerg Infect Dis. 2014;20:728–30. http://dx.doi.org/10.3201/eid2004.131708
35. Lundgren IS, Heltshe SL, Smith AL, Chibwana J, Fried MW, Duffy PE. Bacteremia and malaria in Tanzanian children hospitalized for acute febrile illness. J Trop Pediatr. 2015;61:81–5. http://dx.doi.org/10.1093/tropej/fmu069
36. Fenollar F, Mediannikov O, Socolovschi C, Bassene H, Diatta G, Richet H, et al. *Tropheryma whipplei* bacteremia during fever in rural West Africa. Clin Infect Dis. 2010;51:515–21. http://dx.doi.org/10.1086/655677
37. Stein A, Doutchi M, Fenollar F, Raoult D. *Tropheryma whipplei* pneumonia in a patient with HIV-2 infection. Am J Respir Crit Care Med. 2013;188:1036–7. http://dx.doi.org/10.1164/rccm.201304-0692LE
38. Lagier JC, Fenollar F, Lepidi H, Raoult D. Evidence of lifetime susceptibility to *Tropheryma whipplei* in patients with Whipple's disease. J Antimicrob Chemother. 2011;66:1188–9. http://dx.doi.org/10.1093/jac/dkr032
39. Mayxay M, Castonguay-Vanier J, Chansamouth V, Dubot-Pérès A, Paris DH, Phetsouvanh R, et al. Causes of non-malarial fever in Laos: a prospective study. Lancet Glob Health. 2013;1:e46–54. http://dx.doi.org/10.1016/S2214-109X(13)70008-1

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Outbreak of *Vibrio parahaemolyticus* Sequence Type 120, Peru, 2009

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In 2009, an outbreak of *Vibrio parahaemolyticus* occurred in Piura, Cajamarca, Lambayeque, and Lima, Peru. Whole-genome sequencing of clinical and environmental samples from the outbreak revealed a new *V. parahaemolyticus* clone. All the isolates identified belonged to a single clonal complex described exclusively in Asia before its emergence in Peru.

Vibrio parahaemolyticus is a marine bacterium considered to be one of the major causes of bacterial foodborne outbreaks. Infections caused by *V. parahaemolyticus* have shown a steady expansion in recent years, with a growing number of cases detected worldwide (*1*–7).

The epidemiology of *V. parahaemolyticus* infections in Peru has traditionally been dominated by a characteristic pattern of an increase number of cases during the summer months, corresponding to higher coastal water temperatures (*8*). This seasonality in the epidemic dynamics of *V. parahaemolyticus* infections was only altered during the emergence of cases associated with 2 major outbreaks of illnesses reported in the country, which were caused by the arrival of novel genetic variants coming from Asia (*9,10*). *V. parahaemolyticus* infections in Peru had been predominantly associated with the O4:K8 serotype and sequence type (ST) 88 until 1995 (*11*), when a novel genetic variant of O4:K8 emerged in the country. Infections caused by this novel variant (ST-189a) quickly spread throughout the country, replacing those caused by the ST-88 variant (*10*). ST-189a was replaced in 1997 as the dominant ST by the arrival of a new variant, the pandemic clone ST-3, which also originated in Asia (*8*,*12*). Infections were mostly associated with the pandemic clone throughout 1997 and 1998 and then with a less clear pattern of dominance afterwards because of the presence of multiple serotypes.

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The Study

A new and large *V. parahaemolyticus* outbreak was detected in Peru during the austral summer of 2009. During February–March 2009, a total of 30 isolates were obtained from clinical samples of patients with symptoms of gastroenteritis. Initially illnesses were reported only in the northern cities of Peru (Cajamarca, Chiclayo, and Piura), but subsequently the outbreak extended to Lima.

Thirty *V. parahaemolyticus* strains isolated from this outbreak were initially investigated for the presence of virulence-related genes, serotyped, and subtyped by using pulse-field gel electrophoresis (PFGE). All strains belonged to serotype O3:K59, a serotype not previously identified in Peru; moreover, all were *tdh*-positive, *trh*-negative, and carried genes for the α variant of the type-3 secretion system 2 (T3SS2α). PFGE analysis showed that all the clinical strains shared an indistinguishable PFGE pattern (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/eid/article/22/7/15-1896-Techapp1.pdf).

Environmental strains of *V. parahaemolyticus* isolated from shellfish collected at the central market in Lima over the course of the outbreak were also investigated. These strains (n = 4) were *tdh*-positive, *trh*-negative, T3SS2α-positive, and indistinguishable by PFGE analysis from the outbreak strains.

The genomes of 20 of those strains (18 clinical and 2 environmental) were sequenced by MiSeq (Illumina, San Diego, CA, USA) with 500 (2 × 250) cycles, 2 × 250 pair-end library with a minimum coverage of 40–120×; testing was carried out at the US Food and Drug Administration's Center for Food Safety and Nutrition (College Park, MD, USA). Libraries were prepared with the Nextera XT DNA sample preparation kit (Illumina), according to the manufacturer's instructions. Whole-genome sequence contigs for each strain were de novo assembled by using CLC Genomics Workbench version 7.5.1 (QIAGEN, Valencia, CA, USA).

In silico multilocus sequence typing (MLST) by eBURST (*13*) identified all strains as belonging to a single sequence type profile, ST-120, which is the ancestral founder of clonal complex (CC) 120 (online Technical Appendix Figure 2). All strains deposited in the *V. parahaemolyticus* MLST database belonging to CC120 originated from China. Whole-genome MLST analysis (wgMLST) using Ridom SeqSphere+ version 3.0.0 (http://www.ridom.de/seqsphere) identified 4,265 genes shared among all ST-120 strains from Peru. The genome of strain RIMD 2210633 (*14*) was used

as reference. Ridom SeqSphere+ does a gene-by-gene mapping of the shotgun genomes against the reference genome, identifies the core genes present in all genomes, identifies variants at sequence level (single-nucleotide polymorphisms [SNPs]), and assigns alleles to each unique individual gene sequence. SNPs identified in each allele for each locus were extracted and saved into a SNP matrix to be used for further analysis. Then, Nei's DNA distance method (*15*) was used for calculating the genetic distance matrix by taking the number of same/different alleles scored for each loci in each genome. In some cases, values are not found in certain loci because that gene was either missing or truncated because of its position at either end of the de novo assembled contigs. With these genetic distances, we then built either a neighbor-joining tree or minimum-spanning tree. Among those 4,265 core genes, only 20 were different from the rest. A minimum-spanning tree of these strains showed genetic uniformity among all the outbreak strains, grouping all genomes within a single complex with a central group of 6 strains (Figure). These 6 strains were indistinguishable, and the remaining strains showed minor differences ranging from 1 to 3 alleles and from 1 to 5 SNPs (online Technical Appendix Figure 3). Furthermore, environmental strains showed identical allelic profiles and sequences to the outbreak strains, which represent evidence supporting the domestic source of the seafood originating the infections. The shellfish predominantly comprised bivalve mollusk species collected from warm areas of the north of the country where the outbreak originated and that are shipped daily to the central market in Lima.

A wgMLST analysis of the outbreak isolates with 236 *V. parahaemolyticus* genomes available in GenBank grouped the ST-120 isolates from Peru in a single cluster that exclusively included isolates from China (online Technical Appendix Figure 4). This finding constitutes additional support to the findings observed with the use of the available MLST data (online Technical Appendix Figure 2), which show genetically similar strains in very distant locations. By wgMLST analysis, ST-120 isolates from Peru differed from 2 isolates isolated in China in 1992 (S016) and 1993 (S018) by 48 and 259 alleles, respectively. The fact that these 2 strains were isolated during the 1990s might explain why they are so different from the Peru ST-120 strains. Expanding the analysis to other genomes of ST-120 recently isolated from China or Southeast Asia might identify more closely related strains.

Conclusions

Taken together, our findings reveal another example of the emergence of an Asian variant of *V. parahaemolyticus* in Peru associated with seafood consumption. The arrival of ST-120 strains in Peru represents a third instance of an introduction of Asian populations of pathogenic *V. parahaemolyticus* to the Pacific coasts of South America, and, together with the arrival of strains of the seventh pandemic of cholera in 1991, substantiates the existence of recurrent flux of pathogenic *Vibrio* populations between both sides of the Pacific Ocean. Asian and Peruvian coasts are intermittently interconnected through the movement of water displaced by El Niño episodes. These 4 introduction events of pathogenic *Vibrio* strains in Peru occurred just before the arrival of tropical El Niño waters to the Peruvian coasts, which suggests that the introduction of foreign populations of *Vibrio* could be mediated by El Niño events, as previously suggested (*8*).

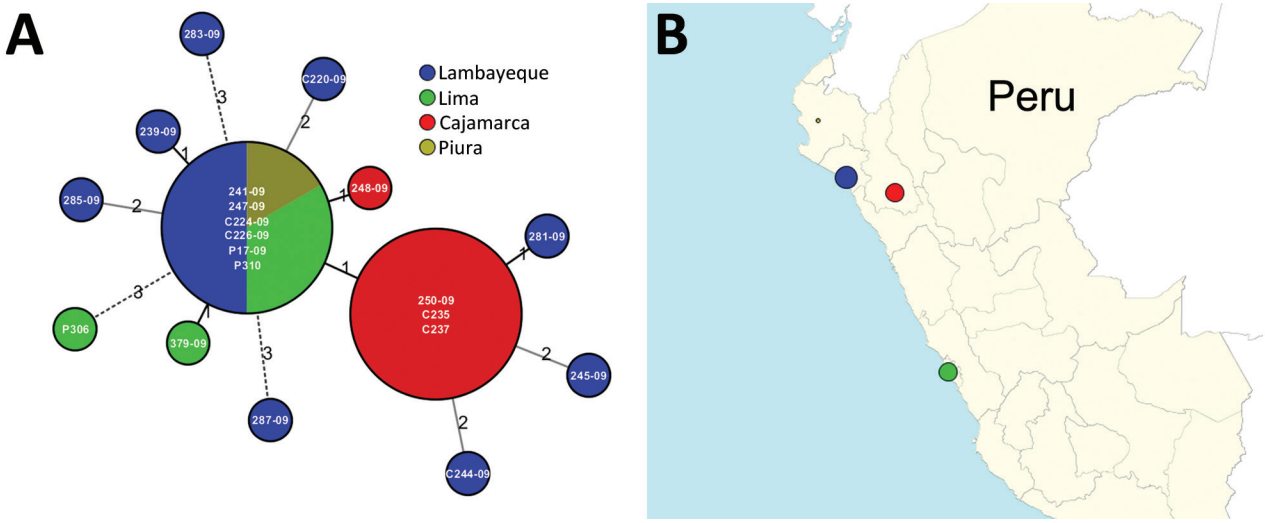

Figure. *V. parahaemolyticus* outbreaks in Peru, 2009. A) Minimum spanning tree showing the loci differences among Peruvian sequence type (ST) 120 strains. Ridom SeqSphere+ version 3.0.0 (http://www.ridom.de/seqsphere) identified 4,265 loci shared among all ST-120 *V. parahaemolyticus* strains. The numbers above the connected lines are loci differences. The lines are not to scale. B) Geographic locations of these ST-120 strains in Peru.

In conclusion, this study stresses the importance of the application of genomic epidemiology for the routine investigation of outbreaks and surveillance as an efficient and high-resolution tool for tracing the dissemination of pathogens and diseases on a global scale. This latter information is critical to detect the emergence of novel genetic variants, understand the colonization history of pathogens, and assess potential sources and scenarios contributing to the emergence of disease.

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References

1. Turner JW, Paranjpye RN, Landis ED, Biryukov SV, González-Escalona N, Nilsson WB, et al. Population structure of clinical and environmental *Vibrio parahaemolyticus* from the Pacific Northwest coast of the United States. PLoS One. 2013;8:e55726. http://dx.doi.org/10.1371/journal.pone.0055726
2. Haendiges J, Timme R, Allard MW, Myers RA, Brown EW, Gonzalez-Escalona N. Characterization of *Vibrio parahaemolyticus* clinical strains from Maryland (2012–2013) and comparisons to a locally and globally diverse *V. parahaemolyticus* strains by whole-genome sequence analysis. Front Microbiol. 2015;6:125 http://dx.doi.org/10.3389/fmicb.2015.00125.
3. Banerjee SK, Kearney AK, Nadon CA, Peterson CL, Tyler K, Bakouche L, et al. Phenotypic and genotypic characterization of Canadian clinical isolates of *Vibrio parahaemolyticus* collected from 2000 to 2009. J Clin Microbiol. 2014;52:1081–8. http://dx.doi.org/10.1128/JCM.03047-13
4. Theethakaew C, Feil EJ, Castillo-Ramírez S, Aanensen DM, Suthienkul O, Neil DM, et al. Genetic relationships of *Vibrio parahaemolyticus* isolates from clinical, human carrier, and environmental sources in Thailand, determined by multilocus sequence analysis. Appl Environ Microbiol. 2013;79:2358–70. http://dx.doi.org/10.1128/AEM.03067-12
5. Martinez-Urtaza J, Baker-Austin C, Jones JL, Newton AE, Gonzalez-Aviles GD, DePaola A. Spread of Pacific Northwest *Vibrio parahaemolyticus* strain. N Engl J Med. 2013;369:1573–4. http://dx.doi.org/10.1056/NEJMc1305535
6. Nair GB, Ramamurthy T, Bhattacharya SK, Dutta B, Takeda Y, Sack DA. Global dissemination of *Vibrio parahaemolyticus* serotype O3:K6 and its serovariants. Clin Microbiol Rev. 2007;20:39–48. http://dx.doi.org/10.1128/CMR.00025-06
7. Haendiges J, Rock M, Myers RA, Brown EW, Evans P, Gonzalez-Escalona N. Pandemic *Vibrio parahaemolyticus*, Maryland, USA, 2012. Emerg Infect Dis. 2014;20:718–20. http://dx.doi.org/10.3201/eid2004.130818
8. Martinez-Urtaza J, Huapaya B, Gavilan RG, Blanco-Abad V, Ansede-Bermejo J, Cadarso-Suarez C, et al. Emergence of Asiatic *Vibrio* diseases in South America in phase with El Niño. Epidemiology. 2008;19:829–37. http://dx.doi.org/10.1097/EDE.0b013e3181883d43
9. Gil AI, Miranda H, Lanata CF, Prada A, Hall ER, Barreno CM, et al. O3:K6 serotype of *Vibrio parahaemolyticus* identical to the global pandemic clone associated with diarrhea in Peru. Int J Infect Dis. 2007;11:324–8 http://dx.doi.org/10.1016/j.ijid.2006.08.003.
10. González-Escalona N, Gavilan RG, Brown EW, Martinez-Urtaza J. Transoceanic spreading of pathogenic strains of *Vibrio parahaemolyticus* with distinctive genetic signatures in the recA gene. PLoS One. 2015;10:e0117485. http://dx.doi.org/10.1371/journal.pone.0117485
11. Gavilan RG, Zamudio ML, Martinez-Urtaza J. Molecular epidemiology and genetic variation of pathogenic *Vibrio parahaemolyticus* in Peru. PLoS Negl Trop Dis. 2013;7:e2210. http://dx.doi.org/10.1371/journal.pntd.0002210
12. González-Escalona N, Gavilan RG, Brown EW, Martinez-Urtaza J. Transoceanic spreading of pathogenic strains of *Vibrio parahaemolyticus* with distinctive genetic signatures in the recA gene. PLoS One. 2015;10:e0117485. http://dx.doi.org/10.1371/journal.pone.0117485
13. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol. 2004;186:1518–30. http://dx.doi.org/10.1128/JB.186.5.1518-1530.2004
14. Makino K, Oshima K, Kurokawa K, Yokoyama K, Uda T, Tagomori K, et al. Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of V cholerae. Lancet. 2003;361:743–9. http://dx.doi.org/10.1016/S0140-6736(03)12659-1
15. Nei M, Tajima F, Tateno Y. Accuracy of estimated phylogenetic trees from molecular data. II. Gene frequency data. J Mol Evol. 1983;19:153–70. http://dx.doi.org/10.1007/BF02300753

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Clinical Manifestations of Senecavirus A Infection in Neonatal Pigs, Brazil, 2015

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We identified new clinical manifestations associated with Senecavirus A infection in neonatal piglets in Brazil in 2015. Immunohistochemical and molecular findings confirmed the association of Senecavirus A with these unusual clinical signs and more deaths. Other possible disease agents investigated were not associated with these illnesses.

Senecavirus A (SVA), formerly called Seneca Valley virus, is the single representative species of the genus *Senecavirus* (family *Picornaviridae*). SVA is a single-stranded, positive-sense, nonenveloped RNA virus with a genome size of ≈7.2 kb (*1*).

SVA infection was associated with porcine idiopathic vesicular disease (PIVD) in pigs in Canada (*2*), the United States (*1*), and Brazil (*3,4*). The clinical manifestations of PIVD are indistinguishable from those of other vesicular virus infections, including foot-and-mouth disease virus (FMDV), vesicular stomatitis virus, swine vesicular disease virus (SVDV), and vesicular exanthema of swine virus (*2,3*). These clinical signs include fluid-filled and ruptured vesicles and ulcerative lesions at the coronary band, hooves, and/or snout (*1–4*). In 2015, we identified new clinical manifestations associated with SVA infections in piglets in Brazil.

The Study

Since early 2015, increased numbers of deaths were recorded in pig herds from different geographic regions of Brazil. Piglets during their first week of life demonstrated clinical signs such as muscular weakness, lethargy, excessive salivation, cutaneous hyperemia, neurologic manifestations, and diarrhea; some died suddenly. Clinical signs lasted for 3–10 days and then disappeared in piglets that survived.

To determine the cause of these illnesses, we investigated 5 farms (A–E). Pig populations per farm varied from 10,000 to 23,000 animals, and piglet death rates during the first week of life ranged from 20% to 30%. Ten piglets that died spontaneously were examined (Table 1).

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Farms A, B, D, and E had gestating and/or farrowing sows with fluid-filled or ruptured vesicles at the coronary bands, hooves, or snouts; reproductive disorders were not observed. We had identified SVA RNA from sows at farms A and B (*3*) a week before the onset of clinical manifestations in these piglets.

Routine necropsies of all piglets were conducted soon after death. Tissues were fixed by immersion in 10% buffered formalin solution and processed for histopathologic evaluation. Selected tissue fragments were used in an immunohistochemical (IHC) assay designed with monoclonal antibodies to detect SVA (*5*). Duplicate sections of the organs and scrapings from oral vesicles and cutaneous lesions were collected for molecular diagnostics. From piglets at farms C, D, and E, we collected diarrheic fecal samples to investigate the possibility of enteric viruses. We analyzed 81 tissue samples and 6 diarrheic fecal samples during this study by a combination of pathologic and molecular diagnostic methods.

Molecular assays were conducted to identify viruses that might be associated with the reported clinical signs; these included SVA (*3*); FMDV, vesicular stomatitis virus, and SVDV (*6*); teschovirus A, sapelovirus A, and enterovirus G (*7*); porcine parvovirus (*8*); and porcine circovirus type 2 (*9*). Feces and fragments of the small intestine from piglets of farms C, D, and E were evaluated for porcine rotavirus species A, B, C (*10*), and H (*11*); porcine epidemic diarrhea virus (*12*); swine deltacoronavirus (*13*); and transmissible gastroenteritis virus (*12*).

Seventeen amplified products were submitted for sequencing. We conducted sequence identity matrix using BioEdit software version 7.1.11 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). A phylogenetic tree based on nucleotide sequences was obtained using MEGA6 software (http://www.megasoftware.net).

The most frequent gross manifestations observed were petechial hemorrhages of the kidney (7 piglets) and ulcerative lesions at the tongue (6 piglets) and coronary bands (4 piglets) (Figure 1, panels A, B). Interstitial pneumonia, the predominant histopathologic alteration, occurred in all the piglets; other frequent lesions were diphtheric glossitis (6 piglets), lymphocytic myocarditis (6 piglets), ballooning degeneration of the transitional epithelium of the urinary bladder (Figure 1, panel C) and the ureters (4 piglets), and lymphoplasmacytic encephalitis (3 piglets).

Consistent SVA IHC staining occurred at the transitional epithelium of the renal pelvis and the urinary bladder

[1]These authors contributed equally to this article.

Table 1. Geographic locations and other characterеristics of pig farms affected by Senecavirus A, Brazil, 2015

Farm	State/region	Month of collection	Animal no.	Age, d	Principal clinical manifestations
A	Paraná/Southern Brazil	February	1	2	Weakness at birth, sudden death at 1–3 d of age
			2	1	
B	Paraná/Southern Brazil	February	3	2	Weakness at birth, sudden death at 1–3 d of age
			4	1	
C	Mato Grosso do Sul/Midwest Brazil	March	5	3	Cutaneous hyperemia, diarrhea, excessive salivation, lethargy, death
D	Santa Catarina/Southern Brazil	March	6	2	Acute diarrhea and/or wasting, death
E	Santa Catarina/Southern Brazil	July	7	2	Diarrhea, neurologic manifestations, sudden death
			8	2	
			9	4	
			10	5	

(Figure 1, panel D) of 4 piglets; within epithelial cells of the choroid plexus of the cerebrum (8 piglets) and the tongue (5 piglets); and at the ependymal cells of the choroid plexus, vascular endothelium, and the enterocytes of the villi of the small intestine (2 piglets) (Table 2, http://wwwnc.cdc.gov/EID/article/22/7/15-1583-T2.htm).

The expected SVA RNA fragment was amplified by reverse transcription PCR from 77.8% (63/81) of all organs; all tissues from piglet 4 were positive for SVA and only 1 tissue sample from 3 piglets (nos. 2, 3, and 10) yielded negative results. Moreover, the nucleic acids of all other viruses investigated during this study were not amplified.

Sequence analysis from the 17 amplicons showed 98.8%–100% nt and aa similarities between each other and other isolates from Brazil available in GenBank (accession nos. KR075677 and KR075678). The SVA isolates we identified had similarities that varied from 87.4% nt (GenBank accession no. EU271760) to 98.5% nt (GenBank

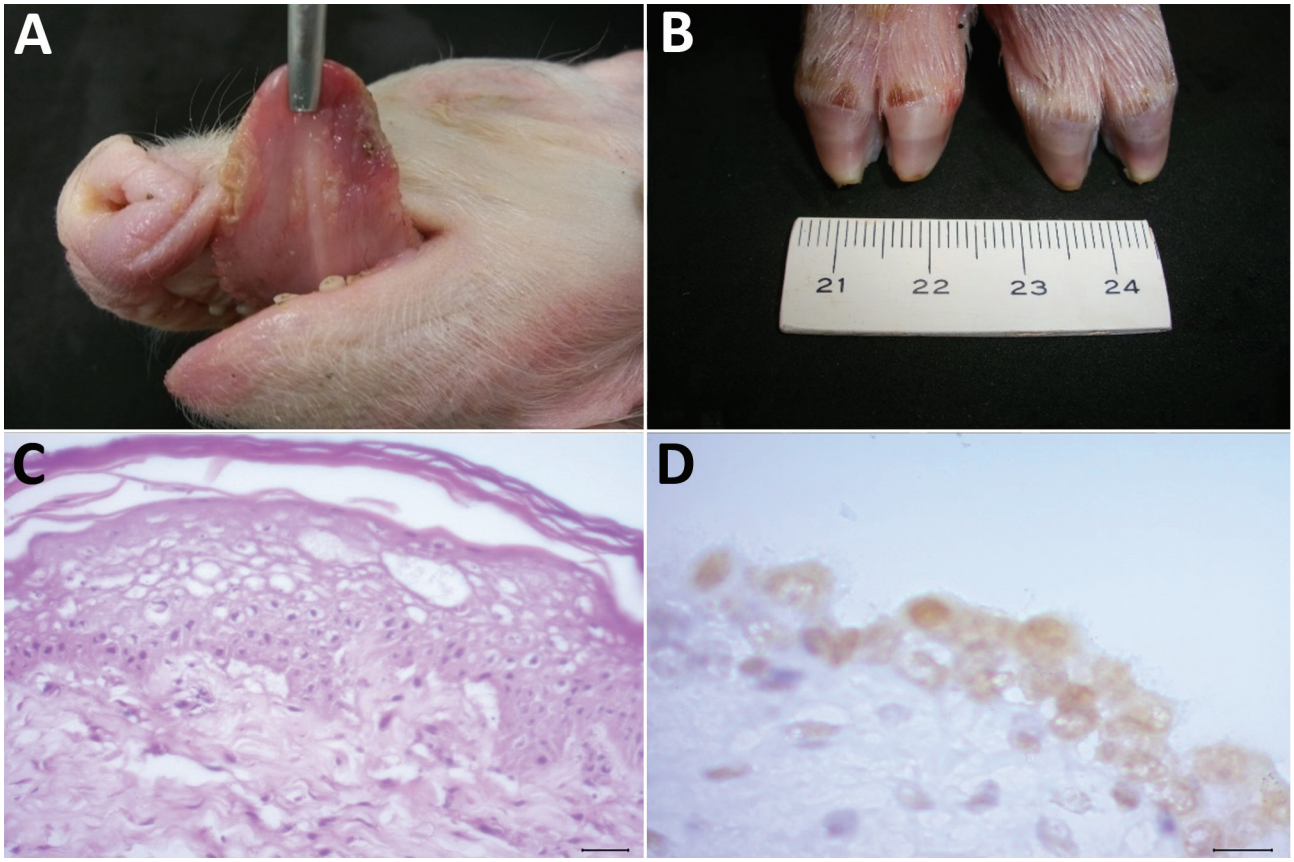

Figure 1. Pathologic alterations in piglets infected with Senecavirus A, Brazil, 2015. Gross examination shows multifocal diphtheric glossitis (A) and ulcerations of the coronary band (B). Histopathologic images demonstrate ballooning degeneration of the epithelium of the tongue (C) and positive immunoreactivity of the uroepithelium of the urinary bladder (D) to Senecavirus A. Panel B, scale shown in centimeters; panel C, hematoxylin and eosin stain; scale bar indicates 20 μm; panel D, immunoperoxidase; scale bar indicates 10 μm.

accession no. KC667560) and 94.4% aa (GenBank accession nos. EU271759 and EU271760) to 99.4% aa (GenBank accession no. KC667560) for isolates identified in North America. Phylogenetic analysis showed that the strains from this study (GenBank accession no. KT445973–KT445977) clustered with other known isolates of SVA and were distant from other picornaviruses associated with vesicular diseases (Figure 2).

Conclusions

SVA has been associated with PIVD in pigs with vesicular lesions at the snout, coronary band, and hooves (*1*–*3*). However, findings from our investigation suggest a new clinical syndrome associated with SVA infection that resulted in disease to multiple tissues and organs of these piglets.

The patterns of the cutaneous lesions identified in this study might be similar to those of other vesicular infections of picornavirus (FMDV and SVDV), in which ballooning degeneration of epithelial cells and the formation of microvesicles are hallmarks (*14*,*15*). In addition, FMDV and SVDV affect different organs of susceptible animals—the heart, lungs, lymph nodes, bone marrow, and central nervous system (*14*,*15*)—suggesting a wide organ tropism of these viruses.

An interesting feature during this study was the constant immunolabelling of SVA within epithelial cells of the choroid plexus of the brain and the surrounding endothelia of blood vessels in piglets with neurologic disease. On the basis of the IHC results and molecular findings in different tissues of the brain, we theorized that the neurologic manifestations of SVA observed during this investigation might be due to early infection of the choroid plexus through alteration of the integrity of the vascular epithelium and subsequent dissemination to the adjacent neuropil. The IHC detection of SVA within the urinary epithelium of all piglets suggests that urine might be a mode of dissemination and a possible source of contamination within affected pig farms.

Another unusual finding associated with SVA infection during this study was the occurrence of diarrhea in piglets. Molecular screening did not detect any of the common enteric viral pathogens of suckling piglets. However, the IHC and reverse transcription PCR identified SVA in the small intestine of piglets with diarrhea, demonstrating the ability of SVA to replicate within the enteric epithelium.

Our results suggest that SVA is a pantropic virus that produces a multisystemic disease entity in pigs infected at an early age. The constant immunolabelling of the uroepithelium of all piglets with SVA antigens might indicate that in-pen contamination, through urine, should be considered as a possible route for the dissemination of this virus.

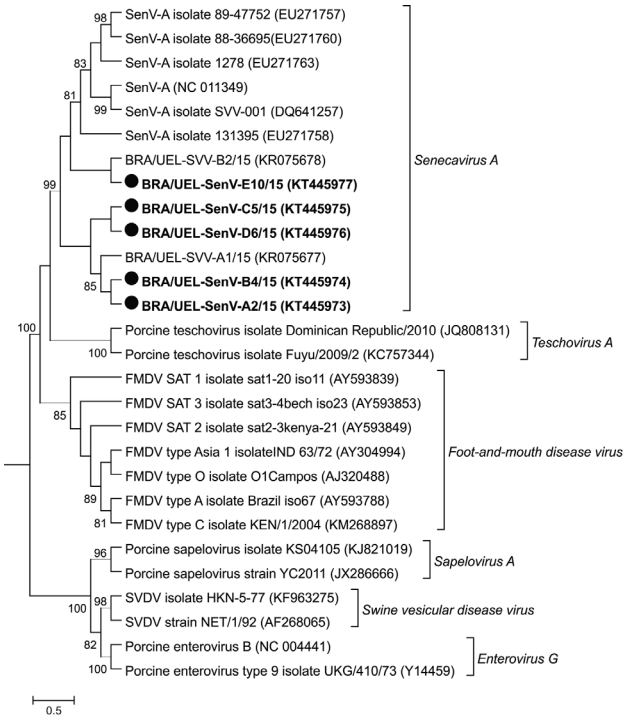

Figure 2. Phylogenetic relationship of strains of Senecavirus A identified in Brazil during 2015 (black circles) and other sequences available in GenBank derived from species of picornavirus associated with vesicular disease. Maximum-likelihood phylogenetic tree construction used the Kimura 2-parameter model with g distribution based on the partial viral protein (VP) 3/VP1 region of the Senecavirus A genome. GenBank accession numbers are given in parentheses. Bootstrap values determined in 1,000 replication. Scale bar indicates nucleotide substitutions per site.

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References

1. Singh K, Corner S, Clark SG, Scherba G, Fredrickson R. Seneca Valley virus and vesicular lesions in a pig with idiopathic vesicular disease. J Vet Sci Technol. 2012;3:1–3.
2. Pasma T, Davidson S, Shaw SL. Idiopathic vesicular disease in swine in Manitoba. Can Vet J. 2008;49:84–5.
3. Leme RA, Zotti E, Alcântara BK, Oliveira MV, Freitas LA, Alfieri AF, et al. Senecavirus A: an emerging vesicular infection

in Brazilian pig herds. Transbound Emerg Dis. 2015;62:603–11. http://dx.doi.org/10.1111/tbed.12430
4. Vannucci FA, Linhares DC, Barcellos DE, Lam HC, Collins J, Marthaler D. Identification and complete genome of Seneca Valley virus in vesicular fluid and sera of pigs affected with idiopathic vesicular disease, Brazil. Transbound Emerg Dis. 2015;62:589–93. http://dx.doi.org/10.1111/tbed.12410
5. Yang M, van Bruggen R, Xu W. Generation and diagnostic application of monoclonal antibodies against Seneca Valley virus. J Vet Diagn Invest. 2012;24:42–50. http://dx.doi.org/10.1177/1040638711426323
6. World Organization for Animal Health. Manual of diagnostic tests and vaccines for terrestrial animals 2015 [cited 2015 Mar 27]. http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/
7. Donin DG, Leme RA, Alfieri AF, Alberton GC, Alfieri AA. First report of porcine teschovirus (PTV), porcine sapelovirus (PSV) and enterovirus G (EV-G) in pig herds of Brazil. Trop Anim Health Prod. 2014;46:523–8. http://dx.doi.org/10.1007/s11250-013-0523-z
8. Soares RM, Durigon EL, Bersano JG, Richtzenhain LJ. Detection of porcine parvovirus DNA by the polymerase chain reaction assay using primers to the highly conserved nonstructural protein gene, NS-1. J Virol Methods. 1999;78:191–8. http://dx.doi.org/10.1016/S0166-0934(98)00177-3
9. McIntosh KA, Tumber A, Harding JC, Krakowka S, Ellis JA, Hill JE. Development and validation of a SYBR green real-time PCR for the quantification of porcine circovirus type 2 in serum, buffy coat, feces, and multiple tissues. Vet Microbiol. 2009;133:23–33. http://dx.doi.org/10.1016/j.vetmic.2008.06.010
10. Médici KC, Barry AF, Alfieri AF, Alfieri AA. Porcine rotavirus groups A, B, and C identified in polymerase chain reaction in a fecal sample collection with inconclusive results by polyacrylamide gel electrophoresis. J Swine Health Prod. 2011;19:146–50.
11. Molinari BL, Lorenzetti E, Otonel RA, Alfieri AF, Alfieri AA. Species H rotavirus detected in piglets with diarrhea, Brazil, 2012. Emerg Infect Dis. 2014;20:1019–22. http://dx.doi.org/10.3201/eid2006.130776
12. Kim SY, Song DS, Park BK. Differential detection of transmissible gastroenteritis virus and porcine epidemic diarrhea virus by duplex RT-PCR. J Vet Diagn Invest. 2001;13:516–20. http://dx.doi.org/10.1177/104063870101300611
13. Wang L, Byrum B, Zhang Y. Detection and genetic characterization of deltacoronavirus in pigs, Ohio, USA, 2014. Emerg Infect Dis. 2014;20:1227–30. http://dx.doi.org/10.3201/eid2007.140296
14. Arzt J, Baxt B, Grubman MJ, Jackson T, Juleff N, Rhyan J, et al. The pathogenesis of foot-and-mouth disease II: viral pathways in swine, small ruminants, and wildlife; myotropism, chronic syndromes, and molecular virus-host interactions. Transbound Emerg Dis. 2011;58:305–26. http://dx.doi.org/10.1111/j.1865-1682.2011.01236.x
15. Escribano-Romero E, Jiménez-Clavero MA, Ley V. Swine vesicular disease virus. Pathology of the disease and molecular characteristics of the virion. Anim Health Res Rev. 2000;1:119–26. http://dx.doi.org/10.1017/S1466252300000104

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Infection with Possible Novel Parapoxvirus in Horse, Finland, 2013

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A horse in Finland exhibited generalized granulomatous inflammation and severe proliferative dermatitis. After euthanization, we detected poxvirus DNA from a skin lesion sample. The virus sequence grouped with parapoxviruses, closely resembling a novel poxvirus detected in humans in the United States after horse contact. Our findings indicate horses may be a reservoir for zoonotic parapoxvirus.

Parapoxviruses (PPVs) are zoonotic viruses that have been known for centuries to cause contagious pustular skin infections in sheep, goats, and cattle worldwide. These viruses also infect other animals, such as red deer, seals, camels, reindeer, and domestic cats (*1*,*2*). In the genus *Parapoxvirus*, 4 species are currently recognized: Orf virus (ORFV), bovine papular stomatitis virus (BPSV), pseudocowpox virus (PCPV), and parapoxvirus of red deer in New Zealand (PVNZ) (*3*). In Finland, ORFV has repeatedly been detected in sheep, PCPV in cattle, and ORFV and PCPV in reindeer and humans (*4*,*5*). PPVs replicate in epidermal keratinocytes and generally produce pustular lesions at the infection site, which is typically around the mouth, tongue, lips, or teats of mammals. Primary lesions can be severe and proliferative but in uncomplicated cases scab within 1 week and resolve in 4–6 weeks. If the disease is complicated by secondary bacteria, the lesions can become ulcerative and necrotic, delaying healing (*6*).

All recognized PPV species except PVNZ have been identified in humans. Manifestations of human PPV infections ("farmyard pox") are typically seen on the hands of persons who had contact with infected ruminants. Recently, Osadebe et al. (*7*) reported novel poxvirus infections in 2 humans who had contact with domestic animals including horses and donkeys.

In Finland, PPV infections are common in ruminants, but unknown in horses; 3.1% of horses are seropositive for orthopoxviruses (OPV), but such infections appear to be subclinical (*8*). We describe a severe disease including dermatitis in a horse and identification of possible novel zoonotic parapoxvirus from a skin lesion.

The Patient

A rapidly progressive disease developed in a 2-year-old Standardbred stallion in Finland; clinical signs were fever, scrotal swelling, and ventral edema (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/22/7/15-1636-Techapp1.pdf); multifocal, hard, nodular skin lesions (Figure 1, panel A) and moderately enlarged lymph nodes were also apparent. The horse was apathetic and lost weight despite a good appetite. The attending clinicians suspected generalized lymphoma. However, a biopsy sample taken from nodular skin lesions showed proliferative dermatitis (Table). The horse had secondary immune-mediated hemolytic anemia 1.5 months after onset of disease; because the prognosis was poor, the horse was euthanized in September 2013. The body was received at the University of Helsinki Faculty of Veterinary Medicine (Helsinki, Finland) for a postmortem examination that month.

In necropsy, the horse was found to be thin and poorly muscled. Multifocal, nodular, dry, hard, proliferative lesions in the skin were mainly on the muzzle, lower forelimbs, and ventral abdomen. Moderate edema was present in the abdomen, scrotum, and all limbs. Thickened and hyperemic mucosa in the small intestine, moderately swollen mesenteric lymph nodes, and ascites were visible.

Histologically, the skin lesions were characterized by severe multifocal lymphohistiocytic dermatitis with intraepidermal vesicles caused by marked ballooning degeneration of the stratum granulosum (Figure 1, panel B). Eosinophilic intracytoplasmic inclusion bodies were seen in keratinocytes. Intestinal tissue, lungs, and mesenteric lymph nodes showed chronic, lymphohistiocytic inflammatory changes (Table). Special stains for mycobacteria were negative.

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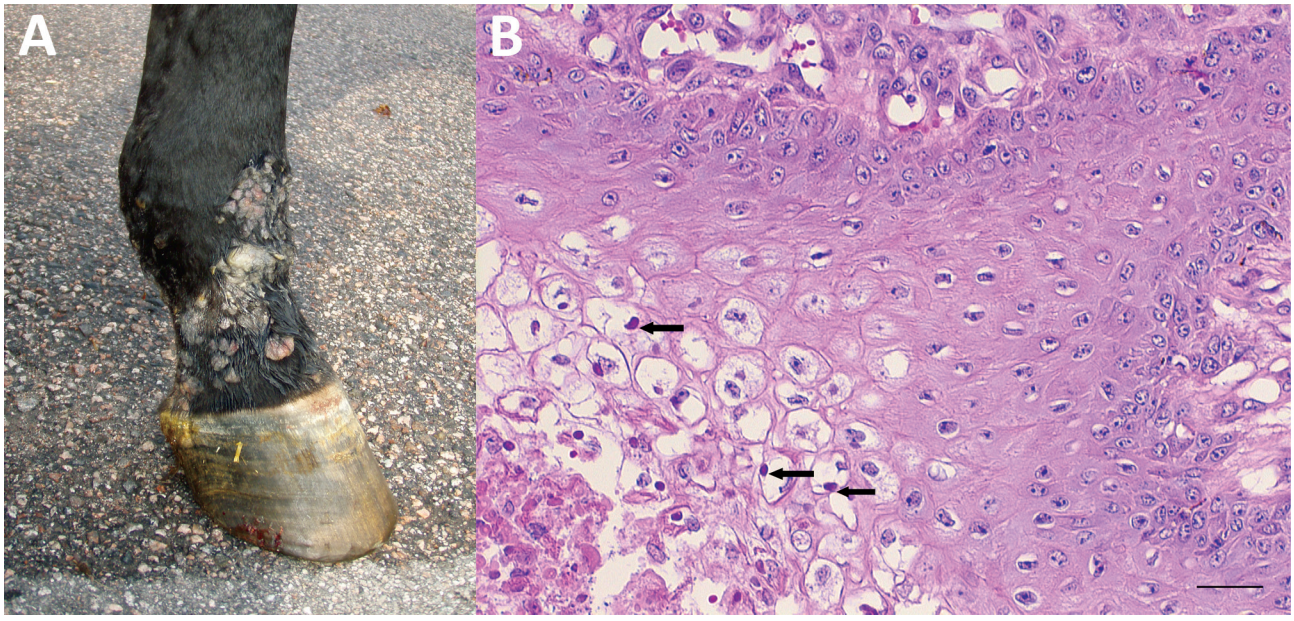

Figure 1. Macroscopic and histologic images of horse infected with possible novel parapoxvirus, Finland, 2013. A) Proliferative and ulcerative skin lesions were seen multifocally on the muzzle, ventral abdomen, and lower limbs (pictured). B) The main histological changes in samples of the skin lesions were severe multifocal lymphohistiocytic dermatitis with marked ballooning degeneration of the stratum granulosum and eosinophilic intrasytoplasmic inclusion bodies in many keratinocytes (arrows). Scale bar indicates 50 μm.

Because the histological findings of the skin samples suggested poxvirus infection, we collected a frozen plain skin sample and slices from formalin-fixed, paraffin-embedded skin, lung, lymph node, and spleen for virological studies. We attempted virus isolation from the skin sample in green monkey and baby hamster kidney cells and saw negative results. DNA was extracted by using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany), but no OPV DNA was detectable by real-time PCR (*9*) (online Technical Appendix Table). However, PPV DNA or that of a closely related virus was present in the skin samples: both the Pan-PPV PCR targeting the PPV envelope phospholipase gene (ENV) (*11*) and the high-GC (guanine-cytosine) pan-pox PCR targeting the large subunit of the poxvirus RNA polymerase gene (RPO147) (*10*) produced amplicons (Table), although several other primer pairs targeting PPV genes were negative (online Appendix Table).

Sequencing of the PCR products showed that the ENV (GenBank accession no. KR863114) and RPO147 (GenBank accession no. KR827441) sequences shared 80%–89% nt and aa identity with other PPVs, depending on the virus species. The RPO147 sequence was 99%–100% identical at nt level and 100% identical at aa level to the sequences of the 2 recent poxvirus isolates (2012_37 and 2013_013 RPO147) from humans in the United States (*7*). In phylogenetic analyses, the sequences from the horse in this study and from these human patients grouped together, forming a different lineage within the PPVs and separate from other related poxviruses, molluscum contagiosum virus and squirrelpox virus (Figure 2). The equine poxvirus was designated F14.1158H.

Table. Histopathologic and PCR findings in samples from a horse infected with parapoxvirus, Finland, 2013*

Source	Histopathology	PPV PCR (*10*), RNA polymerase gene	Pan-PPV PCR (*11*), high GC, ENV gene	Other poxvirus PCRs (*6,10*)†
Skin lesions	Severe multifocal proliferative lymphohistiocytic dermatitis	Positive	Positive	Negative
Postmortem samples				
Skin lesions	Severe multifocal proliferative lymphohistiocytic dermatitis	Positive	Positive	Negative
Lung	Severe diffuse lymphohistiocytic interstitial pneumonia	Negative	Negative	Negative
Intestines	Moderate diffuse lymphohistiocytic enteritis	Negative	Negative	Negative
Intestinal lymph nodes	Moderate multifocal lymphohistiocytic inflammation	Negative	Negative	Negative

*ENV, envelope phospholipase; GC, guanine-cytosine; PPV, parapoxvirus.
†See online Technical Appendix Table (http://wwwnc.cdc.gov/EID/article/22/7/15-1636-Techapp1.pdf).

Although the skin lesions showed poxvirus infection, formalin-fixed samples from internal organs contained no viral inclusion bodies and were negative for PPV by PCR.

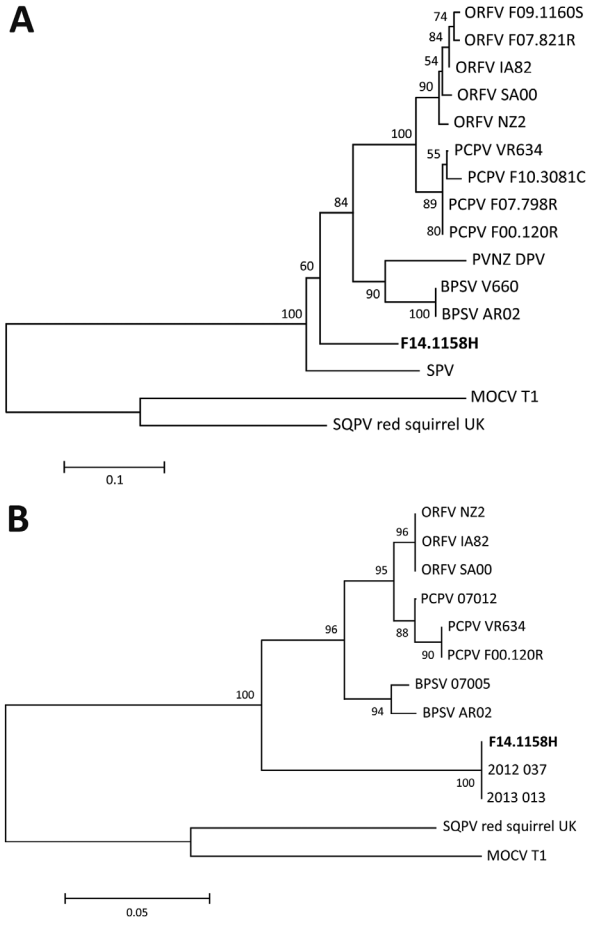

Figure 2. Phylogenetic analyses of sequences amplified from skin lesion of horse infected with possible novel parapoxvirus, Finland, 2013 (poxvirus variant F14.1158H), and other poxviruses. Trees were generated by using the neighbor-joining method in MEGA 6 software (http://www.megasoftware.net) (*12*), based on A) 184 aa of envelope phospholipase gene and B) 195 aa of viral RNA polymerase gene RP0147. GenBank accession numbers for sequences used in the analyses: JF773701 (Orf virus [ORFV] F07.821R), JF773703 (ORFV F09.1160S), AY386263 (ORFV IA82), AY386264 (ORFV SA00), DQ184476 (ORFV NZ2), GQ329670 (pseudocowpox virus [PCPV] VR634), JF773695 (PCPV F10.3081C), JF773692 (PCPV F07.798R), GQ329669 (PCPV F00.120R), AY453655 (parapoxvirus of red deer in New Zealand [PVNZ] DPV), AY453664 (bovine papular stomatitis virus [BPSV] V660), AY386265 (BPSV AR02), AF414182 (sealpoxvirus [SPV]), U60315 (molluscum contagiosum virus [MOCV] subtype 1), HE601899 (squirrelpox virus [SQPV] red squirrel UK), GQ902051.1 (PCPV 07012), GQ902054.1 (BPSV 07005), KM491712 (2013_013), and KM491713 (2012_037). The final 2 sequences originated from recent cases in humans with equine contacts in the United States (*7*). The reliability of the trees was determined by 1,000 dataset bootstrap resampling; the percentage of replicate trees in which the associated taxa clustered together is shown in the branches. Scale bars indicate amino acid substitutions per site.

This finding is in accordance with the fact that PPVs are specialized to replicate in the highly specific immune environment of skin (*13*). Further investigations are required to show whether the poxvirus caused the generalized infection in addition to dermatitis.

The owner, breeder, and trainers of the horses on the farm where this horse became ill were unaware of any other animal or zoonotic cases in the premises and disclosed no contact between the horse and ruminants. The horse had lived in contact with many horses and several dogs and cats in 3 locations in southern parts of western and eastern Finland before being transferred to the last training stable. A few months before onset of clinical signs, the horse had been trained at a farm where cows had been kept 25 years earlier. During the illness, the horse lived in a stable with 17 horses, shared corrals and equipment, and had muzzle contact with 2 horses in adjacent stalls. Despite the direct and indirect contacts, all other horses, the 3 caretakers, and the trainer remained asymptomatic.

Conclusions

We report a clinical equine infection with a novel poxvirus in Finland. The infection is at least of dermatitic relevance for horses, and veterinary awareness is needed. The sequence analysis based on conserved genes revealed a close relationship between this isolate and recent poxvirus isolates from humans with horse contact in the United States (*7*). Although sequence data are limited and the geographic distance between this equine case and the recent cases in humans is remote, the close genetic relatedness suggests that horses have a possible role as reservoir or vector of an emerging zoonotic poxvirus, necessitating medical awareness and emphasizing the importance of the One Health approach (https://www.onehealthcommission.org/). The horse as an origin for zoonoses is not uncommon: as many as 58% of emerging zoonotic pathogens infect ungulates (*14*). As for cowpox virus, horse and human may be infected from a common source, such as rodents, and not necessarily from each other. This case appeared sporadic and not very contagious, and the transmission route remained unresolved. Further studies are needed to elucidate ecology, epidemiology, prevalence, and possible zoonotic transmission.

As our limited sequence analysis suggests, the virus we detected is most closely related to PPVs and may merit being classified as a new *Parapoxvirus* species. However, many of the established PPV primer pairs did not produce PCR product, which suggests that the virus is different from the established PPV species and may represent a new poxvirus genus. More sequence data are needed to validate the taxonomic classification of the equine poxvirus. In conclusion, our results provide further evidence that horses are a possible source of the new poxvirus infection recently observed in humans.

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References

1. Essbauer S, Pfeffer M, Meyer H. Zoonotic poxviruses. Vet Microbiol. 2010;140:229–36. http://dx.doi.org/10.1016/j.vetmic.2009.08.026
2. Fairley RA, Whelan EM, Pesavento PA, Mercer AA. Recurrent localized cutaneous parapoxviruses infection in three cats. N Z Vet J. 2008;56:196–201. http://dx.doi.org/10.1080/00480169.2008.36833
3. Skinner MA, Buller RM, Damon IK, Lefkowitz EJ, McFadden G, Mc Innes CJ, et al. 2012. Poxviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors. Virus taxonomy: classification and nomenclature of viruses. Ninth report of the international committee on taxonomy of viruses. San Diego: Elsevier Academic Press, 2012. p. 291–309.
4. Tikkanen MK, McInnes CJ, Mercer AA, Buttner M, Tuimala J, Hirvela-Koski V, et al. Recent isolates of parapoxvirus of Finnish reindeer (*Rangifer tarandus tarandus*) are closely related to bovine pseudocowpox virus. J Gen Virol. 2004;85:1413–8. http://dx.doi.org/10.1099/vir.0.79781-0
5. Hautaniemi M, Vaccari F, Scagliarini A, Laaksonen S, Huovilainen A, McInnes CJ. Analysis of deletion within the reindeer pseudocowpoxvirus genome. Virus Res. 2011;160:326–32. http://dx.doi.org/10.1016/j.virusres.2011.07.005
6. Haig DM, Mercer AA. Ovine diseases. Orf. Vet Res. 1998; 29:311–26.
7. Osadebe LU, Manhiram K, McCollum AM, Li Y, Emerson GL, Gallardo-Romero NF, et al. Novel poxvirus infection in 2 patients from the United States. Clin Infect Dis. 2015;60:195–202. http://dx.doi.org/10.1093/cid/ciu790
8. Kinnunen PM. Detection and epidemiology of cowpox and Borna disease virus infections [dissertation]. Helsinki (Finland): University of Helsinki; 2011 [cited 2015 Oct 3]. https://helda.helsinki.fi/handle/10138/27343
9. Putkuri N, Piiparinen H, Vaheri A, Vapalahti O. Detection of human orthopoxvirus infections and differentiation of smallpox virus with real-time PCR. J Med Virol. 2009;81:146–52. http://dx.doi.org/10.1002/jmv.21385
10. Li Y, Meyer H, Zhao H, Damon IK. GC content-based pan-pox universal PCR assays for poxvirus detection. J Clin Microbiol. 2010;48:268–76. http://dx.doi.org/10.1128/JCM.01697-09
11. Inoshima Y, Morooka A, Sentsui H. Detection and diagnosis of parapoxvirus by the polymerase chain reaction. J Virol Methods. 2000;84:201–8. http://dx.doi.org/10.1016/S0166-0934(99)00144-5
12. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol. 2013;30:2725–9. http://dx.doi.org/10.1093/molbev/mst197
13. Fleming SB, Wise LM, Mercer AA. Molecular genetic analysis of orf virus: a poxvirus that has adapted to skin. Viruses. 2015;7:1505–39. http://dx.doi.org/10.3390/v7031505
14. Cleaveland S, Laurenson MK, Taylor LH. Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. Philos Trans R Soc Lond B Biol Sci. 2001;356:991–9. http://dx.doi.org/10.1098/rstb.2001.0889

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Vesicular Disease in 9-Week-Old Pigs Experimentally Infected with Senecavirus A

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Senecavirus A has been infrequently associated with vesicular disease in swine since 1988. However, clinical disease has not been reproduced after experimental infection with this virus. We report vesicular disease in 9-week-old pigs after Sencavirus A infection by the intranasal route under experimental conditions.

Senecavirus A (SVA), formerly known as Seneca Valley virus, is a nonenveloped, single-stranded, positive-sense RNA virus that belongs to the family *Picornaviridae* and has recently been proposed to be the prototype species of the genus *Senecavirus* (*1*). Although SVA was first identified as a contaminant in cell culture medium (*2,3*), it has been infrequently associated with cases of idiopathic vesicular disease in pigs in the United States (*3,4*) and Canada (*5*). These findings have led to speculation that SVA infection could be confused with a highly contagious vesicular livestock disease caused by foot-and-mouth disease virus (FMDV), another picornavirus in the genus *Apthovirus*. SVA infection has rarely been reported in other countries.

Beginning in late 2014, vesicular disease was reported in many swine herds in Brazil, and SVA was identified in serum, vesicular fluid, and swab samples from ruptured vesicles collected from affected weaned and adult pigs (*6, 7*). In July 2015, an unprecedented emergence of vesicular disease began in multiple swine herds in the United States, and only SVA was detected in samples from affected animals. Presumably, SVA is the cause of these current epidemics of vesicular disease in Brazil and the United States. However, a causal relationship between the virus and its host has not been made.

We report vesicular disease in nursery-age pigs that were experimentally infected with an SVA isolate obtained from a commercial swine operation in South Dakota, United States. These pigs had idiopathic vesicular disease with lameness.

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The Study

We purchased 17 conventionally raised weaned pigs and housed them until 9 weeks of age at the campus of the Agricultural Research Service, National Animal Disease Center, US Department of Agriculture (Ames, IA, USA), in accordance with Institutional Animal Care and Use Committee protocols (protocol ACUP 2867). At this time, each pig received an intranasal inoculation of a cell culture–propagated SVA isolate (SVA15–41901SD, third passage) (B. Guo, unpub. data) at a dose of 5×10^7 PFU/animal. Challenge virus was grown in a swine testicular cell line (CRL-1746; American Type Culture Collection, Manassas, VA, USA) and tested for extraneous viruses by using PCRs and next-generation sequencing.

We detected no viruses other than SVA in the challenge inoculum. At 2, 4, 6, 8, and 10 days postinfection (dpi), we euthanized a randomly selected pig and conducted necropsy. Although we used the remaining 12 pigs to evaluate the kinetics of virus infection and euthanized them at 36 dpi for the purposes of this study, we describe only the acute phase of infection here.

We collected blood samples at 0, 3, and 15 dpi and monitored all pigs for vesicular and erosive lesions on the snout and hooves. When we detected vesicular lesions, they were swabbed, and vesicular fluid was collected from intact vesicles. We stored serum harvested from blood samples, swab samples, and vesicular fluid at −80°C until testing for SVA RNA by using a primer- and probe-based, quantitative, real-time, reverse transcription PCR. Serum was tested by using an indirect fluorescent antibody test with human lung cancer cells (CRL-5803; American Type Culture Collection) for detection of antibodies against SVA (B. Guo, unpub. data).

At 4 dpi, 7 of 16 pigs had intact or ruptured vesicular lesions on the coronary bands of toes and dewclaws or the interdigital space of ≥1 feet (Figure 1). We observed minimal-to-mild lameness in some animals. After 5 dpi, 14 of 15 pigs had new or previously observed vesicular lesions. Lesion severity ranged from blanched coronary bands to ulcerations and erosions from ruptured vesicles. Focal necrosis and crusting of either the interdigital space (Figure 2, panel A), the coronary band, or both developed in some animals. Severe lesions in a subset of the cohort progressed to multifocal deep ulcers. In general, vesicular lesions were 0.2–2 cm in diameter. However, we observed several pigs with skin abrasions over the carpus (Figure 2, panel B),

[1] These authors contributed equally to this article.

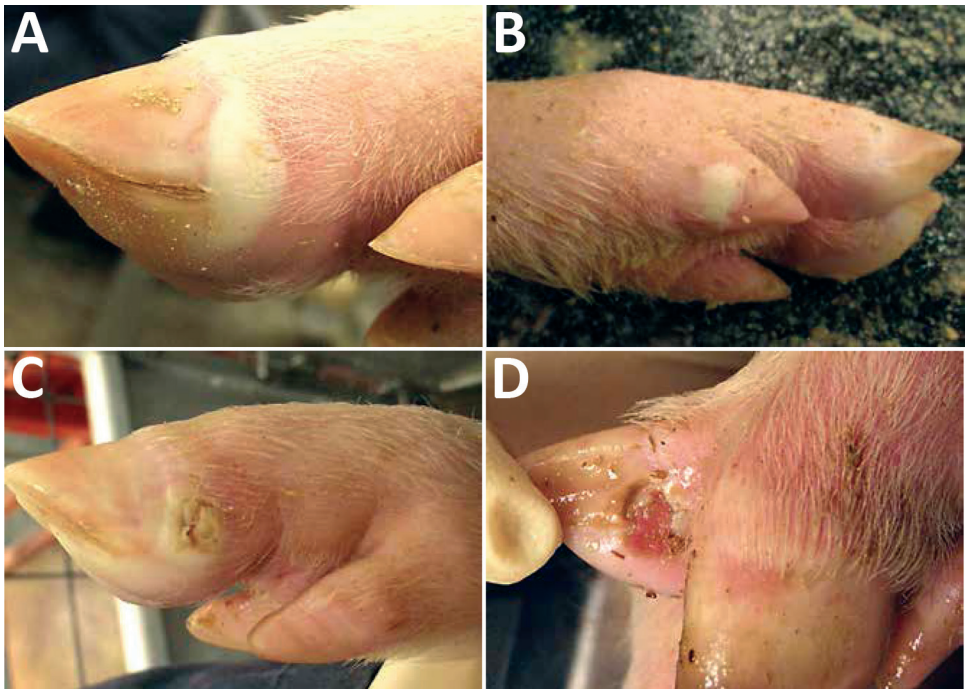

Figure 1. Vesicular lesions on feet of pigs experimentally infected with Senecavirus A. A) Blanched, intact, fluid-filled vesicle on lateral coronary band of toe. B) Intact vesicle on coronary band of medial dewclaw. C) Ruptured vesicle on coronary band of toe. D) Ruptured vesicle with ulceration and erosion in interdigital space.

which indicated that the pigs were moving while knuckling, which was probably caused by having tender feet.

At 5–6 dpi, we observed a single, small, rounded vesicle and erosion on the lower lip (Figure 2, panel C) in 2 pigs. After 10 dpi, no new lesions were detected in any of the extremities or lower lips, and old lesions were healing. We first observed vesicular lesions and erosions on snouts only at 10 dpi in the same animals that had vesicular lesions on the feet (Figure 2, panel D). Of the 5 pigs euthanized, vesicular lesions were visible on coronary bands of ≥1 extremities of pigs subjected to necropsy at 4 and 6 dpi. We observed no other gross abnormalities at necropsy. Water and food consumption was not affected during the course of the disease and no animals died on their own.

Before inoculation, all pig serum samples were negative for SVA RNA and antibody. We detected SVA RNA in serum samples from each pig at 3 dpi (range 1.1×10^2–8×10^5 genomic copies/mL) and in all swab samples collected from pigs with vesicular lesions at 5 dpi (1.9×10^1–7.9×10^4 genomic copies/mL). We also identified SVA by PCR on swab samples from snout ulcers. All surviving pigs seroconverted to SVA by 15 dpi, as determined by indirect fluorescent antibody test (titer ≥1:640).

Conclusions

Idiopathic vesicular disease in swine is a diagnosis made when none of the known etiologies for swine vesicular disease (i.e., vesicular exanthema virus, swine vesicular disease virus, vesicular stomatitis virus, and FMDV) have been detected in a clinical case. SVA has been detected occasionally in cases of idiopathic vesicular disease, which increases the possibility that SVA infection could cause vesicular disease in swine. This assumption was strengthened by the recent emergence of idiopathic vesicular disease in Brazil and the United States in which there was common detection of SVA.

In this study, we experimentally induced clinical signs and gross lesions in nursery-age pigs inoculated with SVA, demonstrating a causative relationship between SVA infection and vesicular disease in susceptible pigs. This finding is noteworthy because SVA disease appears to be clinically indistinguishable from other vesicular diseases of swine (*4,5,8*), especially FMDV (*9–11*), which is a highly transmissible livestock disease that can cause devastating economic losses to the agricultural industry and disruption of the human food supply. However, unlike the typical clinical progression of FMD in swine in which feet and snout lesions develop at about the same time, SVA-induced vesicular disease may have a different temporal pattern. In this study, we observed lesions on the feet several days before any lesions were recognized on the snout, which might be related to the route of inoculation or other factors.

We have begun to elucidate the clinical disease and host responses to SVA infection in swine. However, further investigation is needed to address 1) susceptibility of other age groups to this isolate and other SVA isolates; 2) the contribution to disease of co-infection with other infectious agents and stressful conditions, such as transport and heat; and 3) if viral mutations could explain, at least partially, the increase in recent SVA case reports. A better understanding

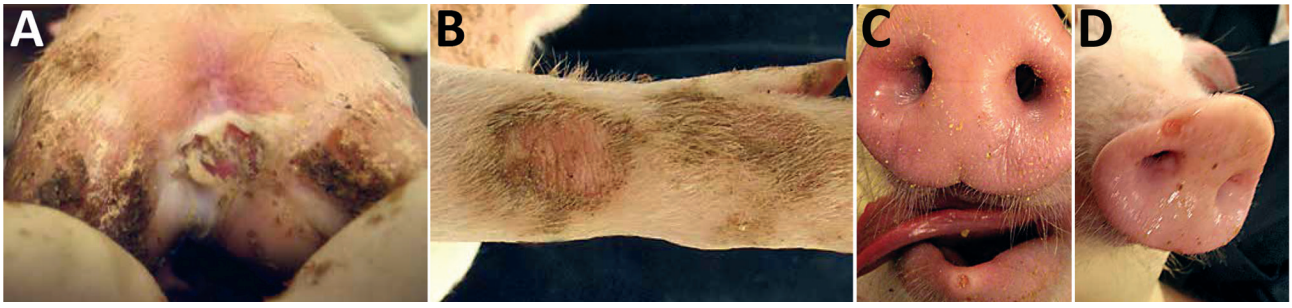

Figure 2. Vesicular and skin lesions on feet and snout of pigs experimentally infected with Senecavirus A. A) Ruptured vesicle with deep ulceration, necrosis, and crusting in interdigital space. B) Skin abrasion on carpus. C) Vesicle and erosion on lower lip. D) Vesicle on snout.

of SVA pathogenesis might help in development of prevention and control measures and differentiation of this virus from those causing other vesicular diseases.

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References

1. Hales LM, Knowles NJ, Reddy PS, Xu L, Hay C, Hallenbeck PL. Complete genome sequence analysis of Seneca Valley virus-001, a novel oncolytic picornavirus. J Gen Virol. 2008;89:1265–75. http://dx.doi.org/10.1099/vir.0.83570-0
2. Willcocks MM, Locker N, Gomwalk Z, Royall E, Bakhshesh M, Belsham GJ, et al. Structural features of the Seneca Valley virus internal ribosome entry site (IRES) element: a picornavirus with a pestivirus-like IRES. J Virol. 2011;85:4452–61. http://dx.doi.org/10.1128/JVI.01107-10
3. Knowles NJ, Hales LM, Jones BH, Landgraf JG, House JA, Skele KL, et al. Epidemiology of Seneca Valley virus: identification and characterization of isolates from pigs in the United States. Presented at: EUROPIC 2006: XIVth Meeting of the European Study Group on the Molecular Biology of Picornaviruses; Nov 26–Dec 1, 2006; Saariselka, Inari, Finland.
4. Singh KC, Clark SG, Scherba G, Fredrickson R. Seneca Valley virus and vesicular lesions in a pig with idiopathic vesicular disease. Journal of Veterinary Science and Technology. 2012;3:1–3.
5. Pasma T, Davidson S, Shaw SL. Idiopathic vesicular disease in swine in Manitoba. Can Vet J. 2008;49:84–5.
6. Leme RA, Zotti E, Alcantara BK, Oliveira MV, Freitas LA, Alfieri AF, et al. Senecavirus A: an emerging vesicular infection in Brazilian pig herds. Transbound Emerg Dis. 2015;62:603–11. http://dx.doi.org/10.1111/tbed.12430
7. Vannucci FA, Linhares DC, Barcellos DE, Lam HC, Collins J, Marthaler D. Identification and complete genome of Seneca Valley virus in vesicular fluid and sera of pigs affected with idiopathic vesicular disease, Brazil. Transbound Emerg Dis. 2015;62:589–93. http://dx.doi.org/10.1111/tbed.12410
8. Alexandersen S, Knowles NJ, Dekker A, Belsham GJ, Zhang Z, Koenen F. Picornaviruses. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, editors. Diseases of swine. 10th ed. New York: John Wiley & Sons, Inc; 2012. p. 587–620.
9. Grubman MJ, Baxt B. Foot-and-mouth disease. Clin Microbiol Rev. 2004;17:465–93. http://dx.doi.org/10.1128/CMR.17.2.465-493.2004
10. Murphy C, Bashiruddin JB, Quan M, Zhang Z, Alexandersen S. Foot-and-mouth disease viral loads in pigs in the early, acute stage of disease. Vet Rec. 2010;166:10–4. http://dx.doi.org/10.1136/vr.b5583
11. Alexandersen S, Oleksiewicz MB, Donaldson AI. The early pathogenesis of foot-and-mouth disease in pigs infected by contact: a quantitative time-course study using TaqMan RT-PCR. J Gen Virol. 2001;82:747–55. http://dx.doi.org/10.1099/0022-1317-82-4-747

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Hepatitis E Virus Infection in Dromedaries, North and East Africa, United Arab Emirates, and Pakistan, 1983–2015

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A new hepatitis E virus (HEV-7) was recently found in dromedaries and 1 human from the United Arab Emirates. We screened 2,438 dromedary samples from Pakistan, the United Arab Emirates, and 4 African countries. HEV-7 is long established, diversified and geographically widespread. Dromedaries may constitute a neglected source of zoonotic HEV infections.

Hepatitis E virus (HEV) is a major cause of acute hepatitis worldwide (*1*). Four HEV genotypes belonging to the species *Orthohepevirus A* are commonly found in humans (HEV-1 through HEV-4). Genotypes 1 and 2 seem to be restricted to humans, whereas genotypes 3 and 4 also occur in domesticated and wild animals. Zoonotic transmission by ingestion of contaminated meat, mainly from pigs, is the most likely zoonotic source of infection (*1*).

Recently, HEV sequences were reported from 3 dromedaries sampled in the United Arab Emirates (UAE) in 2013 and were classified as a new orthohepevirus A genotype, HEV-7 (*2*,*3*). Afterwards a human patient also from the UAE who had chronic hepatitis after liver transplantation was shown to carry HEV-7 (*3*,*4*). Until now, knowledge on HEV-7 and its zoonotic potential relied on these 2 studies, which provide no insight into the prevalence and distribution of HEV-7. To determine the geographic distribution of HEV-7, we conducted a geographically comprehensive study of HEV-7 prevalence in dromedaries by testing 2,438 specimens sampled in 6 countries during the past 3 decades.

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The Study

Serum and fecal samples were collected from dromedary camels in the UAE, Somalia, Sudan, Egypt, Kenya, and Pakistan during 1983–2015 (*5*–*7*). A total of 2,171 serum samples and 267 fecal samples were tested for HEV RNA by using reverse transcription PCR (RT-PCR) as previously described (*8*). Seventeen samples were positive for HEV RNA: 12 (0.6%) of 2,171 serum samples and 5 (1.9%) of 267 fecal samples (Table). Positive samples originated from UAE, Somalia, Kenya, and Pakistan and dated to 1983 (Figures 1, 2**)**. Viral loads were measured by using real-time RT-PCR (*9*) calibrated on the basis of the World Health Organization International Standard for HEV RNA (*10*). Viral RNA concentrations ranged from 3.2×10^4 to 3.6×10^7 IU/g in feces and 6.2×10^2 to 8.3×10^6 IU/mL in serum.

We sequenced a 283-nt fragment of the RNA-dependent RNA polymerase gene of all positive samples for phylogenetic analyses. All camel HEV clustered in a monophyletic clade with the human HEV-7 sequence (Figure 2), supporting the classification of camel-associated HEV to a separate *Orthohepevirus A* genotype (*11*).

African viruses from Somalia and Kenya formed a monophyletic clade, whereas viruses from UAE and Pakistan were intermixed (Figure 2). Distances based on nucleotide identities were calculated for all sequences from this study and 1 reference strain from each orthohepevirus A genotype as defined by Smith et al. (*11*). This subset of references comprised GenBank accession nos. M73218 (HEV-1), M74506 (HEV-2), AF082843 (HEV-3), AJ272108 (HEV-4), AB573435 (HEV-5), AB602441 (HEV-6), and KJ496143 (HEV-7). Nucleotide diversity was remarkable among viral sequences from dromedaries, reaching a maximum distance of 22.7%, compared with a maximum distance of 29.9% among all genotypes. The internal distance among the African viruses was 14.2%, compared with 17.4% distance within viruses from UAE and Pakistan. The African viruses were 16.7%–22.7% distant from UAE and Pakistan viruses, which corresponds to the distance threshold of 22%–25%

Table. Sample characteristics and detection rates of orthohepevirus Agenotype 7 in 6 countries, 1983–2015

Country	Time period	No. positive/no. tested (% positive)		
		Virus RNA		
		Serum	Feces	Antibodies
Sudan	1983	0/60		15/35(42.9)
Somalia	1983–1984	1/105 (0.9)		14/35 (40.0)
Egypt	1997	0/50		22/35 (62.9)
Kenya	1992–2015	2/889 (0.2)		11/35 (31.4)
United Arab Emirates	2013	1/500 (0.2)	5/267 (1.9)	13/35 (37.1)
Pakistan	2012–2015	8/567 (1.4)		21/35 (60.0)
Total		12/2,171 (0.5)	5/267 (1.9)	96/210 (45.7)

that separates the prototype HEV-4 sequence from HEV-5 and HEV-6 prototype sequences. This finding suggests that HEV-7 is a strongly diversified clade of viruses that might need to be further subclassified.

HEV-7 was recently shown to belong to the same serotype as HEV-1–4 (*12*). Therefore, we conducted a preliminary serologic analysis with a subset of 210 specimens (35 per country) by adapting a human HEV ELISA (EUROIMMUN, Lübeck, Germany) for application with camel serum. Serum was tested at a 1:100 dilution. The signal-to-noise ratio was optimized by normalizing the optical density (OD) of test samples against ODs of a reference serum included in every run (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/22/7/16-0168-Techapp1.pdf).

For confirmation of ELISA results and to determine an appropriate ELISA cutoff, we tested 56 samples covering the complete range of OD ratios by adapting the recomLine Immunoblot (MIKROGEN, Neuried, Germany). Thirty-two samples reacted against ≥2 of the presented antigens and were therefore ranked positive in the Immunoblot. All tested samples with ELISA OD ratios >0.46 were positive by immunoblot, whereas only 7 of 31 tested samples below this value were positive by immunoblot (online Technical Appendix Figure). Subsequently we set an ELISA cutoff of 0.46. Using this cutoff, we found 96 (46%) of the 210 serum samples originating from all 6 countries were positive (Table), which is comparable with the seroprevalences typically observed in pigs that are known zoonotic reservoirs for HEV-3 in developed countries (*13*). The percentage of ELISA-positive serum samples ranged from 31% in Kenya to 63% in Egypt but did not differ significantly among all 6 countries ($p = 0.1$, Yates' χ^2 test). These results suggest a wide occurrence and high prevalence of HEV in dromedaries.

Conclusions

We investigated HEV-7 infection in dromedaries. The broad spatial extent, the high diversity of HEV-7 in dromedaries, and the detection of HEV-RNA in a sample collected in 1983 suggest a long evolutionary history of HEV-7 in dromedaries.

Our study has some limitations. First, although most tested dromedaries seemed healthy, no detailed health

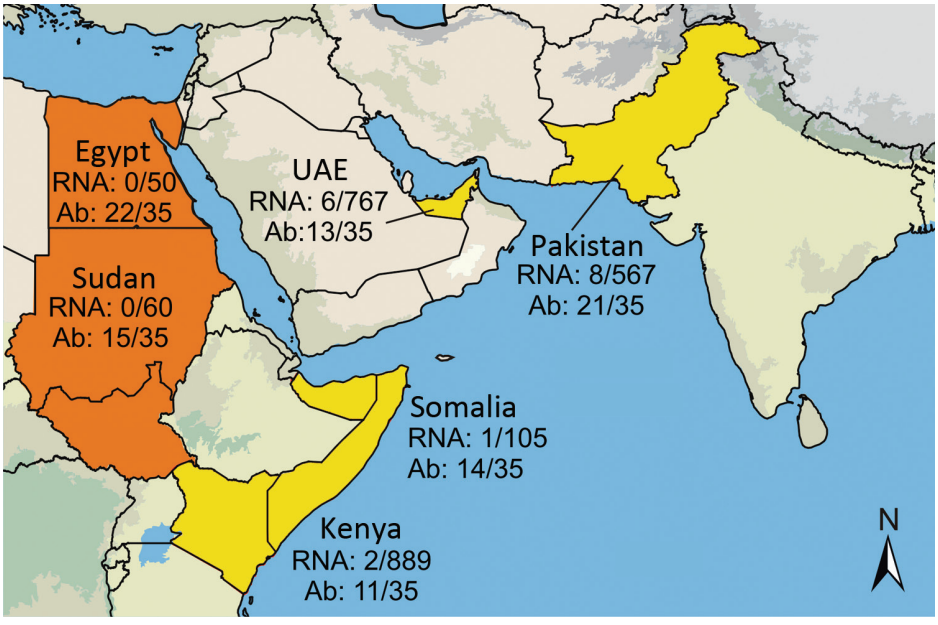

Figure 1. Six countries studied for hepatitis E virus (HEV) infection in dromedary camels, 1983–2015. Number of tested and number of HEV-7 RNA-positive samples or Ab-positive samples are given next to the study sites: Egypt, Sudan (today separated into Sudan and South Sudan), Kenya, Somalia, UAE, and Pakistan. Countries with both HEV-7 RNA and Ab detection are in yellow; countries with only Ab detection are in orange. Ab, antibody; UAE, United Arab Emirates; Map was created by using Quantum GIS (http://qgis.osgeo.org) and data from http://www.naturalearthdata.com.

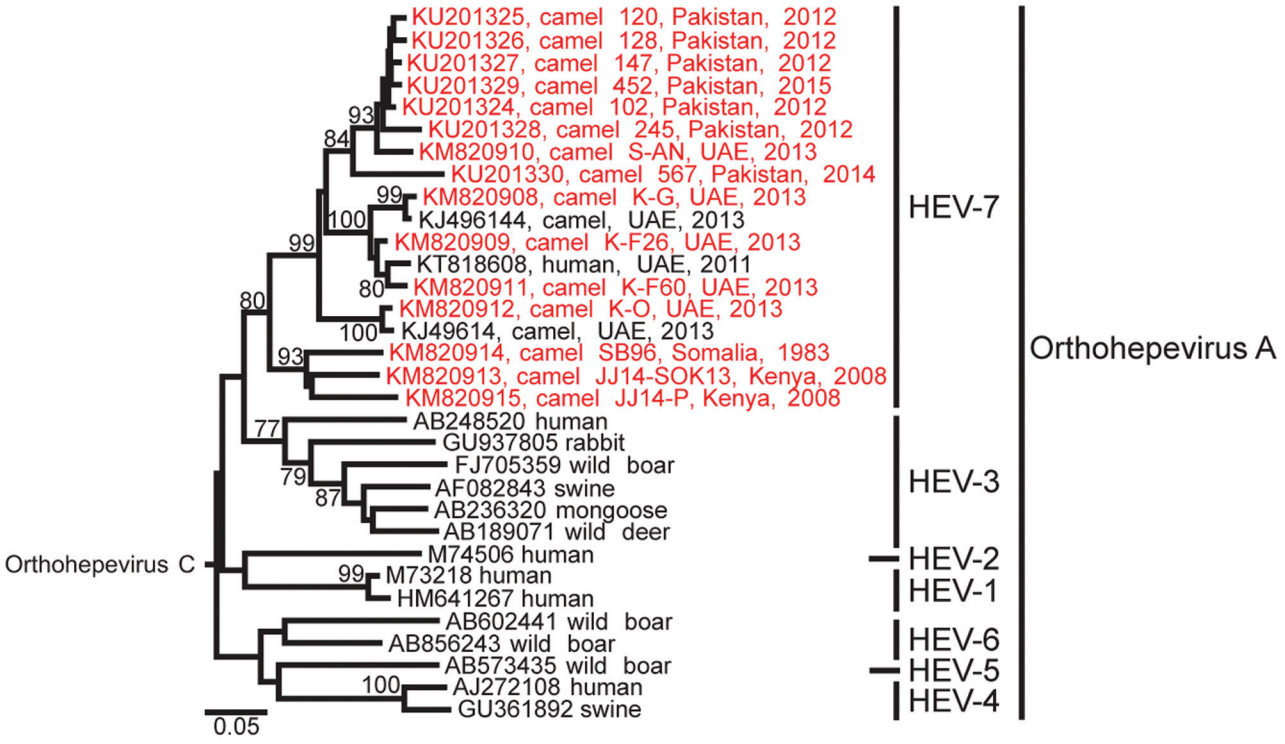

Figure 2. Phylogenetic analysis of *Orthohepevirus A* sequences. The analysis comprised partial hepatitis E virus (HEV) sequences (283 nt from the RNA-dependent RNA polymerase region) from this study, representatives of *Orthohepevirus A* genotypes 1–7 and *Orthohepevirus C* (GenBank accession no. GU345042) as an outgroup. The phylogenetic tree was calculated with MEGA 6.0 (http://www.megasoftware.net) by using the neighbor-joining algorithm and a nucleotide percentage distance substitution model. Bootstrap values (%) of 1,000 repetitive analyses >75 are shown next to the nodes. New camel HEV sequences obtained in this study are in red. Scale bar represents the genetic distance. All sequences obtained in this study are deposited in GenBank (accession nos. KM820907–KM820915 and KU201324–KU201330). UAE, United Arab Emirates.

information from the RNA-positive animals was available. Second, we studied limited genome fragments that prevented formal classification into genome subtypes (*14*). Third, although we used 2 different antibody detection methods, the antibody prevalence in camels should be confirmed by larger studies including virus neutralization studies to determine potential genotype variability.

Investigations of camelids other than dromedaries could help to further elucidate the geographic and evolutionary origin of HEV-7. Furthermore, other wild or domestic ungulates with close contact to dromedaries could be investigated to assess the host range of HEV-7. Human infection with HEV is common in all studied areas (*1*). On the basis of clinical observations and HEV antibody detection tools, several HEV outbreaks mainly linked to water contamination or poor hygienic circumstances have been described for Pakistan, Sudan, Somalia, and Egypt. For Kenya and UAE, data about HEV prevalence is scarce (*1*). In large parts of the Middle East, human infections are unlikely to be caused by contact with swine or consumption of pork for cultural reasons. Even in Saudi Arabia, where pork is absent in diet, blood donors have antibodies at proportions of up to 18.7% (*1*). Thus, most HEV infections in the Middle East are assumed to be caused by nonzoonotic genotypes 1 and 2. However, our study and previous studies (*12*) showed that HEV-7 and other human genotypes form 1 serotype, suggesting a lack of discrimination in seroprevalence studies.

The human HEV seroprevalence in the Middle East region might in fact be caused by HEV-7 infection. Furthermore, human HEV-7 infections might contribute to the HEV prevalence in all studied areas, where camel products are frequent parts of human diet (*15*). A foodborne transmission scenario is further suggested by the fact that 1 of 12 positive serum in the study was actually sampled in a slaughterhouse, documenting that meat from infected animals can enter the food chain (*6*). Detections of HEV-7 RNA in feces in this and a previous study (*2*) point at feces or feces-contaminated camel products, such as milk, as putative additional sources of human infection. Considering the importance of dromedaries as livestock animals (*15*), risk groups, such as slaughterhouse workers, should be screened for HEV-7 infection.

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References

1. Aggarwal R. The global prevalence of hepatitis E virus infection and susceptibility: a systematic review [cited 2016 May 16]. http://whqlibdoc.who.int/hq/2010/WHO_IVB_10.14_eng.pdf
2. Woo PC, Lau SK, Teng JL, Tsang AK, Joseph M, Wong EY, et al. New hepatitis E virus genotype in camels, the Middle East. Emerg Infect Dis. 2014;20:1044–8. http://dx.doi.org/10.3201/eid2006.140140
3. Smith DB, Simmonds P, Jameel S, Emerson SU, Harrison TJ, Meng XJ, et al. Consensus proposals for classification of the family *Hepeviridae*. J Gen Virol. 2014;95:2223–32. http://dx.doi.org/10.1099/vir.0.068429-0
4. Lee GH, Tan BH, Chi-Yuan Teo E, Lim SG, Dan YY, Wee A, et al. Chronic infection with camelid hepatitis E virus in a liver transplant recipient who regularly consumes camel meat and milk. Gastroenterology. 2016;150:355–357.e3. http://dx.doi.org/10.1053/j.gastro.2015.10.048
5. Corman VM, Jores J, Meyer B, Younan M, Liljander A, Said MY, et al. Antibodies against MERS coronavirus in dromedary camels, Kenya, 1992–2013. Emerg Infect Dis. 2014;20:1319–22. http://dx.doi.org/10.3201/eid2008.140596
6. Müller MA, Corman VM, Jores J, Meyer B, Younan M, Liljander A, et al. MERS coronavirus neutralizing antibodies in camels, Eastern Africa, 1983–1997. Emerg Infect Dis. 2014; 20:2093–5. http://dx.doi.org/10.3201/eid2012.141026
7. Meyer B, Müller MA, Corman VM, Reusken CB, Ritz D, Godeke GJ, et al. Antibodies against MERS coronavirus in dromedary camels, United Arab Emirates, 2003 and 2013. Emerg Infect Dis. 2014;20:552–9. http://dx.doi.org/10.3201/eid2004.131746
8. Drexler JF, Seelen A, Corman VM, Fumie Tateno A, Cottontail V, Melim Zerbinati R, et al. Bats worldwide carry hepatitis E virus-related viruses that form a putative novel genus within the family *Hepeviridae*. J Virol. 2012;86:9134–47. http://dx.doi.org/10.1128/JVI.00800-12
9. Girón-Callejas A, Clark G, Irving WL, McClure CP. In silico and in vitro interrogation of a widely used HEV RT-qPCR assay for detection of the species orthohepevirus A. J Virol Methods. 2015;214:25–8. http://dx.doi.org/10.1016/j.jviromet.2014.11.025
10. Baylis SA, Blümel J, Mizusawa S, Matsubayashi K, Sakata H, Okada Y, et al.. World Health Organization International Standard to harmonize assays for detection of hepatitis E virus RNA. Emerg Infect Dis. 2013;19:729–35. http://dx.doi.org/10.3201/eid1905.121845
11. Smith DB, Simmonds P; International Committee on Taxonomy of Viruses Hepeviridae Study Group, Jameel S, Emerson SU, Harrison TJ, et al. Consensus proposals for classification of the family *Hepeviridae*. J Gen Virol. 2014;95(Pt 10):2223–32. http://dx.doi.org/10.1099/vir.0.068429-0
12. Zhou X, Kataoka M, Liu Z, Takeda N, Wakita T, Li TC. Characterization of self-assembled virus-like particles of dromedary camel hepatitis E virus generated by recombinant baculoviruses. Virus Res. 2015;210:8–17. http://dx.doi.org/10.1016/j.virusres.2015.06.022
13. Krumbholz A, Joel S, Neubert A, Dremsek P, Dürrwald R, Johne R, et al. Age-related and regional differences in the prevalence of hepatitis E virus–specific antibodies in pigs in Germany. Vet Microbiol. 2013;167:394–402. http://dx.doi.org/10.1016/j.vetmic.2013.10.001
14. Smith DB, Simmonds P, Izopet J, Oliveira-Filho EF, Ulrich RG, Johne R, et al. Proposed reference sequences for hepatitis E virus subtypes. J Gen Virol. 2016;97:537–42. http://dx.doi.org/10.1099/jgv.0.000393
15. Kadim IT, Mahgoub O, Faye B, Farouk MM, editors. Camel meat and meat products Wallingford (UK): CAB International; 2013.

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Increased Mortality Rates Associated with *Staphylococcus aureus* and Influenza Co-infection, Maryland and Iowa, USA[1]

Jennifer S. McDanel, Eli N. Perencevich, Jeremy Storm,[2] Daniel J. Diekema, Loreen Herwaldt, J. Kristie Johnson, Patricia L. Winokur, Marin L. Schweizer

We retrospectively analyzed data for 195 respiratory infection patients who had positive *Staphyloccocus aureus* cultures and who were hospitalized in 2 hospitals in Iowa and Maryland, USA, during 2003–2009. Odds for death for patients who also had influenza-positive test results were ≥4 times higher than for those who had negative influenza test results.

Staphylococcus aureus is a common cause of respiratory infections, including pneumonia (*1*), and can lead to necrotizing pneumonia and death (*2–4*). Influenza complicated by *S. aureus* co-infection can progress rapidly to death within a week of symptom onset (*3*,*4*). However, few studies have evaluated whether patients who are co-infected with influenza and *S. aureus* are more likely to experience poor outcomes compared with patients who are infected with *S. aureus* alone. We compared patient characteristics and outcomes of patients who had a respiratory culture that grew *S. aureus* and who tested positive for influenza with those who had negative influenza test results.

The Study

This retrospective cohort study included pediatric and adult patients admitted to the University of Iowa Hospitals and Clinics (Iowa City, IA, USA) or to the University of Maryland Medical Center (Baltimore, Maryland, USA) during 2003–2009. First, we used codes from the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM), to identify patients with influenza-like illness (ILI) (*5*). This criterion was part of an initial study investigating influenza-like illness and *S. aureus* pneumonia (J.S. McDanel, unpub. data). Patients were included in the study if they had respiratory cultures (sputum, bronchial specimen, or tracheal aspirate) that grew *S. aureus* and were tested for influenza before or during their admissions. If a patient was admitted ≥1 time, only the admission with the first *S. aureus* positive respiratory culture was included. The University of Iowa institutional review board approved this study.

The primary outcome of interest, 30-day in-hospital mortality, was defined as death occurring in the hospital within 30 days of the first culture that grew *S. aureus*. The adapted Charlson Comorbidity Index served as an aggregate score for co-occurring conditions (*6*). The year of each patient's first positive *S. aureus* culture was dichotomized: 2003–2007 and 2008–2009.

We conducted bivariable analyses using either the χ^2 test or the Fisher exact test for categorical variables and the Student *t*-test or Wilcoxon rank-sum test for continuous variables. We used logistic regression to identify associations between potential predictor variables and 30-day mortality rates. We included variables in the multivariable model using a manual stepwise method. Variables associated with death ($p<0.25$) in the bivariable regression analysis were examined for fit within the multivariable model and were retained if statistically significant ($p<0.05$). The year of each patient's first positive *S. aureus* culture was forced into the model. We analyzed data using SAS software version 9.3 (SAS Institute, Cary, NC, USA).

A total of 195 patients had ≥1 respiratory culture that grew *S. aureus* and were also tested for influenza. Sputum samples (115, 59%) and bronchial washes (50, 26%) were the most common respiratory specimens. Blood cultures of 17 (9%) patients grew *S. aureus*. Respiratory or blood samples of 109 (56%) patients grew methicillin-resistant *S. aureus* (MRSA). Most patients (166, 85%) were admitted to the University of Maryland Medical Center; 116 (59%) were male, and median age was 42 (interquartile range 5–59) years.

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Of the 195 patients, 32 (16%) had positive influenza test results. Patients who had a positive influenza test were more likely to receive quinolones (odds ratio [OR] 3.30, 95% CI 1.51–7.21) than were patients whose influenza tests were negative (Table 1). Patients who had a positive influenza test were significantly more likely to have the positive *S. aureus* respiratory culture collected ≤2 days after hospital admission than were the patients whose influenza tests were negative (OR 3.27, 95% CI 1.39–7.70).

Of the 32 influenza-positive patients, 9 (28%) died; of the 163 influenza-negative patients, 18 (11%) died (OR 3.15, 95% CI 1.27–7.86; p = 0.021) (Table 2). Of the 9 influenza-positive patients who died, 5 had MRSA. Among the 27 patients who died, those with a positive influenza test were more likely to have diabetes than those who had a negative influenza test (33% vs. 0%; p = 0.029). The multivariable logistic regression model found that, after statistically adjusting for year and time from admission to collection of *S. aureus* culture samples, patients whose influenza tests were positive had ≥4-fold increased odds of death compared with patients whose influenza tests were negative (OR 4.31, 95% CI 1.57–11.83; p<0.005) (Table 2).

Conclusions

Our results are consistent with the results of other studies. Other investigators reported poor outcomes among patients who were co-infected with influenza viruses and *S. aureus* (*3*,*4*,*7*). Kallen et al. found a statistically significant increased risk for death among patients who had positive influenza test results and community-acquired *S. aureus* pneumonia, compared with patients who had negative influenza test results and community-acquired *S. aureus* pneumonia (*7*). The Kallen et al. study included patients who had either MRSA or methicillin–susceptible *S. aureus* pneumonia (*7*) but evaluated only 47 patients. The sample size for our study was much larger than previously performed studies, and we were able to examine mortality rates among patients who had a respiratory culture that grew either MRSA or methicillin-susceptible *S. aureus*.

Table 1. Characteristics of patients in cohorts demonstrating increased mortality rates associated with *Staphylococcus aureus* and influenza co-infection, Maryland and Iowa, USA*

Characteristic	Positive influenza test, n = 32	Negative influenza test, n = 163	Odds ratio 95% CI	p value
Female sex	12 (38)	67 (41)	0.86 (0.39–1.88)	0.704
Age ≥18 y	24 (75)	112 (69)	1.37 (0.58–3.25)	0.479
Hospital admission within previous 12 mo	12 (38)	75 (46)	0.70 (0.32–1.53)	0.376
Previous MRSA infection or colonization	4 (13)	33 (20)	0.56 (0.18–1.72)	0.307
Co-occurring conditions				
Cancer	3 (9)	24 (15)	0.60 (0.17–2.12)	0.579
Cerebrovascular disease	0 (0)	6 (4)	UTD	0.592
Chronic pulmonary disease	11 (34)	41 (25)	1.56 (0.69–3.51)	0.282
Heart disease	4 (13)	20 (12)	1.02 (0.32–3.22)	1.000
Diabetes	6 (19)	15 (9)	2.28 (0.81–6.41)	0.123
Liver disease	0 (0)	6 (4)	UTD	0.592
Renal disease	5 (16)	8 (5)	3.59 (1.09–11.79)	0.042
Charlson Comorbidity Index score, median (IQR)	1 (0–2)	1 (0–3)	0.89 (0.75–1.07)	0.641
Methicillin resistance				
MRSA	16 (50)	93 (57)	0.75 (0.35–1.61)	0.462
MSSA	15 (47)	66 (40)	1.30 (0.61–2.78)	0.503
Unknown	1 (3)	4 (2)	1.28 (0.14–11.86)	1.000
First positive *S. aureus* culture collected ≤2 d after hospital admission	24 (75)	78 (48)	3.27 (1.39–7.70)	0.005
Year of first positive *S. aureus* culture				0.038
2003	1 (3)	3 (2)	Reference	
2004	1 (3)	10 (6)	0.30 (0.01–6.38)	
2005	7 (22)	25 (15)	0.84 (0.08–9.38)	
2006	0 (0)	25 (15)	UTD	
2007	2 (6)	14 (9)	0.43 (0.03–6.41)	
2008	12 (38)	26 (16)	1.39 (0.13–14.73)	
2009	9 (28)	60 (37)	0.45 (0.04–4.81)	
Antimicrobial drugs received				
Vancomycin	25 (78)	128 (79)	0.98 (0.39–2.44)	0.960
Linezolid	7 (22)	26 (16)	1.48 (0.58–3.77)	0.414
Quinolone	19 (59)	50 (31)	3.30 (1.51–7.21)	0.002
Macrolide	13 (41)	58 (36)	1.24 (0.57–2.69)	0.588
Aminoglycoside	2 (6)	41 (25)	0.20 (0.05–0.87)	0.018
Cephalosporin	20 (63)	105 (64)	0.92 (0.42–2.02)	0.836
30-d in-hospital deaths	9 (28)	18 (11)	3.15 (1.27–7.86)	0.021

*Values are no. (%) patients except as indicated. MRSA, methicillin-resistant *Staphylococcus aureus*; UTD; unable to determine because calculation includes zero; IQR; interquartile range; MSSA, methicillin-susceptible *S. aureus*.

Table 2. Adjusted regression analysis of the association between influenza and 30-d in-hospital deaths among patients with *Staphylococcus aureus*–positive respiratory cultures, Maryland and Iowa, USA*

Model and variable	Odds ratio (95% CI)	p value
Unadjusted		
Influenza-positive test	3.15 (1.27–7.86)	0.021
Adjusted†		
Influenza-positive test	4.31 (1.57–11.83)	0.005
First positive *S. aureus* culture collected ≤2 d after hospital admission	3.00 (1.18–7.61)	0.021
Year of first positive *S. aureus* culture, 2008–2009†	1.71 (0.70–4.13)	0.237

*Defined as death occurring in the hospital within 30 d of the first respiratory culture that grew *S. aureus*.
†Reference 2003–2007.

Additionally, co-infection with influenza and *S. aureus* has been examined in animal models to identify mechanisms that cause poor outcomes (*8*–*12*). Severity of illness related to co-infection has been associated with a dysfunctional cell repair system and an altered immunologic response such as suppression of macrophage function, inhibition in phagocytic bacterial clearance, and cell damage to the airway system (*8*–*12*). Investigators have hypothesized that influenza damages epithelial cells in the respiratory system, providing opportunity for enhanced bacterial attachment (*8*,*11*). Once bacteria invade, cell destruction and fluid cause dysfunction of the airway system (*8*,*11*).

This study had limitations. First, the investigation might have excluded patients who were tested for influenza at other facilities or who did not have laboratory-confirmed influenza. Second, we could not determine whether the respiratory cultures that grew *S. aureus* represented infections or colonization. However, the information we describe remains clinically relevant because often clinicians do not know whether patients with positive *S. aureus* cultures are infected or colonized. Diagnosing *S. aureus* pneumonia is challenging, and acquiring a lower respiratory culture such as a bronchial specimen or tracheal aspirate can be invasive and difficult to collect. Therefore, if *S. aureus* pneumonia is suspected (e.g., symptoms and positive sputum culture), patients may be treated without a confirmed positive lower respiratory culture. Third, our dataset did not include information about variables such as influenza vaccination status, mechanical ventilation, co-infection with organisms other than influenza and *S. aureus*, and whether the pneumonia was necrotizing. Fourth, misclassification bias may exist based on our definition of influenza infection. Patients with a negative influenza test may be misclassified since we were unable to determine the time interval between the onset of ILI symptoms and the collection of the influenza sample. Therefore, patients may have recovered from influenza before receiving an influenza test. Last, influenza-like illness ICD-9-CM codes were used to identify the cohort because the patients initially were included in a study of influenza-like illness and *S. aureus* pneumonia (J.S. McDanel, unpub. data). Therefore, patients may have been missed if they had a respiratory infection with *S. aureus* and the condition or symptoms were not captured through an ICD-9-CM code.

In conclusion, among patients whose respiratory cultures grew *S. aureus*, patients with influenza were significantly more likely to die than were patients whose influenza tests were negative. Interventions that increase influenza vaccination rates among patients at high risk for *S. aureus* respiratory infections may prevent both co-infection and death.

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References

1. Kollef MH, Shorr A, Tabak YP, Gupta V, Liu LZ, Johannes RS. Epidemiology and outcomes of health-care-associated pneumonia: results from a large US database of culture-positive pneumonia. Chest. 2005;128:3854–62. http://dx.doi.org/10.1378/chest.128.6.3854
2. Centers for Disease Control and Prevention (CDC). Four pediatric deaths from community-acquired methicillin-resistant *Staphylococcus aureus*—Minnesota and North Dakota, 1997–1999. MMWR Morb Mortal Wkly Rep. 1999;48:707–10.
3. Centers for Disease Control and Prevention (CDC). Severe methicillin-resistant *Staphylococcus aureus* community-acquired pneumonia associated with influenza—–Louisiana and Georgia, December 2006-January 2007. MMWR Morb Mortal Wkly Rep. 2007;56:325–9.
4. Hageman JC, Uyeki TM, Francis JS, Jernigan DB, Wheeler JG, Bridges CB, et al. Severe community-acquired pneumonia due to *Staphylococcus aureus*, 2003–04 influenza season. Emerg Infect Dis. 2006;12:894–9. http://dx.doi.org/10.3201/eid1206.051141
5. Marsden-Haug N, Foster VB, Gould PL, Elbert E, Wang H, Pavlin JA. Code-based syndromic surveillance for influenza like

illness by International Classification of Diseases, Ninth Revision. Emerg Infect Dis. 2007;13:207–16. http://dx.doi.org/10.3201/eid1302.060557
6. Deyo RA, Cherkin DC, Ciol MA. Adapting a clinical comorbidity index for use with ICD-9-CM administrative databases. J Clin Epidemiol. 1992;45:613–9. http://dx.doi.org/10.1016/0895-4356(92)90133-8
7. Kallen AJ, Brunkard J, Moore Z, Budge, P. Arnold KE, Fosheim G, et al. *Staphylococcus aureus* community-acquired pneumonia during the 2006 to 2007 influenza season. Ann Emerg Med. 2009;53:358–65. http://dx.doi.org/10.1016/j.annemergmed.2008.04.027
8. Kash JC, Taubenberger JK. The role of viral, host and secondary bacterial factors in influenza pathogenesis. Am J Pathol. 2015; 185:1528–36. http://dx.doi.org/10.1016/j.ajpath.2014.08.030
9. Robinson KM, McHugh KJ, Mandalapu S, Clay ME, Lee B, Scheller EV, et al. Influenza A virus exacerbates *Staphylococcus aureus* pneumonia in mice by attenuating antimicrobial peptide production. J Infect Dis. 2014;209:865–75. http://dx.doi.org/10.1093/infdis/jit527
10. Sun K, Metzger DW. Influenza infection suppresses NADPH oxidase-dependent phagocytic bacterial clearance and enhances susceptibility to secondary methicillin-resistant *Staphylococcus aureus* infection. J Immunol. 2014;192:3301–7. http://dx.doi.org/10.4049/jimmunol.1303049
11. Kash JC, Walters KA, Davis AS, Sandouk A, Schwartzman LM, Jagger BW, Chertow DS, Li Q, Kuestner RE, Ozinsky A, Taubenberger JK. Lethal synergism of 2009 pandemic H1N1 influenza virus and Streptococcus pneumoniae coinfection is associated with loss of murine lung repair responses. MBiol 2011; 2:e00172e11. http://dx.doi.org/10.1128/mBio.00172-11
12. Kostrzewska K, Massalski W, Narbutowicz B, Zielinski W. Pulmonary staphylococcal complications in patients during the influenza epidemic in 1971–1972. Mater Med Pol. 1974;6:207–12.

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Extended-Spectrum Cephalosporin-Resistant *Salmonella enterica* serovar Heidelberg Strains, the Netherlands[1]

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Extended-spectrum cephalosporin-resistant *Salmonella enterica* serovar Heidelberg strains (JF6X01.0022/XbaI.0251, JF6X01.0326/XbaI.1966, JF6X01.0258/XbaI.1968, and JF6X01.0045/XbaI.1970) have been identified in the United States with pulsed-field gel electrophoresis. Our examination of isolates showed introduction of these strains in the Netherlands and highlight the need for active surveillance and intervention strategies by public health organizations.

Salmonella enterica serovar Heidelberg is among the most prevalent causes of human salmonellosis in the United States and Canada but has been reported infrequently in Europe (*1*–*3*). Although most nontyphoidal *Salmonella* infections are self-limiting and resolve within a few days, *Salmonella* ser. Heidelberg tends to provoke invasive infections (e.g., myocarditis and bacteremia) that require antimicrobial drug therapy (*4*). To treat systemic nontyphoidal *Salmonella* infections, third-generation cephalosporins are preferred drugs for children or for adults with fluoroquinolone contraindications (*5*). Resistance to third-generation cephalosporins is increasing in *S. enterica* infections, mainly because of production of plasmid-mediated extended-spectrum or AmpC β-lactamases (*6*).

Resistance to extended-spectrum cephalosporins (ESCs) among *Salmonella* Heidelberg strains found in human infections, food-producing animals, and poultry meat indicates zoonotic and foodborne transmission of these strains and potential effects on public health (*7*,*8*). Unlike in Canada and the United States, few ESC-resistant *Salmonella* Heidelberg strains have been documented in Europe (*9*–*13*). However, increased occurrence of ESC resistance in *S. enterica* infections and decreased susceptibility to fluoroquinolones compromise the use of these drugs and constitute a serious public health threat (*6*,*14*).

Few data are available regarding prevalence of ESC-resistant *Salmonella* Heidelberg isolates in Europe, their underlying antimicrobial drug resistance gene content, and genetic platforms (i.e., plasmids and insertion sequence [IS] elements) associated with resistance genes. We attempted to determine the occurrence and molecular characteristics of *Salmonella* Heidelberg isolates recovered from human patients, food-producing animals, and poultry meat in the Netherlands during 1999–2013.

The Study

During 1999–2013, the Netherlands National Institute of Public Health and the Environment collected 437 *Salmonella* Heidelberg isolates from human infections (n = 77 [17.6%]), food-producing animals (n = 138 [31.6%]), poultry meat (n = 170 [38.9%]), and other sources (n = 52 [11.9%]). From this collection, we selected 200 epidemiologically unrelated isolates for further analysis (Table; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/15-1377-Techapp.pdf).

MICs for antimicrobial agents were determined with the broth microdilution method (online Technical Appendix) and showed a higher frequency of multidrug non–wild-type susceptibility phenotype in isolates from poultry meat (n = 44 [68.8%]) than in isolates from food-producing animals (n = 14 [31.8%]) and human infections (n = 16 [19.5%]). Most human infections exhibited wild-type MICs to most antimicrobial agents tested (Table).

Of the 200 *Salmonella* Heidelberg isolates in the study, 47 (23.5%) were ESC resistant. ESC resistance in *Salmonella* Heidelberg isolates increased from 33.3% in 2011 to 60.0% in 2012 to 75.0% in 2013, after which *Salmonella* Heidelberg was the predominant serotype in ESC-resistant *Salmonella* isolates in the Netherlands (Figure 1).

These isolates showed MICs for cefotaxime and ceftazidime of 2 to >4 mg/L and 4 to >16 mg/L, respectively; non–wild-type susceptibility to fluoroquinolones was 87.2%. The emergence of isolates with decreased

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Table. Characteristics of *Salmonella enterica* serovar Heidelberg isolates recovered from human infections, food-producing animals, poultry meat, and other sources, the Netherlands, 1999–2013*

Source	1999–2001	2002–2004	2005–2007	2008–2010	2011–2013
Human infections					
No. isolates studied	13	10	22	23	15
Resistance phenotypes (no.)	Amp (1), AmpCol (1), AmpSmxTmpStr (1), AmpTetSmxTmpStr (1), SmxStr (1), Str (5), TetSmxTmpStr (1), WT (2)	AmpSmxStr (1), AmpTetSmx (1), SmxStr (3), Str (1), TetSmxStr (1), WT (3)	AmpFotTazStr (1), AmpSmxTmpNalCip (1), AmpTet (1), NalCip (2), SmxStr (1), Tet (1), TetSmxNalCip (1), WT (14)	ChlCol (1), Col (10), Str (1), StrCol (5), TetCol (1), TetNalCip (1), TetSmxTmp StrCol (1), TetStr KanCol (1), TetStr SmxCol (1), WT (1)	Col (1), Str (3), TetSmxStr (2), TetSmxTmp (1), WT (8)
No. ESCR isolates	0	0	1	0	0
Food-producing animals					
No. isolates studied	5	16	5	7	13
Resistance phenotypes (no.)	NalCip (1), WT (4)	Amp (3), AmpSmxTmpNalCipStr (2), AmpStr (2), NalCip (5), SmxStrTmp (1), WT (3)	AmpTetSmxTmpNalCip (1), WT (4)	AmpCol (1), AmpFotTazNalCip (1), AmpFotTazTetSmxGenStrKanCol (1), Col (4)	AmpCol (1), AmpFotTazTetSmx (1), AmpFotTazTetSmxNalCip (4), Col (2), TetSmxNalCip (2), TetSmxNalCipGenStrKan (1), WT (2)
No. ESCR isolates	0	0	0	2	5
Poultry meat					
No. isolates studied	3	3	15	6	40
Resistance phenotypes (no.)	AmpTetSmxTmpNalCipStr (1), SmxTmpStr (1), WT (1)	AmpSmxStr (1), WT (2)	NalCip (3), SmxCipGen (1), SmxGen (1), SmxTmpNalCip (1), TetSmxTmp (1), WT (8)	AmpFotTaz (1), AmpFotTazSmxTmpChlStrCol (1), AmpFotTazStrCol (1), Col (2), NalCipCol (1)	AmpFotTazTetSmxNalCip (26), AmpFotTazTetSmxNalCipCol (1), AmpFotTazTetSmxNalCipGenStrKan (1), AmpFotTazTetSmxNalCipStr (6), AmpFotTazTetSmxTmpNalCipChl (1), Col (2), TetSmxNalCip (1), TetSmxNalCipGenStr (1), TetSmxNalCipStr (1)
No. ESCR isolates	0	0	0	3	35
Other					
No. isolates studied	0	1	0	6	4
Resistance phenotypes (no.)		WT (1)		Col (2), NalCipCol (1), Str (1), StrCol (2)	AmpFotTazTetSmxNalCip (1), NalCipCol (1), Str (1), TetSmxNalCipGenStr (1)
No. ESCR isolates	0	0	0	0	1

*Amp, ampicillin; Cip, ciprofloxacin; Chl, chloramphenicol; Col, colistin; ESCR, extended-spectrum cephalosporin-resistant; Fot, cefotaxime; Gen, gentamicin; Kan, kanamycin; Nal, nalidixic acid; Smx, sulfamethoxazole; Str, streptomycin; Taz, ceftazidime; Tet, tetracycline; Tmp, trimethoprim; WT, wild type.

susceptibility to these first-line antimicrobial drugs limits effective treatment options for potential human infections.

ESC typing of the 47 isolates, performed by microarray analysis followed by PCR and sequencing (online Technical Appendix), revealed the presence of the $bla_{\text{CMY-2}}$ gene in 41 ESC-resistant *Salmonella* Heidelberg isolates that exhibited an AmpC β-lactamase phenotype. The other 6 isolates exhibited an extended-spectrum β-lactamase phenotype and encoded $bla_{\text{CTX-M-2}}$ (n = 4), $bla_{\text{CTX-M-1}}$ (n = 1), or $bla_{\text{CTX-M-14}}$ (n = 1) genes (Figure 2).

We assessed the genetic relatedness of the 47 cephalosporin-resistant *Salmonella* Heidelberg isolates by using the standardized *Xba*I–pulsed-field gel electrophoresis (PFGE) (online Technical Appendix), which identified 2 major PFGE types: *Xba*I.1968 and *Xba*I.1973 (PFGE numbers assigned by the European Centre for Disease Prevention and Control, Solna, Sweden). Of the 47 isolates, 26 (55.3%) belonged to *Xba*I.1968 and 5 (10.6%) belonged to *Xba*I.1973. Forty-one of the isolates were $bla_{\text{CMY-2}}$ carriers, 31 (75.6%) of which belonged to these 2 PFGE types; 10 (24.4%) were distributed equally among other PFGE types. Six of the 47 isolates were $bla_{\text{CTX-M}}$ carriers associated with 5 PFGE types (Figure 2). Comparing these isolates with those in the PulseNet database (http://www.cdc.gov/pulsenet/index.html) revealed the

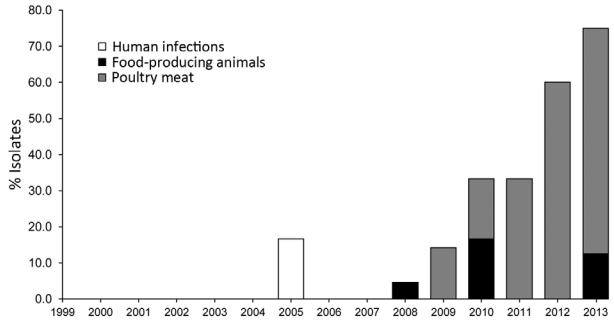

Figure 1. Occurrence of extended-spectrum cephalosporin-resistant *Salmonella enterica* serovar Heidelberg isolates, the Netherlands, 1999–2013.

introduction of 4 epidemic clones of ESC-resistant *Salmonella* Heidelberg strains in the Netherlands (JF6X01.0022/*Xba*I.0251, JF6X01.0326/*Xba*I.1966, JF6X01.0258/*Xba*I.1968, and JF6X01.0045/*Xba*I.1970). To raise awareness and determine whether related ESC-resistant *Salmonella* Heidelberg isolates had been observed in other European countries, the Epidemic Intelligence Information System (European Centre for Disease Prevention and Control) issued an alert on September 18, 2014.

We successfully transferred plasmids carrying extended-spectrum or AmpC β-lactamases from ESC-resistant *Salmonella* Heidelberg isolates to the recipient *E. coli* DH10B strain (online Technical Appendix). PCR-based Inc/Rep typing and multilocus or double-locus sequence typing (ST) of the plasmids revealed that the $bla_{\text{CMY-2}}$ or $bla_{\text{CTX-M}}$ genes were located on plasmids for 46 (97.8%) of the 47 isolates. ESC-resistant *Salmonella* Heidelberg isolates encoding $bla_{\text{CMY-2}}$ on IncI1/ST12 plasmids were associated predominantly with the *Xba*I.1968 (n = 26 [78.8%]) PFGE type; those encoding $bla_{\text{CMY-2}}$ on IncA/C plasmids were associated with *Xba*I.1973 (n = 5 [71.4%]). Isolates encoding $bla_{\text{CTX-M-2}}$ on IncHI2P/ST2, $bla_{\text{CTX-M-1}}$ on IncI1/ST49, and $bla_{\text{CTX-M-14}}$ on IncI1/ST80 plasmids were associated with *Xba*I.1964, *Xba*I.1963, and *Xba*I.1966, respectively (Figure 2).

The $bla_{\text{CMY-2}}$ gene was present in 12 different PFGE types and was carried on plasmids of 2 different incompatibility groups (IncI1/ST12 and IncA/C) or on the chromosome. This gene's diverse genetic background suggests that emergence of the $bla_{\text{CMY-2}}$–producing *Salmonella* Heidelberg strain in the Netherlands results not only from expansion of a single clone but from multiclonal dissemination of the strain and horizontal transfer of plasmids encoding the $bla_{\text{CMY-2}}$ gene. IncI1/ST12 and IncA/C plasmids have been associated with the $bla_{\text{CMY-2}}$ gene in *Salmonella* Heidelberg isolates in the United States and Canada (*8*,*15*).

We analyzed a subset of ESC-resistant *Salmonella* Heidelberg isolates to determine the size and conjugation frequency of plasmids carrying extended-spectrum and AmpC β-lactamases. We also assessed a subset of *Salmonella* Heidelberg isolates (n = 17) for each PFGE type, including isolates for each type if they showed variation in extended-spectrum and AmpC β-lactamase genes or in gene location. This assessment sought to detect the upstream presence of resistance genes ($bla_{\text{CTX-M}}$ and bla_{CMY}) of frequently encountered insertion sequences (IS*Ecp1*, IS*CR1*, and IS*26*) (Figure 2; online Technical Appendix).

We attribute the increase of ESC-resistant *Salmonella* Heidelberg isolates in the Netherlands to the frequent occurrence of isolates carrying IncI1/ST12 plasmids encoding $bla_{\text{CMY-2}}$ in food-producing animals and poultry products imported from Brazil. Isolates from imported poultry products are associated predominantly with PFGE types *Xba*I.1968 and *Xba*I.1973 (Figure 2). A similar introduction of ESC-resistant *Salmonella* Heidelberg strains in Ireland was associated with imported poultry meat from Brazil (R. Slowey, pers. comm.). Although ESC-resistant *Salmonella* Heidelberg strains are rarely reported in Europe, their introduction through imported poultry meat could pose a public health risk; Brazil is among the world's leading countries for exporting poultry meat.

Conclusions

Most ESC-resistant *Salmonella* Heidelberg isolates in our study had profiles (*Xba*I.0251, *Xba*I.1966, *Xba*I.1968, and *Xba*I.1970) indistinguishable from those of previous epidemic types (JF6X01.0022, JF6X01.0326, JF6X01.0258, and JF6X01.0045) that caused outbreaks and showed potency for bloodstream infections (*16*). Our identification of clonal clusters shared by ESC-resistant *Salmonella* Heidelberg strains in food-producing animals or poultry meat that can cause human infections underscores the risk for potential zoonotic or foodborne transmission of these strains to humans.

Although we observed a frequent occurrence of ESC-resistant *Salmonella* Heidelberg isolates in poultry products, no human infections linked to these contaminated products have been yet documented in the Netherlands. Nevertheless, the risk of potential zoonotic or foodborne transmission of ESC-resistant *Salmonella* Heidelberg strains highlights the necessity for active surveillance and intervention strategies by public health organizations.

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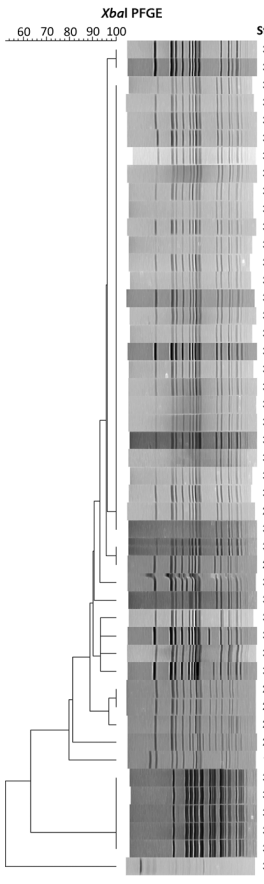

Strain ID	Year	Source	Origin	Resistance phenotype	PFGE types*	β-lactamase gene	Location	Inc/ Rep types	pST/ pCC	Size, kb	Conjugation frequency	Upstream region
365.49	2013	FPA	ND	AmpFotTazTetSmx	XbaI.0251/ JF6X01.0022	$bla_{CTX-M-2}$	Plasmid	IncHI2P	2	242	1.8×10^{-6}	IS*CR1*
364.04	2013	PM	ND	AmpFotTazTetSmxNalCipGenStrKan	XbaI.0251/ JF6X01.0022	$bla_{CTX-M-2}$	Plasmid	IncHI2P	2			
358.26	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
326.49	2012	PM	Brazil	AmpFotTazTetSmxNalCipStr	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12	97	1.2×10^{-3}	IS*Ecp1*
353.78	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
353.53	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
308.62	2011	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
326.76	2012	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
360.50	2013	FPA	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
359.76	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
353.34	2013	PM	Brazil	AmpFotTazTetSmxNalCipStr	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
359.39	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
357.52	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
361.23	2013	PM	Brazil	AmpFotTazTetSmxNalCipStr	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
305.11	2011	PM	ND	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
326.22	2012	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
361.17	2013	FPA	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
364.06	2013	PM	Brazil	AmpFotTazTetSmxTmpNalCipChl	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
360.40	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
335.04	2012	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
357.49	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
350.25	2012	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
362.10	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
318.28	2011	PM	ND	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
361.06	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
357.62	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
299.58	2011	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
370.43	2013	PM	ND	AmpFotTazTetSmxNalCipCol	XbaI.1968/ JF6X01.0258	bla_{CMY-2}	Plasmid	IncI1	12/12			
370.16	2013	PM	ND	AmpFotTazTetSmxNalCip	XbaI.1976	bla_{CMY-2}	Plasmid	IncI1	12/12	97	1.8×10^{-6}	IS*Ecp1*
254.46	2009	PM	Netherlands	AmpFotTazStrCol	XbaI.1970/ JF6X01.0045	bla_{CMY-2}	Plasmid	IncI1	12/12	76	7.0×10^{-6}†	IS*Ecp1*
353.63	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1969	bla_{CMY-2}	Chromosome‡	NA	NA	NA	0§	IS*Ecp1*
364.07	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1975	bla_{CMY-2}	Plasmid	IncI1	12/12	97	1.3×10^{-3}	IS*Ecp1*
360.16	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.0252	bla_{CMY-2}	Plasmid	IncI1	12/12	97	3.8×10^{-3}	IS*Ecp1*
366.65	2013	FPA	ND	AmpFotTazTetSmxNalCip	XbaI.1965	bla_{CMY-2}	Plasmid	IncA/C	NA	218	1.0×10^{-9}†	IS*Ecp1*
311.07	2011	PM	Brazil	AmpFotTazTetSmxNalCipStr	XbaI.1992	bla_{CMY-2}	Plasmid	IncI1	12/12	97	8.1×10^{-3}	IS*Ecp1*
362.81	2013	PM	Brazil	AmpFotTazTetSmxNalCipStr	XbaI.1971	bla_{CMY-2}	Plasmid	IncI1	12/12	194	2.3×10^{-4}	IS*Ecp1*
269.16	2010	PM	ND	AmpFotTaz	XbaI.1966/ JF6X01.0326	$bla_{CTX-M-14}$	Plasmid	IncI1	80	105	1.2×10^{-3}	¶
274.39	2010	FPA	Argentina	AmpFotTazNalCip	XbaI.1966/ JF6X01.0326	bla_{CMY-2}	Plasmid	IncI1	12/12	110	5.3×10^{-3}	IS*Ecp1*
243.23	2008	FPA	ND	AmpFotTazTetSmxGenStrKanCol	XbaI.1967	$bla_{CTX-M-2}$	Plasmid	IncHI2P	2	242	0§	¶
249.37	2009	PM	ND	AmpFotTazSmxTmpChlStrCol	XbaI.1964	$bla_{CTX-M-2}$	Plasmid	IncHI2P	2	290	1.4×10^{-8}	IS*CR1*
133.52	2005	HI	Netherlands	AmpFotTazStr	XbaI.1963	$bla_{CTX-M-1}$	Plasmid	IncI1	49/9	83	1.7×10^{-3}	IS*Ecp1*
365.07	2013	PM	Brazil	AmpFotTazTetSmxNalCipStr	XbaI.1973	bla_{CMY-2}	Plasmid	IncA/C	NA			
327.32	2012	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1973	bla_{CMY-2}	Plasmid	IncA/C	NA			
369.05	2013	FPA	ND	AmpFotTazTetSmxNalCip	XbaI.1973	bla_{CMY-2}	Plasmid	IncA/C	NA			
363.78	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1973	bla_{CMY-2}	Plasmid	IncA/C	NA	218	3.5×10^{-9}†	IS*Ecp1*
359.42	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.1973	bla_{CMY-2}	Plasmid	IncA/C	NA			
353.62	2013	PM	Brazil	AmpFotTazTetSmxNalCip	XbaI.X#	bla_{CMY-2}	Plasmid	IncA/C	NA	218	7.0×10^{-9}†	IS*Ecp1*

Figure 2. Characteristics of extended-spectrum cephalosporin-resistant *Salmonella enterica* serovar Heidelberg isolates, the Netherlands, 1999–2013. The dendrogram was generated by using BioNumerics version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium) and indicates results of a cluster analysis on the basis of *Xba*I–pulsed-field gel electrophoresis (PFGE) fingerprinting. Similarity between the profiles was calculated with the Dice similarity coefficient and used 1% optimization and 1% band tolerance as position tolerance settings. The dendrogram was constructed with the UPGMA method based on the resulting similarity matrix. Amp, ampicillin; Cip, ciprofloxacin; Chl, chloramphenicol; Col, colistin; Fot, cefotaxime; FPA, food-producing animals; Gen, gentamicin; HI, human infection; Kan, kanamycin; Nal, nalidixic acid; ND, not determined (i.e., refers to isolates recovered in the Netherlands but with unknown origin of the sample); pCC, plasmid clonal complex; PM, poultry meat; pST, plasmid sequence type; Smx, sulfamethoxazole; Str, streptomycin; Taz, ceftazidime; Tet, tetracycline; Tmp, trimethoprim. *Pattern numbers assigned by The European Surveillance System molecular surveillance service of the European Centre for Disease Prevention and Control database and corresponding pattern numbers from the PulseNet database (http://www.cdc.gov/pulsenet/index.html). †Results refer to the conjugation frequencies during filter-mating experiments. ‡Chromosomal location confirmed by I-*Ceu*I PFGE of total bacterial DNA, followed by Southern blot hybridization. §No transconjugants were obtained after liquid and filter-mating experiments, suggesting the presence of nonconjugative plasmids or conjugation frequencies below detection limits. ¶Insertion sequences IS*Ecp1*, IS*CR1*, or IS26 were not found upstream of the extended-spectrum β-lactamase genes for these PFGE types. #This PFGE fingerprint was not submitted to The European Surveillance System molecular surveillance service of the European Centre for Disease Prevention and Control database for name assignment.

from the PulseNet database; we also thank John Egan and Rosemarie Slowey for providing information about the ESC-resistant *S. enterica* ser. Heidelberg strains detected in Ireland.

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References

1. European Centre for Disease Prevention and Control. Technical document. EU protocol for harmonised monitoring of antimicrobial resistance in human Salmonella and Campylobacter isolates. Stockholm: The Centre; 2014.
2. Public Health Agency of Canada. National Enteric Surveillance Program (NESP). Annual summary 2012. Ottawa (Ontario, CA): The Agency; 2014.

3. Centers for Disease Control and Prevention. National Antimicrobial Resistance Monitoring System: enteric bacteria. 2012 Human isolates final report. Atlanta: The Centers; 2014.
4. Hoffmann M, Zhao S, Pettengill J, Luo Y, Monday SR, Abbott J, et al. Comparative genomic analysis and virulence differences in closely related *Salmonella enterica* serotype Heidelberg isolates from humans, retail meats, and animals. Genome Biol Evol. 2014;6:1046–68. http://dx.doi.org/10.1093/gbe/evu079
5. Acheson D, Hohmann EL. Nontyphoidal salmonellosis. Clin Infect Dis. 2001;32:263–9. http://dx.doi.org/10.1086/318457
6. Miriagou V, Tassios PT, Legakis NJ, Tzouvelekis LS. Expanded-spectrum cephalosporin resistance in non-typhoid *Salmonella*. Int J Antimicrob Agents. 2004;23:547–55. http://dx.doi.org/10.1016/j.ijantimicag.2004.03.006
7. Dutil L, Irwin R, Finley R, Ng LK, Avery B, Boerlin P, et al. Ceftiofur resistance in *Salmonella enterica* serovar Heidelberg from chicken meat and humans, Canada. Emerg Infect Dis. 2010;16:48–54. http://dx.doi.org/10.3201/eid1601.090729
8. Folster JP, Pecic G, Singh A, Duval B, Rickert R, Ayers S, et al. Characterization of extended-spectrum cephalosporin-resistant *Salmonella enterica* serovar Heidelberg isolated from food animals, retail meat, and humans in the United States 2009. Foodborne Pathog Dis. 2012;9:638–45. http://dx.doi.org/10.1089/fpd.2012.1130
9. Aarestrup FM, Hasman H, Olsen I, Sørensen G. International spread of bla_{CMY-2}–mediated cephalosporin resistance in a multiresistant *Salmonella enterica* serovar Heidelberg isolate stemming from the importation of a boar by Denmark from Canada. Antimicrob Agents Chemother. 2004;48:1916–7. http://dx.doi.org/10.1128/AAC.48.5.1916-1917.2004
10. Miriagou V, Filip R, Coman G, Tzouvelekis LS. Expanded-spectrum cephalosporin-resistant *Salmonella* strains in Romania. J Clin Microbiol. 2002;40:4334–6. http://dx.doi.org/10.1128/JCM.40.11.4334-4336.2002
11. González-Sanz R, Herrera-León S, de la Fuente M, Arroyo M, Echeita MA. Emergence of extended-spectrum β-lactamases and AmpC-type β-lactamases in human *Salmonella* isolated in Spain from 2001 to 2005. J Antimicrob Chemother. 2009;64:1181–6. http://dx.doi.org/10.1093/jac/dkp361
12. Batchelor M, Hopkins KL, Threlfall EJ, Clifton-Hadley FA, Stallwood AD, Davies RH, et al. Characterization of AmpC-mediated resistance in clinical *Salmonella* isolates recovered from humans during the period 1992 to 2003 in England and Wales. J Clin Microbiol. 2005;43:2261–5. http://dx.doi.org/10.1128/JCM.43.5.2261-2265.2005
13. Burke L, Hopkins KL, Meunier D, de Pinna E, Fitzgerald-Hughes D, Humphreys H, et al. Resistance to third-generation cephalosporins in human non-typhoidal *Salmonella enterica* isolates from England and Wales, 2010–12. J Antimicrob Chemother. 2014;69:977–81. http://dx.doi.org/10.1093/jac/dkt469
14. Piddock LJ. Fluoroquinolone resistance in *Salmonella* serovars isolated from humans and food animals. FEMS Microbiol Rev. 2002;26:3–16. http://dx.doi.org/10.1111/j.1574-6976.2002.tb00596.x
15. Andrysiak AK, Olson AB, Tracz DM, Dore K, Irwin R, Ng LK, et al.; Canadian Integrated Program for Antimicrobial Resistance Surveillance Collaborative. Genetic characterization of clinical and agri-food isolates of multi drug resistant *Salmonella enterica* serovar Heidelberg from Canada. BMC Microbiol. 2008;8:89. http://dx.doi.org/10.1186/1471-2180-8-89
16. Centers for Disease Control and Prevention. Investigation update: multistate outbreak of human *Salmonella* Heidelberg infections linked to "kosher broiled chicken livers" from Schreiber Processing Corporation. 2012 Jan 11 [cited 2015 Jul 23]. http://www.cdc.gov/salmonella/2011/chicken-liver-1-11-2012.html

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Identification of *Streptococcus suis* Meningitis through Population-Based Surveillance, Togo, 2010–2014

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During 2010–2014, we enrolled 511 patients with suspected bacterial meningitis into surveillance in 2 districts of northern Togo. We identified 15 persons with *Streptococcus suis* infection; 10 had occupational contact with pigs, and 12 suffered neurologic sequelae. *S. suis* testing should be considered in rural areas of the African meningitis belt.

Streptococcus suis, an encapsulated, gram-positive bacterium, commonly colonizes the respiratory, genital, and intestinal tracts of pigs and may cause severe disease, including meningitis, sepsis, and bronchopneumonia (*1*). Zoonotic cases of *S. suis* invasive disease have been documented in Europe, Asia, the Americas, and Oceania among persons in direct contact with pigs or pork meat; the case-fatality rate is 10%–20%, and neurologic sequelae frequently occur (*2*–*4*). Two large outbreaks have occurred in China (*5*,*6*), but little is known about the disease among humans in Africa.

We began surveillance in May 2010 for acute bacterial meningitis in hospitals in 2 rural districts in Togo. The National Ethical Committee of Togo reviewed and approved our study protocols.

The Study

During 2010–2014, we conducted surveillance for patients with signs and symptoms of meningitis at 5 hospitals in northern Togo: 4 in Dapaong, Tône District, and 1 in Cinkassé, Cinkassé District. These districts are within the African meningitis belt and experience annual outbreaks of hyperendemic bacterial meningitis during the dry season (November–April) and generalized epidemics every 4–6 years (*7*). Lumbar puncture was performed at admission on all patients with suspected meningitis, provided informed consent had been given. Cerebrospinal fluid (CSF) specimens were transferred to the regional bacteriology laboratory in Dapaong for cytologic testing, Gram staining, latex agglutination, and culture. CSF samples were further tested by conventional PCR at Centre Muraz Laboratory (Bobo-Dioulasso, Burkina Faso) or Institut National d'Hygiène (Lomé, Togo) for identification of *S. pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* type b (Hib).

Beginning in August 2011, *Streptococcus* isolates from Dapaong were stored at –80°C in STGG (skim milk, tryptone, glucose, glycerol) medium and sent to the National Reference Center for Streptococci (Aachen, Germany) for confirmatory testing. In April 2013, after the reference laboratory identified several cases of *S. suis* infection, the bacteriology laboratory in Dapaong implemented additional diagnostic testing using the API Kit (bioMérieux, Marcy l'Etoile, France) to enable rapid case detection by culture. For species confirmation and molecular typing, we sequenced the genome of *S. suis* isolates by using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and the MiSeq Reagent Kit v3 (Illumina) for 2 × 300-bp paired-end reads. We deposited sequence data in the European Nucleotide Archive (accession no. PRJEB12952).

In June 2014, we visited all identified *S. suis* meningitis case-patients and used a questionnaire to collect data on their environments and contact with pigs and pork meat. Thereafter, we prospectively administered the questionnaire to new case-patients. We used Stata 12 (StataCorp LP, College Station, TX, USA) to analyze the data.

During August 2010–July 2014, we enrolled 511 persons with suspected bacterial meningitis, of whom 126 (24.6%) were <5 years of age. We performed lumbar puncture on 489 enrollees: 89 were positive for *S. pneumoniae*, 60 for *N. meningitidis*, 15 for *S. suis*, 9 for *Streptococcus* sp., 8 for Hib, and 7 for other identified pathogens; 301 had no identified etiology. *S. suis*–positive cases were confirmed by genome sequence analysis of the isolates, using

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Kraken (*8*) and comparative analyses with reference *S. suis* genomes (*9*) and other outlier species. We predicted that all 15 *S. suis* isolates were serotype 2 due to 100% coverage of the associated capsular polysaccharide loci sequence (*10*); 6 were sequence type 1, and 11 were a single-locus variant of sequence type 1 exhibiting a new *rec*A allele sequence (http://ssuis.mlst.net/).

S. suis cases peaked in April through August each year (Figure). Of the 15 *S. suis* patients, 3 were 5–14 years of age, 7 were 30–49 years of age, and 5 were ≥50 years of age; 12 (80%) patients were male (Table 1). Median time from symptom onset to hospitalization was 2 days (interquartile range 1–4 days), similar to the time for patients with meningitis caused by other pathogens. One (6.7%) patient died; 12 (85%) of the 14 survivors had neurologic sequelae (Table 1). Resistance to antimicrobial drugs was relatively uncommon (Table 2).

All 15 *S. suis* meningitis patients were involved in pig farming or slaughtering or had a family member who was: 3 each were pig farmers only or butchers only, 3 were pig farmers and involved in slaughtering, and 6 had a family member engaged in 1 of these activities. Twelve patients reported handling pig meat during cooking; 14 reported eating pork (9 at least once per week). Three patients reported that a family member or neighbor had also contracted meningitis and had subsequent hearing loss.

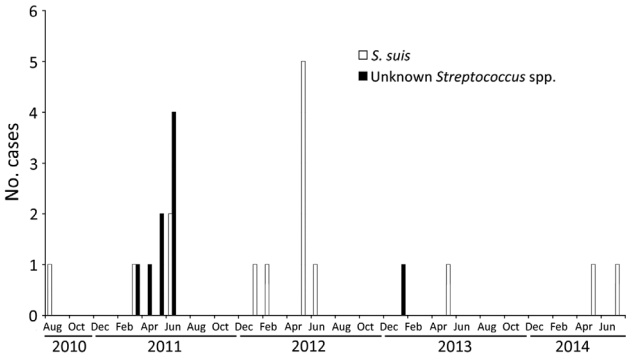

Figure. *Streptococcus suis* and other *Streptococcus* spp. infections identified per month through acute bacterial meningitis surveillance in northern Togo, 2010–2014.

Conclusions

In the area of Togo under surveillance, meningitis cases peak during the dry season, and *S. pneumoniae* and *N. meningitidis* have been the leading causal agents since the introduction of Hib conjugate vaccine in 2008. During 2010–2014, we identified 15 cases of *S. suis* meningitis, representing 3.1% of all suspected bacterial meningitis cases and 8.0% of etiologically confirmed cases. These numbers are probably an underestimate because to identify *S. suis*, we relied on culture only, whereas we used CSF PCR to test for other bacteria. Furthermore, 9 *Streptococcus* isolates did not survive to be sent for confirmatory testing and may represent additional *S. suis* cases.

Our investigation showed that two thirds of *S. suis* meningitis patients were involved in pig farming or slaughtering, and the remainder had a family member who was involved in these activities. Most case-patients regularly cooked and ate pork. *S. suis* meningitis cases appear to have a seasonal pattern, clustering in May–July, a period of intensive slaughtering, during which pigs are confined to pens, and run-off water accumulates nearby in open-air pits. Slaughtering is conducted in butcher shops, where carcasses remain for several days. Approximately 30 pork

Table 1. Characteristics of patients from 2 rural districts with meningitis caused by various pathogens, Togo, 2010–2014

Characteristic	No. (%) case-patients infected with					No. (%) all case-patients, N = 489
	S. suis, n = 15	Other *Streptococcus* sp., n = 9	*S. pneumoniae*, n = 89	Other infections, n = 75*	No etiologic agent, n = 301	
Age, y						
<5	0	3 (33)	12 (13)	22 (29)	84 (28)	121 (25)
5–14	3 (20)	0	34 (38)	36 (48)	74 (25)	147 (30)
15–29	0	2 (22)	23 (26)	7 (9)	65 (22)	97 (20)
30–49	7 (47)	4 (44)	13 (15)	8 (11)	52 (17)	84 (17)
≥50	5 (33)	0	7 (8)	2 (3)	26 (9)	40 (8)
Sex						
M	12 (80)	6 (67)	42 (47)	39 (52)	156 (52)	255 (52)
F	0 (0)	3 (33)	47 (53)	36 (48)	145 (48)	244 (48)
Died	1 (7)	1 (11)	29 (33)	5 (7)	41 (14)	77 (16)
Sequelae						
Any†	12 (80)	2 (22)	17 (19)	5 (7)	27 (9)	63 (13)
Hearing loss‡	8 (67)	1 (50)	8 (47)	3 (60)	4 (15)	24 (5)
Paralysis‡	2 (17)	1 (50)	3 (18)	2 (40)	6 (22)	14 (3)
Visual impairment‡	5 (42)	0	2 (12)	0	1 (4)	8 (2)
Seizure disorder‡	0	0	0	0	2 (7)	2 (0)

*Includes 60 case-patients with *Neisseria meningitidis*, 11 with *Haemophilus influenzae*, 1 with *Escherichia coli*, 1 with *Staphylococcus aureus*, 1 with *Streptococcus pyogenes*, and 1 undetermined.
†Percentages calculated among all patients.
‡Percentages calculated among patients with sequelae.

Table 2. Antimicrobial resistance patterns of *Streptococcus suis* and other *Streptococcus* spp. isolates from meningitis patients in 2 rural districts in northern Togo, 2010–2014

Antimicrobial drug	No. *S. suis* samples tested/no. (%) susceptible		No. other *Streptococcus* spp. samples tested/no. (%) susceptible
	Tested at local laboratory*	Tested at reference laboratory†	
Amoxicillin	15/15 (100)	11/10 (91)	9/5 (56)
Cefotaxime	0	11/10 (91)	0
Ceftriaxone	15/15 (100)	0	9/8 (89)
Chloramphenicol	15/12 (80)	11/11 (100)	9/8 (89)
Clindamycin	0	11/10 (91)	0
Cotrimoxazole	15/14 (93)	0	9/6 (67)
Gentamicin	15/7 (47)	0	9/4 (44)
Levofloxacin	0	11/10 (91)	0
Oxacillin	12/3 (25)	11/11 (100)	1/0
Penicillin	15/15 (100)	11/10 (91)	9/5 (56)
Rifampin	15/15 (100)	0	9/6 (67)
Tetracycline	0	11/0	0

*The regional bacteriology laboratory in Dapaong, Togo.
†National Reference Center for Streptococci in Aachen, Germany.

butchers work in Dapaong, and overall, >1,000 pigs are slaughtered in Tône District each year (G. A. Boukaya, Direction Régionale de l'Agriculture, de l'Elevage et de la Pêche des Savanes, pers. comm., 2015 Aug 1). Based on these data, the cumulative incidence of *S. suis* meningitis among Dapaong butchers during 2010–2014 was 20% (6 cases/30 butchers), compared with 0.00375% (15 cases/400,000 total population) in Tône and Cinkassé Districts (incidence rate ratio 5,333). Although this was not a rigorously controlled prospective study, our results are highly suggestive of an association between butchering pigs and acquisition of *S. suis* meningitis; pig contact through farming or cooking may also be a risk factor. Three patients reported that a close contact had also contracted meningitis; these cases probably reflect acquisition from a shared environmental source.

We plan to continue laboratory testing for *S. suis* among patients enrolled in bacterial meningitis surveillance in northern Togo and investigating risk factors among confirmed case-patients. Biochemical testing for speciation of *Streptococcus* spp. is rarely part of routine bacteriologic evaluation of CSF in Africa and was not done at our site before the initial identification of several *S. suis* cases. *S. suis* testing should be considered for meningitis patients in areas of rural Africa where pig farming is common. All *S. suis* isolates in our study were susceptible to ceftriaxone, the presumptive therapy for nonepidemic meningitis in the meningitis belt; consequently, our findings do not suggest a need for altering current therapeutic guidelines. To reduce the incidence of *S. suis* meningitis, future efforts should first more fully delineate the practices that increase the risk for infection and then be directed toward educational campaigns targeting groups at high risk.

Dr. Tall is an epidemiologist and project coordinator at AMP in Ouagadougou, Burkina Faso. She has coordinated a meningitis and pneumonia surveillance study in northern Togo since 2011 and focuses on building country capacity for bacterial meningitis case–based surveillance in the African meningitis belt.

References

1. Gottschalk M. Streptococcosis. In: Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ, editors. Diseases of swine. 10th ed. Ames (IA): Blackwell Publishing; 2012. p. 841–55.
2. Goyette-Desjardins G, Auger JP, Xu J, Segura M, Gottschalk M. *Streptococcus suis*, an important pig pathogen and emerging zoonotic agent—an update on the worldwide distribution based on serotyping and sequence typing. Emerg Microbes Infect. 2014;3:e45. http://dx.doi.org/10.1038/emi.2014.45
3. Segura M, Zheng H, de Greeff A, Gao GF, Grenier D, Jiang Y, et al. Latest developments on *Streptococcus suis*: an emerging zoonotic pathogen: part 1. Future Microbiol. 2014;9:441–4. http://dx.doi.org/10.2217/fmb.14.14
4. Segura M, Zheng H, de Greeff A, Gao GF, Grenier D, Jiang Y, et al. Latest developments on *Streptococcus suis*: an emerging zoonotic pathogen: part 2. Future Microbiol. 2014;9:587–91. http://dx.doi.org/10.2217/fmb.14.15
5. World Health Organization. Outbreak associated with *Streptococcus suis* in pigs, China. Wkly Epidemiol Rec. 2005;80:269–70.
6. Yu H, Jing H, Chen Z, Zheng H, Zhu X, Wang H, et al. Human *Streptococcus suis* outbreak, Sichuan, China. Emerg Infect Dis. 2006;12:914–20. http://dx.doi.org/10.3201/eid1206.051194
7. Greenwood B. Editorial: 100 years of epidemic meningitis in West Africa—has anything changed? Trop Med Int Health. 2006;11:773–80. http://dx.doi.org/10.1111/j.1365-3156.2006.01639.x
8. Wood DE, Salzberg SL. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol. 2014;15:R46. http://dx.doi.org/10.1186/gb-2014-15-3-r46
9. Holden MT, Hauser H, Sanders M, Ngo TH, Cherevach I, Cronin A, et al. Rapid evolution of virulence and drug resistance in the emerging zoonotic pathogen *Streptococcus suis*. PLoS ONE. 2009;4:e6072. http://dx.doi.org/10.1371/journal.pone.0006072
10. Okura M, Takamatsu D, Maruyama F, Nozawa T, Nakagawa I, Osaki M, et al. Genetic analysis of capsular polysaccharide synthesis gene clusters from all serotypes of *Streptococcus suis*: potential mechanisms for generation of capsular variation. Appl Environ Microbiol. 2013;79:2796–806. http://dx.doi.org/10.1128/AEM.03742-12

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Postbooster Antibodies from Humans as Source of Diphtheria Antitoxin

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Diphtheria antitoxin for therapeutic use is in limited supply. A potential source might be affinity-purified antibodies originally derived from plasma of adults who received a booster dose of a vaccine containing diphtheria toxoid. These antibodies might be useful for treating even severe cases of diphtheria.

Although diphtheria is an almost forgotten disease in industrialized countries, sporadic cases still occur. Possible reasons for these cases include partial failure of vaccine compliance, antivaccine campaigns, inadequate booster regimens, and immunosenescence. Health authority interest in this disease was rekindled after a nonvaccinated boy in Spain died of systemic diphtheria in June 2015 and 9 cases of cutaneous diphtheria among refugees were notified by Denmark, Sweden, and Germany in 2015 (*1*). According to the World Health Organization, 7,321 cases of diphtheria were reported worldwide in 2014. In the early 1890s, Emil von Behring used serum from a hyperimmune horse (challenged with sublethal dose of *Corynebacterium diphtheriae*) to develop equine diphtheria antitoxin (DAT), which seemed to confer passive immunity to patients with diphtheria (*2*). Subsequently, use of equine DAT to treat this disease became common. Uncontrolled but large studies of mortality rates from that time suggested effectiveness of equine DAT use; however, double-blinded randomized studies conducted by Adolf Bingel in 1918 concluded that equine DAT offered no benefit over serum from nonhyperimmune horses (not challenged with *C. diphtheriae*) (*2*). Although modern efficacy studies are lacking, equine DAT is still the recommended treatment for diphtheria, listed among the World Health Organization essential medicines (*3*). When administered early in the clinical course of disease, treatment with DAT can be lifesaving for patients with toxin-induced systemic symptoms.

A large proportion of European countries do not stockpile DAT, and many countries have experienced difficulties replacing expired stockpiles (*3*,*4*). As highlighted by the European Centre for Disease Prevention and Control (*1*), the current lack of DAT in the European Union is a concern. DAT is not produced or licensed in the United States or in most European countries; it is imported from Brazil under an Investigational New Drug protocol (*5*).

Equine DAT can induce anaphylactic reactions (a test for sensitivity to DAT should be conducted before each administration) (*5*). The European Centre for Disease Prevention and Control and the US Centers for Disease Control and Prevention encourage searching for new providers of equine DAT and promote the development of alternative antitoxins of human origin. The definitive solution will probably come from monoclonal antibodies (*4*) or synthetic molecules such as nucleic acid aptamers. These new molecules could constitute an unlimited source of DAT, with a low risk for hypersensitivity reactions. Unfortunately, these alternatives are not yet available and will need to undergo thorough regulatory processes before being approved for use in humans. We therefore describe the potential role of human plasma from vaccinated volunteers as a source of DAT.

Plasma from vaccinated persons is used to produce Anthrasil (Cangene Corporation, Winnipeg, Manitoba, Canada), a fully human polyclonal antianthrax intravenous immunoglobulin (IVIG) licensed in the United States. Antitetanus immunoglobulin is produced from plasma of young volunteers who received a booster dose of the tetanus–diphtheria vaccine.

The successful implementation of vaccination programs in industrialized and many developing countries indicates that most of these populations have antibodies against the diphtheria toxin. Nonetheless, the geometric mean concentration of IgG against diphtheria toxin in plasma of vaccinated adults who received the last dose of tetanus–diphtheria vaccine in their adolescence is not much over 0.3 IU/mL (*6*). For diphtheria treatment, 20,000–100,000 IU of DAT is needed; the dose depends on disease severity (*5*). In consequence, producing DAT from plasma obtained from the general population could not be cost-effective because large volumes would be needed to obtain a dose of DAT with enough potency for clinical use.

An alternative could be to obtain plasma from adult donors who recently received a booster dose of vaccine. Researchers have observed that during the diphtheria epidemic that emerged in the newly independent states of the

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former Soviet Union from 1991 through 1994, booster vaccination of convalescent patients led to enhanced antidiphtheria toxin titers (*3*,*7*). Seroepidemiologic studies evaluating the effect of booster vaccination of adults against diphtheria support this finding. Booster vaccination of adults induces up to 10 IU/mL of IgG against diphtheria toxin in plasma 4 weeks after vaccination (*8*–*13*) (Table). The use of conjugate vaccines, or a high vaccine dose, could yield the highest plasma concentrations of DAT after a booster dose of vaccine (*9*).

Assuming use of revaccinated donor plasma with the highest titer, IVIG with a DAT potency up to 60–100 IU/mL could be obtained by using the standard methods for producing IVIG (*3*). This concentration could be enough to treat moderate forms of diphtheria (those with skin lesions only, laryngeal disease, or nasopharyngeal disease) (*5*). The European Pharmacopoeia recommends that the potency of equine-derived DAT be no less than 1,000 IU/mL (*3*). To treat severe diphtheria, a dose of 100,000 IU, obtained by using a 5% IVIG solution with potency of 100 IU/mL, would require 1.6 liters of product, a substantially high volume that would be very difficult to administer to a child.

This major drawback could be solved by using antigen-specific antibody purification. The process is simple: the antigen is immobilized in a solid phase so that the antibodies that bind specifically to it are retained during addition of plasma. Bound antibody can be recovered by acid elution (*14*). This method has been successfully used to purify specific antibodies from plasma or normal IVIG for research and development purposes (*15*). In 1988, also in an experimental context, M. Sutjita et al. demonstrated that this approach was useful for concentrating DAT from human serum; they used a diphtheria toxoid-Sepharose 4B (Sigma Aldrich, St. Louis, MO, USA) affinity column (*14*). In consequence, this approach could be used to purify DAT from plasma of revaccinated persons or from commercial immunoglobulins (i.e., the antitetanus immunoglobulin itself or nonspecific IVIG), which contains variable concentrations of DAT (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/15-1670-Techapp1.pdf). This concentrated DAT could be useful for treating diphtheria of any severity in adults and children, with very low risk of inducing hypersensitivity reactions.

A potential drawback of affinity purification is that the obtained DAT could be denatured by acid elution. This risk could be minimized by immediately neutralizing pH by adding 1 mol/L Tris, followed by dialysis with phosphate-buffered saline. The obtained product should undergo the same biological agent removal processes as those used for standard IVIG (i.e., chemical inactivation, heat inactivation, nanofiltration, and precipitations). Neutralization potency of DAT obtained from human plasma should be assigned according to the Vero cell cytotoxicity assay and the guinea pig lethality model; the 1st International Standard for Diphtheria Antitoxin Human should be used as the reference antitoxin (National Institute for Biological Standards and Control code 10/262).

A limitation of using DAT obtained from human plasma is the potential cost. Some developing countries, where most cases of diphtheria occur, could not afford it. Production costs and the price of each dose of human DAT could be reduced by using as source the same plasma obtained from the donors recruited to produce the antitetanus immunoglobulin. Industrialized countries could also donate doses of this human DAT to developing countries.

Table. Seroepidemiologic studies assessing levels of antidiphtheria antibodies in adults who received a booster dose of vaccine*

	Study population			Immunogenicity, GMC IU/mL (95% CI)	
Ref.	Mean age, y (SD or range)	No.	Vaccine	Before booster	After booster
(*8*)	40.1 (13.63)	1,448	0.5 mL Tdap (Boostrix; GlaxoSmithKline Biologicals, Rixensart, Belgium)	0.4 (0.4–0.4)	4.7 (4.4–5.1)
	40.4 (13.48)	728	0.5 mL Tdap (Adacel; Sanofi Pasteur, Swiftwater, PA, USA)	0.5 (0.4–0.5)	5.0 (4.6–5.4)
(*9*)	31.7 (15–69)	64	0.5 mL of Tdap (Sanofi Pasteur Limited, Toronto, ON, Canada) after previous vaccination with MCV4D (Menactra; Sanofi Pasteur, Swiftwater, PA, USA)	4.45 (2.77–7.15)	8.70 (6.59–11.5)
		379	0.5 mL Tdap (Sanofi Pasteur Limited, Toronto)	0.13 (0.11–0.16)	2.17 (1.84–2.56)
(*10*)	19.4 (1.2)	55	0.2 mL DTap (Kaketsuke, Kumamoto, Japan)	0.22 (0.16–0.30)	4.29 (3.53–5.21)
	19.4 (0.8)	56	0.5 mL DTap (Kaketsuke)	0.21 (0.15–0.30)	6.28 (4.86–8.11)
(*11*)	66.0 (59–91)	252	0.5 mL Tdap (Repevax; Sanofi Pasteur MSD GmbH, Leimen, Germany)	0.04 (0.03–0.06)	1.09 (0.81–1.46)
	24.0 (20–33)	21		0.14 (0.05–0.33)	4.16 (2.36–7.34)
(*12*)	21.1 (0.31)	74	0.5 mL Tdap (Boostrix)	0.3 (0.2–0.4)	6.0 (4.7–7.7)
(*13*)	26.5 (18–52)	401	0.5 mL TdaP (Statens Serum Institut, Copenhagen, Denmark)	0.11 (0.9–0.14)	4.60 (4.03–5.26)
	26.1 (18–55)	399	0.5 mL diTeBooster (Statens Serum Institut)	0.11 (0.09–0.14)	5.54 (4.00–5.15)

*DTaP, diphtheria, tetanus, and pertussis vaccine (for children ≥6 years of age); GMC, geometric mean concentration; Ref., reference; Tdap, tetanus, diphtheria, and pertussis vaccine (for children >11 years of age and adults).

Plasma from young adults receiving a booster dose of vaccine could represent a potential source of human DAT. Antigen-affinity antibody purification could help to produce a highly concentrated DAT from this plasma, useful for treating even the most severe forms of diphtheria. This approach could help mitigate the limited access to this essential medicine.

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References

1. European Centre for Disease Prevention and Control. Rapid risk assessment: a case of diphtheria in Spain [cited 2015 Jul 7]. http://ecdc.europa.eu/en/publications/Publications/diphtheria-spain-rapid-risk-assessment-june-2015.pdf
2. Opinel A, Tröhler U, Gluud C, Gachelin G, Smith GD, Podolsky SH, et al. Commentary: the evolution of methods to assess the effects of treatments, illustrated by the development of treatments for diphtheria, 1825–1918. Int J Epidemiol. 2013;42:662–76. http://dx.doi.org/10.1093/ije/dyr162
3. Wagner KS, Stickings P, White JM, Neal S, Crowcroft NS, Sesardic D, et al. A review of the international issues surrounding the availability and demand for diphtheria antitoxin for therapeutic use. Vaccine. 2009;28:14–20. http://dx.doi.org/10.1016/j.vaccine.2009.09.094
4. Both L, White J, Mandal S, Efstratiou A. Access to diphtheria antitoxin for therapy and diagnostics. Euro Surveill 2014;19:pii:20830
5. Centers for Disease Control and Prevention. Use of diphtheria antitoxin (DAT) for suspected diphtheria cases [cited 2015 Aug 19]. http://www.cdc.gov/diphtheria/downloads/protocol.pdf
6. Wagner KS, White JM, Andrews NJ, Borrow R, Stanford E, Newton E, et al. Immunity to tetanus and diphtheria in the UK in 2009. Vaccine. 2012;30:7111–7. http://dx.doi.org/10.1016/j.vaccine.2012.09.029
7. Bissumbhar B, Rakhmanova AG, Berbers GA, Iakolev A, Nosikova E, Melnick O, et al. Evaluation of diphtheria convalescent patients to serve as donors for the production of anti-diphtheria immunoglobulin preparations. Vaccine. 2004;22:1886–91. http://dx.doi.org/10.1016/j.vaccine.2003.11.006
8. Blatter M, Friedland LR, Weston WM, Li P, Howe B. Immunogenicity and safety of a tetanus toxoid, reduced diphtheria toxoid and three-component acellular pertussis vaccine in adults 19–64 years of age. Vaccine. 2009;27:765–72. http://dx.doi.org/10.1016/j.vaccine.2008.11.028
9. Halperin SA, McNeil S, Langley J, Blatter M, Dionne M, Embree J, et al. Tolerability and antibody response in adolescents and adults revaccinated with tetanus toxoid, reduced diphtheria toxoid, and acellular pertussis vaccine adsorbed (Tdap) 4–5 years after a previous dose. Vaccine. 2011;29:8459–65. http://dx.doi.org/10.1016/j.vaccine.2011.07.068
10. Hara M, Okada K, Yamaguchi Y, Uno S, Otsuka Y, Shimanoe C, et al. Immunogenicity and safety after booster vaccination of diphtheria, tetanus, and acellular pertussis in young adults: an open randomized controlled trial in Japan. Clin Vaccine Immunol. 2013;20:1799–804. http://dx.doi.org/10.1128/CVI.00490-13
11. Kaml M, Weiskirchner I, Keller M, Luft T, Hoster E, Hasford J, et al. Booster vaccination in the elderly: their success depends on the vaccine type applied earlier in life as well as on pre-vaccination antibody titers. Vaccine. 2006;24:6808–11. http://dx.doi.org/10.1016/j.vaccine.2006.06.037
12. Mertsola J, Van Der Meeren O, He Q, Linko-Parvinen A, Ramakrishnan G, Mannermaa L, et al. Decennial administration of a reduced antigen content diphtheria and tetanus toxoids and acellular pertussis vaccine in young adults. Clin Infect Dis. 2010;51:656–62. http://dx.doi.org/10.1086/655825
13. Thierry-Carstensen B, Jordan K, Uhlving HH, Dalby T, Sørensen C, Jensen AM, et al. A randomised, double-blind, non-inferiority clinical trial on the safety and immunogenicity of a tetanus, diphtheria and monocomponent acellular pertussis (TdaP) vaccine in comparison to a tetanus and diphtheria (Td) vaccine when given as booster vaccinations to healthy adults. Vaccine. 2012;30:5464–71. http://dx.doi.org/10.1016/j.vaccine.2012.06.073
14. Sutjita M, Hohmann A, Comacchio R, Bradley J. Polyspecific human and murine antibodies to diphtheria and tetanus toxoids and phospholipids. Clin Exp Immunol. 1988;73:191–7.
15. Estabrook MM, Jarvis GA, McLeod Griffiss J. Affinity-purified human immunoglobulin G that binds a lacto-N-neotetraose-dependent lipooligosaccharide structure is bactericidal for serogroup B *Neisseria meningitidis*. Infect Immun. 2007;75:1025–33. http://dx.doi.org/10.1128/IAI.00882-06

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Travel-Associated Rabies in Pets and Residual Rabies Risk, Western Europe

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In 2015, countries in western Europe were declared free of rabies in nonflying mammals. Surveillance data for 2001–2013 indicate that risk for residual rabies is not 0 because of pet importation from countries with enzootic rabies. However, the risk is so low (7.52×10^{-10}) that it probably can be considered negligible.

Although western and northern Europe and most countries in central Europe have eliminated rabies in nonflying animals (https://zenodo.org/record/49670#) (*1*,*2*), alerts are regularly issued because of importation of rabid pets. Policy makers recommend postexposure prophylaxis (PEP) after exposure in Western Europe to bats or pet bites in areas with rabies alerts. However, the policy after exposure to these pets is unclear (https://zenodo.org/record/49670#).

Residual risk for rabies in pets in Western Europe is defined as no risk (no PEP necessary) or low risk (PEP recommended after exposure), depending on recommendations (e.g., no risk according to Public Health England and low risk according to the World Health Organization) (*3*). Thus, evaluation of residual rabies risk in western Europe caused by pet movement is needed. We evaluated residual rabies risk caused by pet movement in western Europe.

The Study

We calculated the risk that a given pet in western Europe is contagious for rabies on a given day by the equation

$$P_A = \Sigma \frac{\text{No. contagious days for pets in the area during 2001–2013}}{\text{No. pets in the area} \times \text{no. days during 2001–2013}}$$

We describe factors associated with rabid pets (https://zenodo.org/record/49670#) and define pet transport as any noncommercial movement of a live cat, dog, or ferret and its owner or an authorized person across an administrative border.

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During 2001–2013, a total of 21 animal rabies cases attributed to pets from rabies-enzootic countries were reported in western Europe (https://zenodo.org/record/49670#), which represented 1.6 pets/year and 23 days/year of potential contagiousness. Fifteen dogs and 1 kitten originated from rabies-endemic countries outside western Europe. Five dogs raised in western Europe acquired rabies outside this region. One dog subsequently infected 2 indigenous dogs in France (*4*). All pet owners were identified. All owners except 1 (a Spanish man living in a van) were official residents of western Europe. Circumstances that led to pet examination and rabies diagnosis were clinical suspicion (14 pets), bitten humans (3 pets), border quarantine (2 pets), and retrospective data (2 pets with indigenous secondary cases during the alert in France in 2008).

Average contagious period was 16 days/pet: 14 days in western Europe (8 days without signs of rabies and 6 days with signs of rabies) and 2 days before arriving in western Europe. For 1 dog, signs of rabies appeared before the animal entered western Europe. For each rabid animal, an average of 34 (range 0–187) persons and other animals received PEPs. The maximum value of this range corresponds to an alert in France in 2004. After this alert, 1,200 animals were tested and 759 were observed for 1 year. Human and pet vaccinations led to vaccine shortages that required importing of vaccines not authorized for use in France (*5*).

We identified animal origin and mode of entry into western Europe (Table 1). Most rabies cases originated in Morocco and were recorded in France. Three cases were imported from eastern Europe to Germany, 1 from The Gambia to France, and 1 from Sri Lanka to the United Kingdom. Customs officials could not identify any of 11 cases in animals transported mainly by road (e.g., after a ferry trip from Morocco to Spain, Portugal, or France). Seven pets were transported through other countries in western Europe before arriving in the country of diagnosis (https://zenodo.org/record/49670#). Six puppies and 1 kitten were transported by air, of which only 2 were identified by customs officials (in the United Kingdom and Germany).

Of 19 transported rabid pets, 8 (42%) had no rabies vaccination, pet passport, or health certificate. Only 6 were vaccinated (0/2 infected in France, 3/3 imported but raised in western Europe, 3/7 imported by air, and 0/8 imported by road). Most vaccinated pets did not comply with recommended age for vaccination (≥12 weeks of age) or time between vaccination, serologic analysis, and transport. No reports mentioned valid rabies serologic analysis included in

Table 1. Transport mode, country of origin, and country of diagnosis of 21 pets reported with rabies related to travel, 2000–2013

Transport mode	No (%) rabid pets	Country of origin (no.)	Country of diagnosis (no.)
Road	11 (52)	Morocco (9), Croatia (1), Bosnia and Herzegovina (1)	France (8), Germany (2), Spain (1)
Air	7 (33)	Morocco (4), Azerbaijan (1), Sri Lanka (1), The Gambia (1)	Germany (2), Belgium (1), France (2), The Netherlands (1), UK (1)
Unknown	1 (5)	Morocco (1)	Switzerland (1)
None	2 (10)	Dogs from France (secondary cases) (2)	France (2)

European Pet Movement Policy (Figure) for unlisted third countries (e.g., Morocco, the Gambia, Sri Lanka, or Azerbaijan) (*6*). Using data for 2001–2013, we calculated that, for contact on a given day with a pet in western Europe, the probability of the pet being contagious for rabies attributed to pet transport was 7.52×10^{-10} (Table 2).

We observed a significant correlation between number of contagious days for dogs in a country and number of tourists traveling from this country to Morocco ($r = 0.73$, $p = 0.017$). We found no correlation with other variables tested (total dog population, dog population density, number of dogs per inhabitant).

Conclusions

Risk for indigenous rabies has decreased in western Europe. During 2001–2013, because of appropriate control of imported rabid pets, only 4 indigenous cases of human rabies were reported (3 in recipients of organs from a donor infected in India and 1 from a rabid bat in Scotland) (https://zenodo.org/record/49670#). Since 2011, no indigenous rabies cases have been reported in terrestrial mammals in western Europe. Because of increased travel (*7*), rabies imported by trips to rabies-enzootic countries has increased, and travel became the main source of rabies in humans (1.46 patients/year) (*8*) and pets (1.6 rabid pets/year) in 2001–2013. However, because of improved surveillance, although the number of imported rabies cases increased, the number of secondary cases decreased (https://zenodo.org/record/49670#).

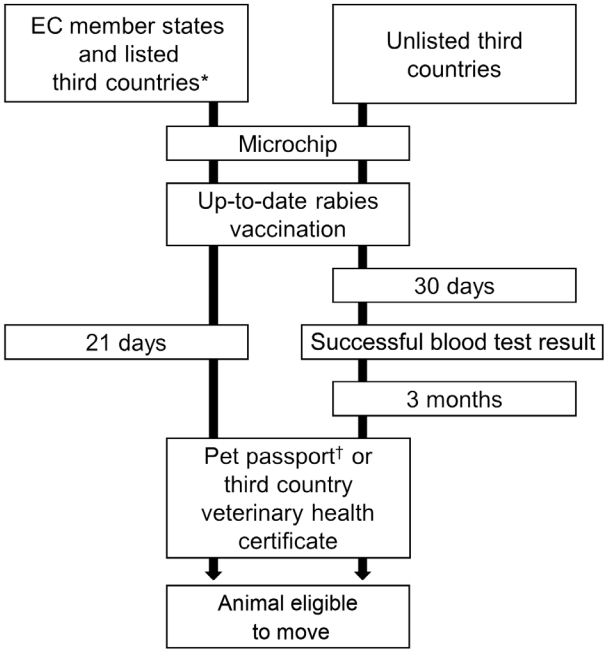

Figure. European Union (EU) regulations (no. 998/2003 and no. 576/2013, http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32013R0576) on movement of cats, dogs, and ferrets, 2003–2013. Before 2003, national rules applied (e.g., animal checked at destinations, rabies vaccination, animal identification, quarantine, health certification). EC, European community. *http://ec.europa.eu/food/animal/liveanimals/pets/list_third_en.htm. †A pet passport is required for pets transported in the EU. A health certificate provided by an official veterinarian is mandatory for pets transported from outside the EU.

Illegal importation of rabid animals is not limited to western Europe (*9*) or dogs and cats (*10*). This finding highlights the need for a global approach for regulation of animal movement worldwide and strengthening real-time reporting for animal and human rabies.

Risk for dog rabies being reintroduced into the European Union from Morocco was estimated as 0.21 cases/year (*11*). However, we estimate that 1.1 pets/year are entering western Europe after being infected in Morocco. Morocco has become the main source of pet rabies in western Europe, often through Ceuta and Melilla (Spanish enclaves in northern Morocco). Because no prophylaxis or specific vaccinations are needed for travel to northern Africa, few travelers seek pretravel advice and most have little knowledge of pet rabies (*12*,*13*).

Lack of awareness also increases importation of human rabies. Despite an efficient policy for preventing entry of rabid pets, the United Kingdom reported the highest number of patients with imported rabies during the study period (https://zenodo.org/record/49670#). Patients returning to this country did not believe that a correct PEP was needed after exposure abroad. None of the transported rabid pets fully satisfied European Pet Movement Policy, which raised questions about how to improve the current regulation application. Increasing international travel, expansion of the Schengen area (26 countries in Europe that have a common visa policy) into rabies-enzootic countries in eastern Europe, and development of internet animal trade (source of illegal importation) (*14*) are new challenges for ensuring compliance.

Because bat rabies is more difficult to control than dog rabies, and some developing countries still have difficulties

Table 2. Risk that given dogs or cats are rabid on a given day in 10 countries in western Europe relative to pet transport, 2001–2013*

Country†	No. pets without signs of rabies			No. days of pet contagiousness for rabies attributed to pet transport						Estimation of probability in the event of contact on a given day with a pet that is contagious for rabies attributed to pet transport		
				Pets without rabies signs			Total			Total		
	Total	Dogs	Cats	Total	Dogs	Cats	Total	Dogs	Cats	Total, P_A‡	Dogs, P_{AD}§	Cats, P_{AC}¶
Belgium	16	16	0	5	5	0	21	21	0	1.38×10^{-9}	3.32×10^{-9}	0
France	91	81	10	67	63	4	158	144	14	1.79×10^{-9}	3.99×10^{-9}	2.69×10^{-10}
Germany	32	32	0	31	31	0	63	63	0	9.83×10^{-10}	2.50×10^{-9}	0
Ireland	0	0	0	0	0	0	0	0	0	0	0	0
Italy	0	0	0	0	0	0	0	0	0	0	0	0
The Netherlands	1	1	0	4	4	0	5	5	0	2.41×10^{-10}	7.05×10^{-10}	0
Portugal	0	0	0	0	0	0	0	0	0	0	0	0
Spain	22	22	0	6	6	0	28	28	0	7.28×10^{-10}	1.25×10^{-9}	0
Switzerland	10	10	0	5	5	0	15	15	0	1.62×10^{-10}	7.10×10^{-9}	0
United Kingdom	6	6	0	3	3	0	9	9	0	1.18×10^{-10}	2.37×10^{-10}	0
Total	178	168	10	121	117	4	299	285	14	NA	NA	NA
Mean	18	17	1	12	12	0	30	29	1	7.52×10^{-10}	1.57×10^{-9}#	6.48×10^{-11}

*NA, not applicable.
†We considered only countries in western Europe with a population >1 million persons.
‡P_A, calculated risk that a given pet is rabid on a given day in a country in western Europe relative to pet transport.
§P_{AC}, calculated risk that a given cat is rabid on a given day in a country in western Europe relative to pet transport.
¶P_{AD}, calculated risk that a given dog is rabid on a given day in a country in western Europe relative to pet transport.
#P_{AD} was lower if the dog had no signs of rabies (9.25×10^{-10}–1.03×10^{-10} for dogs with no signs of rabies and 6.44×10^{-9}–6.44×10^{-6} for dogs with signs of rabies assuming that 90%–99.99% had no signs of rabies on a given day).

controlling rabies, eradication of rabies is not a realistic objective. Awareness should be increased, and current regulations for pet transport should be applied to reduce rabies importation and ensure that risk in western Europe remains low.

To avoid unnecessary and costly PEP and optimize resource allocation, it should be clearly stated which WHO recommendations, Public Health England recommendations, or other practices most relevant after pet exposure should be applied. Low risks ($<10^{-6}$) are usually considered acceptable or essentially 0 (*3,15*). The risk of a fatal car crash while traveling to PEP consultations was higher than the risk of rabies after exposure to a pet in France in 2001–2011 (*3*). The most pertinent policy in areas at low risk for rabies is probably that of the United Kingdom (i.e., no PEP outside alert areas that do not have asymptomatic animals or exposure to bats) (https://zenodo.org/record/49670#).

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References

1. Cliquet F, Picard-Meyer E, Robardet E. Rabies in Europe: what are the risks? Expert Rev Anti Infect Ther. 2014;12:905–8. http://dx.doi.org/10.1586/14787210.2014.921570
2. Freuling CM, Hampson K, Selhorst T, Schröder R, Meslin FX, Mettenleiter TC, et al. The elimination of fox rabies from Europe: determinants of success and lessons for the future. Philos Trans R Soc Lond B Biol Sci. 2013;368:20120142. http://dx.doi.org/10.1098/rstb.2012.0142
3. Ribadeau Dumas F, N'Diaye DS, Paireau J, Gautret P, Bourhy H, Le Pen C, et al. Cost-effectiveness of rabies post-exposure prophylaxis in the context of very low rabies risk: a decision-tree model based on the experience of France. Vaccine. 2015;33:2367–78. http://dx.doi.org/10.1016/j.vaccine.2015.02.075
4. French multidisciplinary investigation team. Identification of a rabid dog in France illegally introduced from Morocco. Euro Surveill. 2008;13:pii: 8066.
5. Servas V, Mailles A, Neau D, Castor C, Manetti A, Fouquet E, et al. An imported case of canine rabies in Aquitaine: investigation and management of the contacts at risk, August 2004–March 2005. Euro Surveill. 2005;10:222–5.
6. Regulation (EC) no. 998/2003 of the European parliament and of the council, 2003 [cited 2015 Apr16]. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32013R0576
7. World Tourism Organization. UNWTO annual report 2014. Madrid: UNWTO 2015 [cited 2015 Jun 10]. http://www2.unwto.org/annualreport2014
8. Carrara P, Parola P, Brouqui P, Gautret P. Imported human rabies cases worldwide, 1990–2012. PLoS Negl Trop Dis. 2013;7:e2209. http://dx.doi.org/10.1371/journal.pntd.0002209
9. Lankau EW, Tack DM, Marano N. Crossing borders: one world, global health. Clin Infect Dis. 2012;54:v–vi. http://dx.doi.org/10.1093/cid/cis429
10. Metlin AE, Holopainen R, Tuura S, Ek-Kommonen C, Huovilainen A. Imported case of equine rabies in Finland: clinical course of the disease and the antigenic and genetic characterization of the virus. Journal of Equine Veterinary Science. 2006;26:584–7. http://dx.doi.org/10.1016/j.jevs.2006.11.001

11. Napp S, Casas M, Moset S, Paramio JL, Casal J. Quantitative risk assessment model of canine rabies introduction: application to the risk to the European Union from Morocco. Epidemiol Infect. 2010;138:1569–80. http://dx.doi.org/10.1017/S0950268810000415
12. Altmann M, Parola P, Delmont J, Gautret P. Knowledge, attitudes, and practices of French travelers from Marseille regarding rabies risk and prevention. J Travel Med. 2009;16:107–11. http://dx.doi.org/10.1111/j.1708-8305.2008.00283.x
13. Gautret P, Ribadeau-Dumas F, Parola P, Brouqui P, Bourhy H. Risk for rabies importation from north Africa. Emerg Infect Dis. 2011;17:2187–93. http://dx.doi.org/10.3201/eid1712.110300
14. RSPCA. Pushing at an open door: how the present UK controls on rabies are failing [cited 2015 Apr 24]. http://www.rspca.org.uk/80/search?searchKey=Pushing+at+an+open++door+%E2%80%93+how+the++present+UK+controls+on+rabies+are+failing&x=0&y=0
15. Kelly KE, Cardon NC. The myth of 10^{-6} as a definition of acceptable risk. Presented at: 84th Annual Meeting and Exhibition of the Air and Waste Management Association; 1991 Jun 16–21; Vancouver, British Columbia, Canada.

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Natural Norovirus Infections in Rhesus Macaques

Tibor Farkas

Using a recently developed real-time reverse transcription PCR, I retested 500 fecal samples from rhesus macaques collected in 2008. Previous conventional reverse transcription PCR testing identified 1 isolate of GII norovirus; retesting found GI, GII, and possible GIV noroviruses in the samples, indicating the natural circulation of noroviruses in nonhuman primate colonies.

Noroviruses are a leading cause of acute gastroenteritis in humans and have also been described in several animal species. Although the pathologic role of most animal noroviruses is not clearly established, evidence of the close genetic relatedness of animal and human noroviruses and the detection of animal norovirus–specific antibodies in humans and of human norovirus specific–antibodies in different animal species strongly suggest zoonotic or interspecies transmission of noroviruses (*1*–*4*). However, zoonotic norovirus infections have not yet been reported, perhaps because zoonotic transmission of noroviruses is rare or the routine norovirus detection techniques are not designed to detect animal strains. On the other hand, experimental infection of several animal species has clearly shown that human norovirus strains are able to replicate in animals, including gnotobiotic pigs and calves and nonhuman primates (*5*–*8*). In addition, molecular detection of GII.12 and GII.4 noroviruses, which are usually human strains, has been reported in swine, cattle, and pet dogs (*9,10*).

The Study

A previous study evaluated samples that were collected during 2008 from the rhesus macaque (*Macaca mulatta*) colony housed at the Tulane National Primate Research Center (Covington, LA, USA) to determine the presence of caliciviruses (*11*). Using a broadly reactive primer pair (P289/P290), this study detected diverse recovirus strains and 1 GII norovirus (FT244, GenBank accession no. HM035148) in 1 macaque (*11*). Recently, a quantitative real-time reverse transcription PCR (RT-PCR) for the detection of GI, GII, and GIV noroviruses was developed (*12*). In 2015, I used this highly specific and sensitive assay to retest the 500 rhesus macaque fecal samples from the previous study, including the sample that contained FT244, to determine the presence of noroviruses.

Of the 500 samples, 41 (8.2%) showed a cycle threshold (C_t) lower than the assay's cutoff value. These positive samples included 30 (6%) GI (C_t 29.60–39.98), 8 (1.6%) GII (C_t 32.96–38.76), and 3 (0.6%) GIV (C_t 38.30–39.88) noroviruses. The viral load in the GI (mean 3.2×10^6 copies/g) and GII (mean 7.5×10^4 copies/g) norovirus-positive samples were in the range reported for human fecal samples (*13*). All GIV-positive samples had viral loads close to the assay's detection limit (1.3×10^3 copies/g) (Figure 1). The sample that was positive for the GII norovirus (FT244) in the previous study had a viral

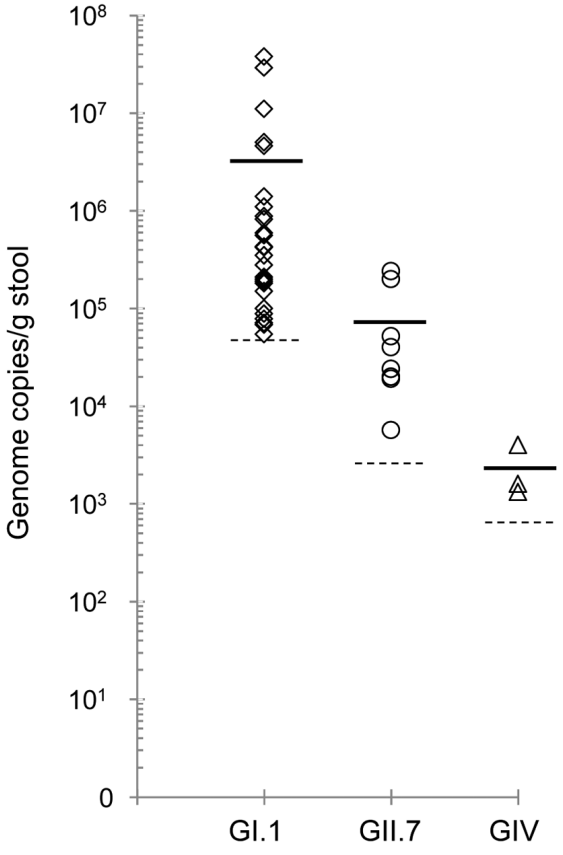

Figure 1. Norovirus genome copies per gram of rhesus macaque fecal samples collected in 2008 (*11*) and retested in 2015 by using a highly sensitive and specific real-time reverse transcription PCR. Shown are samples positive for GI (n = 30; diamonds), GII (n = 8; circles), and GIV (n = 3; triangles) noroviruses. Solid lines represent mean viral load; dashed lines represent the corresponding detection limits of the multiplex assay.

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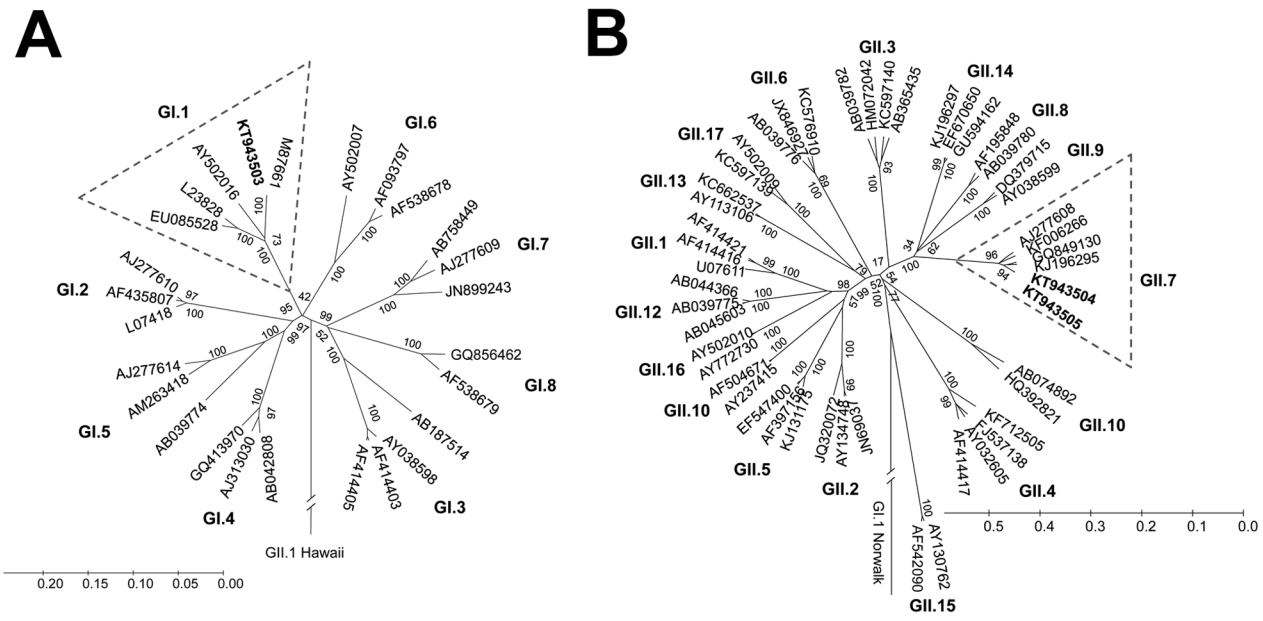

Figure 2. Phylogenetic trees showing GI and GII noroviruses from rhesus macaque fecal samples collected in 2008 (*11*) and retested in 2015 by using a highly sensitive and specific real-time reverse transcription PCR. GenBank accession numbers or other isolate identifiers are shown. Bold indicates isolates detected in this study. A) GI noroviruses share 100% nucleotide homology with the prototype Norwalk virus GI.1 strain (M87661). B) GII noroviruses group with GII.7 human noroviruses. Three of the 4 GII norovirus open reading frame (ORF) 2 sequences obtained in this study were identical. Only nonidentical sequences are shown. Phylogenetic trees were constructed on the basis of alignments of full length ORF2 nucleotide sequences, by using the unweighted pair group method with arithmetic mean and the neighbor-joining clustering methods of the Molecular Evolutionary Genetics Analysis (MEGA version 6.1; http://mega.software.informer.com/6.1/) software with Jukes-Cantor distance calculations. The confidence values of the internal nodes were obtained by performing 1,000 bootstrap analyses. Scale bars represent nucleotide substitutions per site.

load of 2.0×10^5 genome copies/g. Further analysis by sequencing of region D amplicons (*14*) revealed that the GI-positive samples contained GI.1 noroviruses and the GII-positive samples contained GII.7 noroviruses. However, I could not obtain RT-PCR products and sequence information for the GIV-positive samples, even by nested RT-PCR. Consequently, I concluded that these samples either gave a false-positive result (C_t values close to the detection limit) by real-time RT-PCR or contained a novel GIV norovirus that could not be amplified efficiently by primers designed on the basis of currently available GIV norovirus sequences.

In addition, I obtained full-length open reading frame (ORF) 2 sequences from 2 GI-positive and 4 GII-positive samples. The GI.1 norovirus ORF2 sequences (1,593 nt) had 100% nucleotide homology with each other and with the prototype Norwalk virus (M87661) (Figure 2, panel A). The GII.7 norovirus ORF2 sequences (1,623 nt) exhibited 99%–100% nucleotide homology with each other and 95% nucleotide homology with the closest GII.7 strain in the GenBank database (accession no. KJ196295) (Figure 2, panel B). I deposited 3 ORF2 sequences obtained in this study into the GenBank database under accession nos. KT943503–KT943505.

Conclusions

A previous study reported the molecular detection of a GII norovirus in 1 of 500 rhesus macaques tested (*11*). Although this detection rate was extremely low (0.2%), the finding indicated the occurrence of natural norovirus infections in colony macaques. In this study, retesting the 500 samples by a more sensitive and specific real-time RT-PCR confirmed the previously detected presence of the GII norovirus in 1 sample but also identified additional samples positive for GI, GII, and GIV noroviruses. Furthermore, additional amplification of the viral genome and sequencing confirmed the presence of GI.1 and GII.7 noroviruses but not GIV noroviruses.

The detection of a GI.1 norovirus in samples collected in 2008, with 100% homology to the prototype Norwalk virus, is somewhat surprising. The prototype virus was originally described in an outbreak occurring during 1968 (*15*). According to outbreak surveillance data, GI.1 norovirus infections are extremely rare; consequently, establishing whether the Norwalk strain is still in circulation or has completely disappeared is difficult. Data on human norovirus strains circulating in the community or present in environmental samples at the time of the rhesus macaque fecal sample collection were not available from the local

health department. The fecal samples were collected by rectal loops from individual macaques, so environmental contamination of samples, which might be an issue in other studies where manure is collected (*9*), can be ruled out. Samples positive for Norwalk virus were not present in the laboratories involved in the study, and rhesus macaque samples were stored in a separate freezer under Biosafety Level 2+ protocol. This finding is supported by a report of recent circulation of noroviruses with 100% nt homology to the Norwalk virus (GenBank accession nos. JX455860, JX455863, JX455870, and JX455872) that were found in sewage samples in Tunisia during 2007–2009.

The observations from this study and the previous study (*11*) indicate a strong plausibility for nonhuman primate reservoirs of human norovirus infections and the genetic mixing of animal and human caliciviruses under natural conditions from which new strains or emerging pathogens may arise. Additional studies are needed to establish the frequency, identity, and relevance of norovirus infections in nonhuman primates.

At the time of the study, Dr. Farkas was an assistant professor at the Division of Infectious Diseases, Cincinnati Children's Hospital Medical Center. Currently, he is an assistant professor at the School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana, USA. His research interests include human and animal enteric viral diseases.

References

1. Tse H, Lau SK, Chan WM, Choi GK, Woo PC, Yuen KY. Complete genome sequences of novel canine noroviruses in Hong Kong. J Virol. 2012;86:9531–2. http://dx.doi.org/10.1128/JVI.01312-12
2. Caddy SL, de Rougemont A, Emmott E, El-Attar L, Mitchell JA, Hollinshead M, et al. Evidence for human norovirus infection of dogs in the United Kingdom. J Clin Microbiol. 2015;53:1873–83. http://dx.doi.org/10.1128/JCM.02778-14
3. Farkas T, Nakajima S, Sugieda M, Deng X, Zhong W, Jiang X. Seroprevalence of noroviruses in swine. J Clin Microbiol. 2005;43:657–61. http://dx.doi.org/10.1128/JCM.43.2.657-661.2005
4. Widdowson MA, Rockx B, Schepp R, van der Poel WH, Vinje J, van Duynhoven YT, et al. Detection of serum antibodies to bovine norovirus in veterinarians and the general population in the Netherlands. J Med Virol. 2005;76:119–28. http://dx.doi.org/10.1002/jmv.20333
5. Cheetham S, Souza M, Meulia T, Grimes S, Han MG, Saif LJ. Pathogenesis of a genogroup II human norovirus in gnotobiotic pigs. J Virol. 2006;80:10372–81. http://dx.doi.org/10.1128/JVI.00809-06
6. Souza M, Azevedo MS, Jung K, Cheetham S, Saif LJ. Pathogenesis and immune responses in gnotobiotic calves after infection with the genogroup II.4–HS66 strain of human norovirus. J Virol. 2008;82:1777–86. http://dx.doi.org/10.1128/JVI.01347-07
7. Wyatt RG, Greenberg HB, Dalgard DW, Allen WP, Sly DL, Thornhill TS, et al. Experimental infection of chimpanzees with the Norwalk agent of epidemic viral gastroenteritis. J Med Virol. 1978;2:89–96. http://dx.doi.org/10.1002/jmv.1890020203
8. Rockx BH, Bogers WM, Heeney JL, van Amerongen G, Koopmans MK. Experimental norovirus infections in non-human primates. J Med Virol. 2005;75:313–20. http://dx.doi.org/10.1002/jmv.20273
9. Mattison K, Shukla A, Cook A, Pollari F, Friendship R, Kelton D, et al. Human noroviruses in swine and cattle. Emerg Infect Dis. 2007;13:1184–8. http://dx.doi.org/10.3201/eid1308.070005
10. Summa M, von Bonsdorff CH, Maunula L. Pet dogs—a transmission route for human noroviruses? J Clin Virol. 2012;53:244–7. http://dx.doi.org/10.1016/j.jcv.2011.12.014
11. Farkas T, Cross RW, Hargitt E 3rd, Lerche NW, Morrow AL, Sestak K. Genetic diversity and histo-blood group antigen interactions of rhesus enteric caliciviruses. J Virol. 2010;84:8617–25. http://dx.doi.org/10.1128/JVI.00630-10
12. Farkas T, Singh A, Le Guyader FS, La Rosa G, Saif L, McNeal M. Multiplex real-time RT-PCR for the simultaneous detection and quantification of GI, GII and GIV noroviruses. J Virol Methods. 2015;223:109–14. http://dx.doi.org/10.1016/j.jviromet.2015.07.020
13. Furuya D, Kuribayashi K, Hosono Y, Tsuji N, Furuya M, Miyazaki K, et al. Age, viral copy number, and immunosuppressive therapy affect the duration of norovirus RNA excretion in inpatients diagnosed with norovirus infection. Jpn J Infect Dis. 2011;64:104–8.
14. Vinjé J, Hamidjaja RA, Sobsey MD. Development and application of a capsid VP1 (region D) based reverse transcription PCR assay for genotyping of genogroup I and II noroviruses. J Virol Methods. 2004;116:109–17. http://dx.doi.org/10.1016/j.jviromet.2003.11.001
15. Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM. Visualization by immune electron microscopy of a 27-nm particle associate with acute infectious nonbacterial gastroenteritis. J Virol. 1972;10:1075–81.

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Red Fox as Sentinel for *Blastomyces dermatitidis*, Ontario, Canada

Nicole M. Nemeth, G. Douglas Campbell, Paul T. Oesterle, Lenny Shirose, Beverly McEwen, Claire M. Jardine

Blastomyces dermatitidis, a fungus that can cause fatal infection in humans and other mammals, is not readily recoverable from soil, its environmental reservoir. Because of the red fox's widespread distribution, susceptibility to *B. dermatitidis*, close association with soil, and well-defined home ranges, this animal has potential utility as a sentinel for this fungus.

Blastomyces dermatitidis (family Ajellomycetaceae) is a fungal pathogen that causes blastomycosis, a life-threatening disease in humans, canids, and other mammals (*1*,*2*). Infection usually occurs through inhalation of conidia released from an environmental reservoir (soil) (*3*). In North America, high incidences of infection are reported in humans and dogs from around the Great Lakes (*4*). Recently, increased numbers of human blastomycosis cases have been detected in the provinces of Quebec, Saskatchewan, Manitoba, and Ontario, Canada (*2*,*5*–*7*). Despite this increased detection, human blastomycosis is probably underdiagnosed (*2*,*8*).

The geographic range of *B. dermatitidis* is based on reported clinical human cases and is therefore not clearly defined (*4*). *B. dermatitidis* is not readily recoverable or uniformly distributed within the environment, and identification has been problematic (*4*,*9*). Therefore, identifying high-risk areas for exposure has been difficult, yet this this information is crucial to minimize the number of infections.

We evaluated the utility of wild and domestic canids as potential sentinels of *B. dermatitidis* in the environment. We retrospectively reviewed blastomycosis case data for wildlife and companion animals in Ontario, which contains areas where blastomycosis is endemic and areas of likely emergence (*2*,*3*,*5*,*8*). Once a candidate sentinel species is identified, a targeted surveillance system can be developed to identify high-risk areas and assess risk factors associated with disease.

The Study

We analyzed blastomycosis cases diagnosed at the Animal Health Laboratory, University of Guelph (Guelph, Ontario), during 1998–2014; at 2 private diagnostic services (Guelph) during 1996–2006; and at the Canadian Wildlife Health Cooperative (Ontario regional center) during 1991–2014. Case data included date of sample collection, species, and location of carcass (wildlife) or veterinary clinic or diagnostic laboratory (companion animals). Personal privacy legislation in Canada prevented use of home addresses for companion animals. We compared animal blastomycosis data with those for 309 published human cases in Ontario during 1994–2003 (*2*).

Diagnoses in companion animals were made using impression smears, cytology, histopathology, serology (agar gel immunodiffusion test), and antigen detection (sandwich enzyme immunoassay). Diagnoses in wildlife were made postmortem by gross pathology and histopathology.

Blastomycosis was diagnosed in 250 companion animals (222 dogs [88.8%], 27 cats [10.8%], 1 ferret [0.4%]) and in 14 wild canids (11 red foxes [*Vulpes vulpes*; 78.6%], 3 gray wolves [*Canis lupus*; 21.4%]). Diagnoses in wild canids represent 7.4% of 149 red foxes and 1.6% of 185 wolves submitted to the Canadian Wildlife Health Cooperative (Ontario regional center) during the same period. Lungs of wild canids with blastomycosis consistently had nodules of inflammatory cells and *B. dermatitidis* yeasts. Less commonly, lymph nodes and skin were also affected. In red foxes found dead, *B. dermatitidis* was associated with severe, multifocal to coalescing, granulomatous pneumonia, whereas trapper-killed red foxes had small numbers of well-circumscribed pulmonary lesions.

Most infected companion animals were from central regions of Ontario, as previously defined (*2*); however, all regions were represented (Figure). All infected wild canids were in the north region, where most human blastomycosis cases (61%; 188/309) originated during 1994–2003 (*2*). An additional study traced 74% (20/27) of human cases to north and east of Lake Superior (*3*).

Dogs were most commonly diagnosed with blastomycosis (64.4%) during July–December; the fewest (14%) were diagnosed during January–March. Most wildlife with blastomycosis (85.7%, 10/11 red foxes and 2/3 wolves) were diagnosed during November–March; the remaining 2 animals were diagnosed in September and April. Most human cases (59%, 181/309) were diagnosed during October–March (*2*).

B. dermatitidis infections in humans and other mammals are opportunistic and associated with contact with aerosolized conidia. Habitat sharing among humans, wildlife, and domestic animals is increasingly common (*10*)

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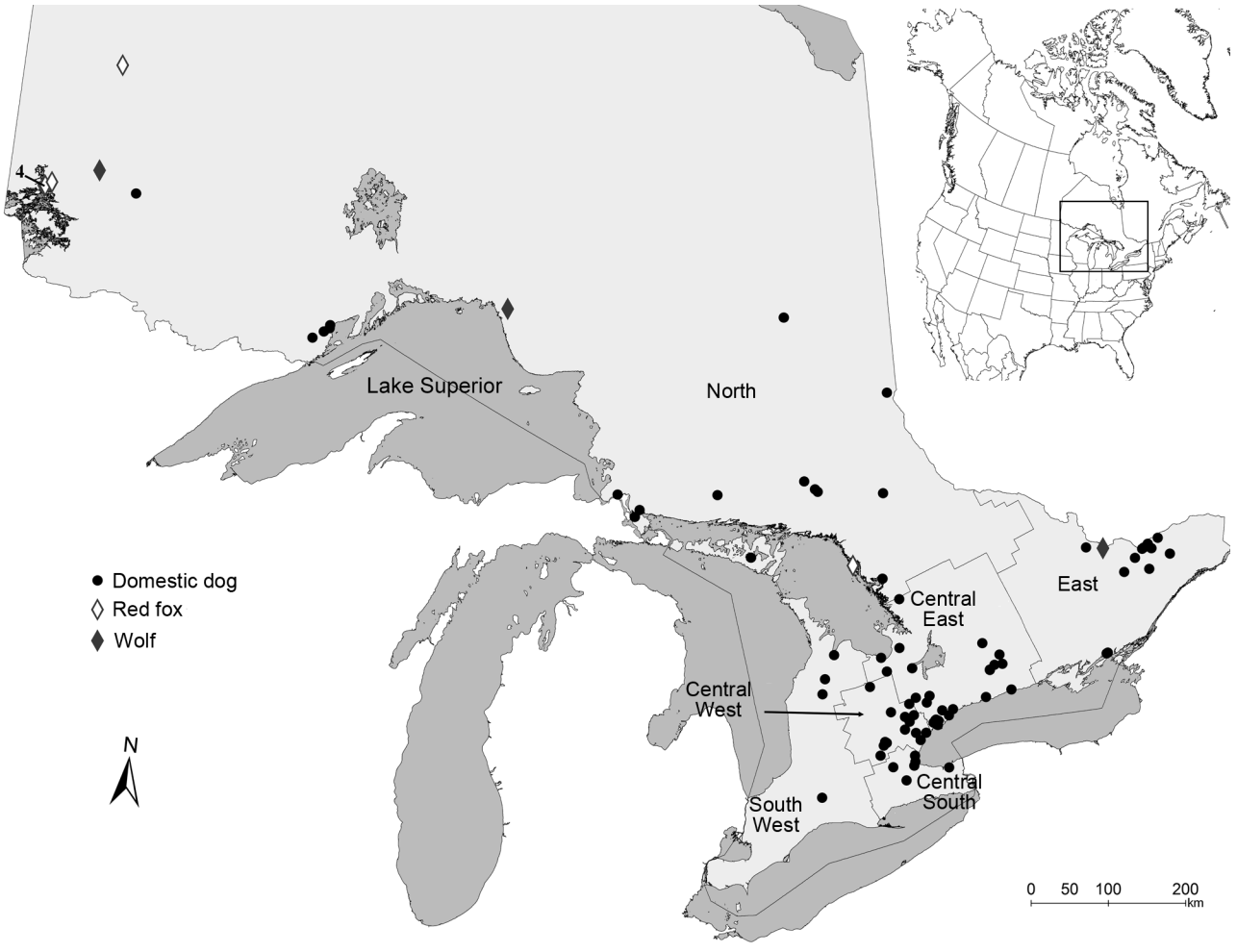

Figure. Locations of wild and domestic canids infected with *Blastomyces dermatitidis* during 1996–2014, Ontario, Canada. Inset map shows the location of Ontario in Canada. Health regions within the province consist of grouped public health units as defined by the Ontario Ministry of Public Health and are named according to Morris et al. (*2*). Dark gray shading indicates lakes; the Great Lakes are shown in the lower part of the figure.

and provides communal opportunities for *B. dermatitidis* exposure. Wildlife with limited and relatively well-defined home ranges (e.g., the red fox) offer a unique opportunity to assess the distribution and, therefore, the potential risks of exposure to pathogens with an environmental reservoir.

The red fox is the most widely distributed carnivore worldwide and is common throughout much of North America, including regions with endemic as well as emerging *B. dermatitidis* (*11*). This species is highly adaptive and coexists with humans in various habitats, including recreational, residential, commercial, industrial, and urban open areas (*10*). Furthermore, the home range of the red fox is compact, nonoverlapping, and relatively well-defined with year-round occupancy, unlike the range of other wild canids (*10*,*12*), humans, and dogs, which can have varied and long-distance movements. The close proximity of red foxes to the ground, along with their digging, denning, and foraging behaviors, probably increases the likelihood of their close and continuous exposure to *B. dermatitidis* in the soil. In addition, soil in red fox dens is often sandy and acidic, a condition that, along with moisture, decaying vegetation, and animal feces, is conducive to *B. dermatitidis* growth (*1*,*3*,*11*).

Based on the co-occurrence of clinical disease in dogs and their owners, canine blastomycosis cases are a potential epidemiologic marker for the risk for human disease (*9*). However, in our study, pinpointing environmental exposure sources for dogs was impossible due to undisclosed travel, privacy legislation, and location variation between exposure sites and clinics. The time lag between exposure and disease onset can vary by months (*13*), further hindering accurate identification of exposure sources. In our study, dog blastomycosis cases mapped to Guelph are overrepresented because this southwestern Ontario city has a large veterinary hospital and diagnostic laboratory.

Although relatively few wild, compared with domestic, canids were diagnosed with blastomycosis in our study, the utility of an abundant and widespread wild canid such as the red fox as a sentinel for the risk for *B. dermatitidis* infection in humans should be further explored. In our study, *B. dermatitidis* was readily detected by gross pathology and histopathology in the lungs of red foxes, in which pulmonary lesions ranged from severe, diffuse pneumonia in foxes found dead to focal and well-circumscribed lesions in trapper-killed foxes. These findings suggest that *B. dermatitidis*–associated lesions in red foxes would be easily identifiable, regardless of time of year and disease manifestation. Future sampling should target foxes in northern Ontario, where risk for human infections is highest, the annual incidence is increasing, and diagnostic testing is less available (*2*,*8*,*14*). Furthermore, in remote northern regions, defining the range and prevalence of blastomycosis could have positive public health effects on healthcare workers and indigenous human populations.

Limitations for using wildlife for passive disease surveillance include difficulty in finding and recovering carcasses, leading to inconsistent sampling efforts. To circumvent the limitations, we suggest using carcasses of licensed fur trapper–killed red foxes for testing in targeted areas and seasons (i.e., fall to early spring). Red fox trapping is permitted year-round in southern Ontario and from September to late February in the remainder of the province (*15*). Temporal detections of blastomycosis in humans in Ontario (*2*) more closely followed detections in red foxes than in dogs.

Conclusions

Documenting the prevalence, distribution, seasonality, and disease manifestations of blastomycosis in red foxes in southern Ontario could help elucidate the epidemiology of this regionally emerging disease, delineate geospatial differences in exposure risks, and explore the utility of this wild canid as a sentinel for the risk to public health. Multidisciplinary research such as this provides opportunities for the development of partnerships among public health and medical researchers, physicians, veterinarians, biologists, epidemiologists, natural resource managers, and hunter and trapper federations with the common goal of reducing disease risks.

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References

1. Kerl ME. Update on canine and feline fungal diseases. Vet Clin North Am Small Anim Pract. 2003;33:721–7. http://dx.doi.org/10.1016/S0195-5616(03)00035-4
2. Morris SK, Brophy J, Richardson SE, Summerbell R, Parkin PC, Jamieson F, et al. Blastomycosis in Ontario, 1994–2003. Emerg Infect Dis. 2006;12:274–9. http://dx.doi.org/10.3201/eid1202.050849
3. Kane J, Righter J, Krajden S, Lester RS. Blastomycosis: a new endemic focus in Canada. Can Med Assoc J. 1983;129:728–31.
4. Reed KD, Meece JK, Archer JR, Peterson AT. Ecologic niche modeling of *Blastomyces dermatitidis* in Wisconsin. PLoS ONE. 2008;3:e2034. http://dx.doi.org/10.1371/journal.pone.0002034
5. Lester RS, DeKoven JG, Kane J, Simor AE, Krajden S, Summerbell RC. Novel cases of blastomycosis acquired in Toronto, Ontario. CMAJ. 2000;163:1309–12.
6. Litvinov IV, St-Germain G, Pelletier R, Paradis M, Sheppard DC. Endemic human blastomycosis in Quebec, Canada, 1988–2011. Epidemiol Infect. 2013;141:1143–7. http://dx.doi.org/10.1017/S0950268812001860
7. Davies JL, Epp T, Burgess HJ. Prevalence and geographic distribution of canine and feline blastomycosis in the Canadian prairies. Can Vet J. 2013;54:753–60.
8. Dalcin D, Ahmed Z. Blastomycosis in northwestern Ontario, 2004 to 2014. Can J Infect Dis Med Microbiol. 2015;26:259–62.
9. MacDonald PDM, Langley RL, Gerkin SR, Torok MR, MacCormack JN. Human and canine pulmonary blastomycosis, North Carolina, 2001–2002. Emerg Infect Dis. 2006;12:1242–4. http://dx.doi.org/10.3201/eid1708.050781
10. Rosatte R, Allan M. The ecology of red foxes, *Vulpes vulpes*, in metropolitan Toronto, Ontario: disease management implications. Can Field Nat. 2009;123:215–20.
11. Lariviere S, Pasitschniak-Arts M. *Vulpes vulpes*. Mammalian Species. 1996;537:1–11. http://dx.doi.org/10.2307/3504236
12. Trewhella WJ, Harris S, McAllister FE. Dispersal distance, home range size and population density in the red fox (*Vulpes vulpes*): a quantitative analysis. J Appl Ecol. 1988;25:423–34. http://dx.doi.org/10.2307/2403834
13. DiSalvo AF. Blastomycosis. In: Al-Doory Y, DiSalvo AF, editors. The ecology of *Blastomyces dermatitidis*. New York: Plenum Medical Book Co.; 1992. p. 42–73.
14. McIntyre L. Blastomycosis in northwestern Ontario. Can Fam Physician. 1985;31:161–4.
15. Ontario Ministry of Natural Resources. Hunting regulations summary. 2013 [cited 2015 Mar 31]. https://www.ontario.ca/environment-and-energy/2013-furbearing-mammals-seasons-hr-summary

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Surveillance for Highly Pathogenic Avian Influenza Virus in Wild Birds during Outbreaks in Domestic Poultry, Minnesota, USA, 2015

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In 2015, a major outbreak of highly pathogenic avian influenza virus (HPAIV) infection devastated poultry facilities in Minnesota, USA. To understand the potential role of wild birds, we tested 3,139 waterfowl fecal samples and 104 sick and dead birds during March 9–June 4, 2015. HPAIV was isolated from a Cooper's hawk but not from waterfowl fecal samples.

Wild birds of the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls and shorebirds) are believed to be the predominant reservoir for avian influenza viruses (AIVs) (*1*), and most AIV subtypes are low pathogenicity (LPAIV) (*2*). Only subtypes H5 and H7 are commonly associated with highly pathogenic AIVs (HPAIVs), which sometimes arise from mutation after introduction of LPAIV in domestic poultry (*3*). The main transmission route of AIVs in birds is fecal-oral, with viral shedding in both feces and through the upper respiratory tract (*4*). Transmission involves direct or indirect contact between susceptible birds and infectious birds or fomites (*5*). A novel HPAIV (H5N2) strain discovered in North America in 2014, a reassortant with Eurasian (EA) and North American (AM) lineage genes (*6*), had been detected in domestic poultry and wild birds as far east as Kentucky, USA, through January 2016. Of 7,084 wild birds sampled by US federal and state agencies during December 2014–June 2015, a total of 98 (1.4%) tested positive for HPAIV (EA/AM H5N1, EA/AM H5N2, EA H5N8, or other EA H5); these birds were 68 dabbling ducks, 20 geese, 7 raptors, 2 passerines, and 1 diving duck (*7*).

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In Minnesota, USA, HPAIV subtype H5N2 was first confirmed in a poultry facility (hereafter termed facility) in Pope County on March 4, 2015. The scope of the outbreak in Minnesota was unprecedented, and by mid-June 2015, the virus had been found in 23 counties with confirmed cases at 104 sites (98 turkey facilities, 5 chicken facilities, 1 backyard flock). The outbreak resulted in the depopulation of 9 million birds (*8*) and an economic loss of at least $650 million (*9*). Given that wild waterfowl are reservoirs for AIVs and that their movement could contribute to HPAIV spread, we conducted surveillance to detect HPAIV in wild waterfowl feces, selected dead birds, and live birds displaying neurologic impairment.

The Study

On March 6, 2015, we conducted an aerial survey covering a 24-km radius around the Pope County facility and identified ≈100 resident mallards (*Anas platyrhynchos*) and 21 trumpeter swans (*Cygnus buccinator*). During March 9–12, 2015, we collected 148 representative waterfowl fecal samples, pooled in groups of up to 3, to determine whether wild birds were actively shedding HPAIV. We did not detect HPAIV, although 2 pooled samples contained LPAIV (detailed methods in the online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/15-2032-Techapp1.pdf).

In March 2015, we chose 5 counties with infected facilities (Kandiyohi, Lac Qui Parle, Meeker, Nobles, and Stearns) and 5 waterfowl production areas (online Technical Appendix 1) where facilities were uninfected (Figure 1) to test for a spatial difference in HPAIV shedding. Within these areas, we compiled a list of wetlands and lakes and scouted those areas for waterfowl activity and sampled feces. For each area, our goal was to collect 300 fecal samples. In counties with infected poultry, we choose sites within 16 km of infected facilities. We collected ≈20 samples from a given spatiotemporal point to obtain representation within a target area.

We solicited agency staff and the public to report any deceased wild birds or live birds exhibiting neurologic signs consistent with HPAIV infection, including raptors, wild turkeys, and groups of ≥5 dead birds from which we obtained samples. We refer to these as morbidity and mortality samples, and our collection efforts targeted birds that had died <24 h previously.

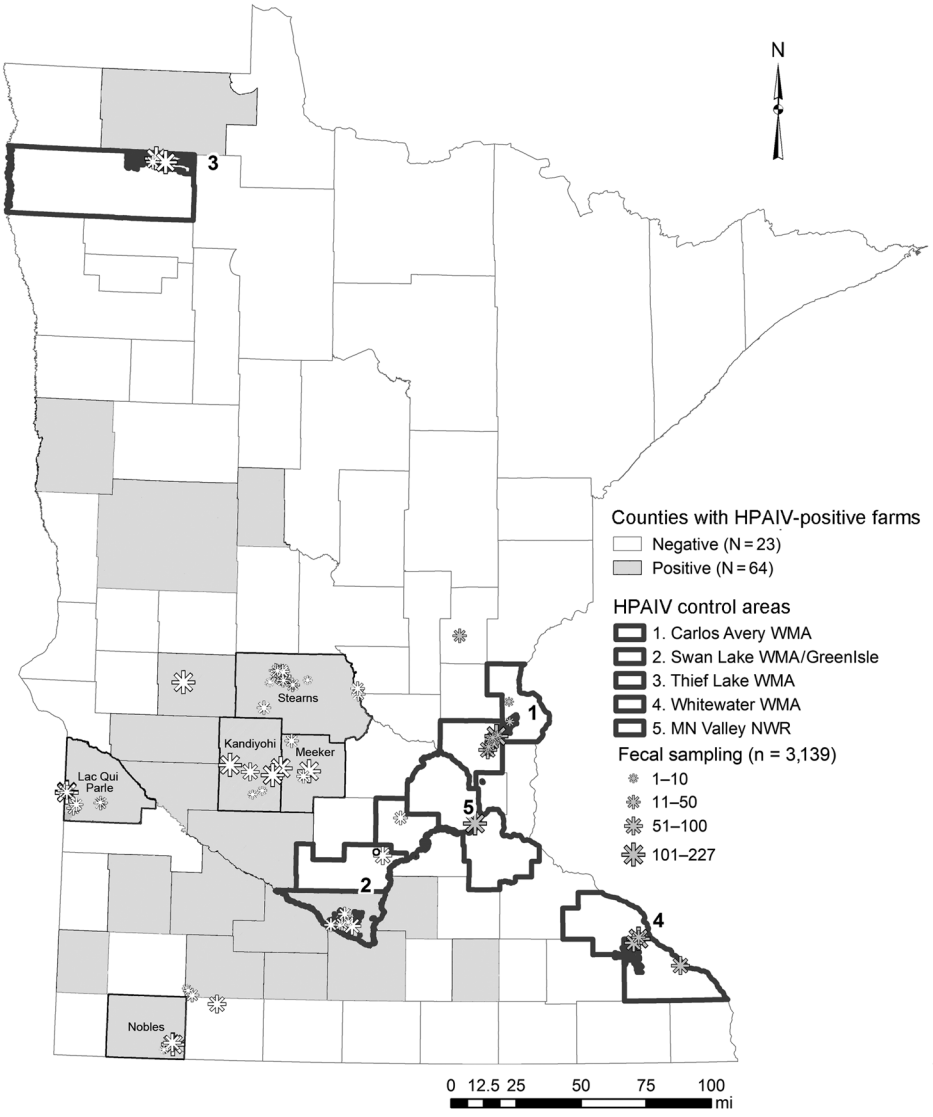

Figure 1. Minnesota collection sites for waterfowl feces sampled for highly pathogenic avian influenza virus (HPAIV) in spring 2015 (N = 3,139). Although HPAIV was confirmed in a Nicollet County poultry facility on May 5, 2015, our sampling occurred during April 22–April 27, 2015, and we consider this a control area (control no. 2). WMA, wildlife management area; NWR, national wildlife refuge.

In April 2015, which coincided with the peak rates of infection in Minnesota facilities (*8*), we collected 2,991 waterfowl fecal samples and pooled them into 1,027 brain-heart–infusion media vials; 1,591 samples (548 pooled) were obtained from counties with infected facilities, and 1,400 samples (479 pooled) were collected from waterfowl production areas without facilities (Figure 1). Although HPAIV was not detected in these samples, 30 pooled samples (representing 85 individual birds) tested positive for LPAIV. Apparent LPAIV fecal prevalence was 0.012 (95% CI 0.007–0.018) in counties with infected poultry, 0.008 (95% CI 0.004–0.014) in counties without infection, and 0.010 (95% CI 0.007–0.014) in the combined study area. Given that HPAIV was not detected and that we could not sample every individual bird in the waterfowl population, if HPAIV were present, there was a 95% probability that fecal prevalence was between 0 and 0.181% in areas with infection and 0 and 0.224% in areas without infection.

Through June 4, 2015 (last confirmed positive facility), we collected and tested 104 morbidity and mortality samples (Table) and detected a single HPAIV-positive bird, a Cooper's hawk (*Accipiter cooperii*) from Yellow Medicine County (20 km from an infected facility); this infection was confirmed on April 29, 2015 (Figure 2). We suspect that this woodland predator and opportunistic scavenger was exposed to HPAIV through a food item. Although not discovered as part of Minnesota Department of Natural Resources surveillance, 3 black-capped chickadees (*Poecile atricapillus*) were found in an urban neighborhood exhibiting neurologic signs and submitted to the University of Minnesota Veterinary Diagnostic Laboratory by the Minnesota Wildlife Rehabilitation Center in June

Table. Wild birds collected (n = 104) for highly pathogenic avian influenza virus screening as part of MNDNR morbidity and mortality sampling efforts, Minnesota, USA, March 9–June 4 2015

Order*	Family	Genus and species	Common name	Count
Anseriformes	Anatidae	*Branta canadensis*	Canada goose	8
		Cygnus buccinators	Trumpeter swan	3
		Aix sponsa	Wood duck	2
		Anas platyrhynchos	Mallard	2
Galliformes	Phasianidae	*Phasianus colchicus*	Ring-necked pheasant	8
		Meleagris gallopavo	Wild turkey	17
Pelicaniformes	Pelicanidae	*Pelicanus erythrorhynchos*	American white pelican	1
Accipitriformes	Cathartidae	*Cathartes aura*	Turkey vulture	1
	Accipitridae	*Haliaeetus leucocephalus*	Bald eagle	5
		Accipiter striatus	Sharp-shinned hawk	8
		Accipiter cooperii†	Cooper's hawk	6
		Buteo platypterus	Broad-winged hawk	1
		Buteo jamaicensis	Red-tailed hawk	3
Gruiformes	Rallidae	*Rallus limicola*	Virginia rail	1
		Porzana carolina	Sora	1
		Fulica americana	American coot	9
	Gruidae	*Grus canadensis*	Sandhill crane	1
Charadriiformes	Laridae	*Larus delawarensis*	Ring-billed gull	1
		Larus argentatus	Herring gull	1
Columbiformes	Columbidae	*Columba livia*	Rock pigeon	2
		Zenaida macroura	Mourning dove	1
Strigiformes	Strigidae	*Bubo virginianus*	Great horned owl	3
Caprimulgiformes	Caprimulgidae	*Chordeiles minor*	Common nighthawk	1
Passeriformes	Sturnidae	*Sturnus vulgaris*	European starling	10
	Parulidae	*Setophaga striata*	Blackpoll warbler	1
		Setophaga palmarum	Palm warbler	1
	Emberizidae	*Melospiza lincolnii*	Lincoln's sparrow	1
	Icteridae	*Euphagus carolinus*	Rusty blackbird	3
		Quiscalus quiscula	Common grackle	1

*1 sparrow not listed was identified to order Passeriformes.
†1 HPAIV-positive Cooper's hawk confirmed on April 29, 2015.

2015; in 1 bird there was weak detection of Eurasian H5 RNA, but no virus was recovered and no sequence could be obtained directly from the sample (*7*). All 3 birds demonstrated multifocal encephalitis, which was likely the cause for the neurologic signs (A. Armien, pers. comm.).

Conclusions

Morbidity and mortality samples yielded the only HPAIV detected in our surveillance of Minnesota wild birds, despite the relatively small number of samples. This sample type has proven valuable for HPAIV detection in wild birds in other states; 32% of HPAIV detections nationwide and 90% of HPAIV detections within the Mississippi flyway were derived from this source during December 2014–June 2015 (*7*). Evolving HPAIV strains can elicit clinical signs and death in young immunologically naive ducks (*10*), and targeted sampling of waterfowl postbreeding areas for dead or neurologically impaired hatch-year birds might prove useful for future HPAIV surveillance (*11*).

Careful thought has been given to the design of surveillance programs for avian influenza (*12*). The study objectives, coupled with the methodologic limitations of available approaches, drive the sampling tool ultimately applied. Although opportunistic sampling (e.g., morbidity and mortality surveillance) is accessible to most agencies, it is not suited for formal population-level inferences. For estimating AIV shedding prevalence, swab sampling of oropharyngeal and cloacal cavities in live birds or the trachea and cloaca in recently deceased birds is optimal because AIV replicates and sheds through the digestive tract (*13*) and the upper respiratory system (*14*). For investigating exposure history, sampling blood from live or recently dead birds for serologic testing would be more appropriate, although timing, location, and mechanism of exposure cannot be determined.

Most of our samples were obtained from waterfowl feces. The outbreak's speed required a quickly deployable method to collect adequate sample sizes and implement spatial design elements that would allow a meaningful comparison between known areas with infection and areas of the state apparently without infection. Modeling has shown that AIV maintenance in wild bird populations is mediated by environmental transmission (*15*), and the detection of LPAIV in waterfowl fecal samples supports that conclusion. No HPAIV was detected in waterfowl feces, although there was 95% probability of apparent fecal prevalence throughout the study area of 0 to 0.1%. Thus, we conclude that during the 2015 HPAIV (H5N2) outbreak in Minnesota poultry, HPAIV contamination in wild waterfowl feces was not widespread.

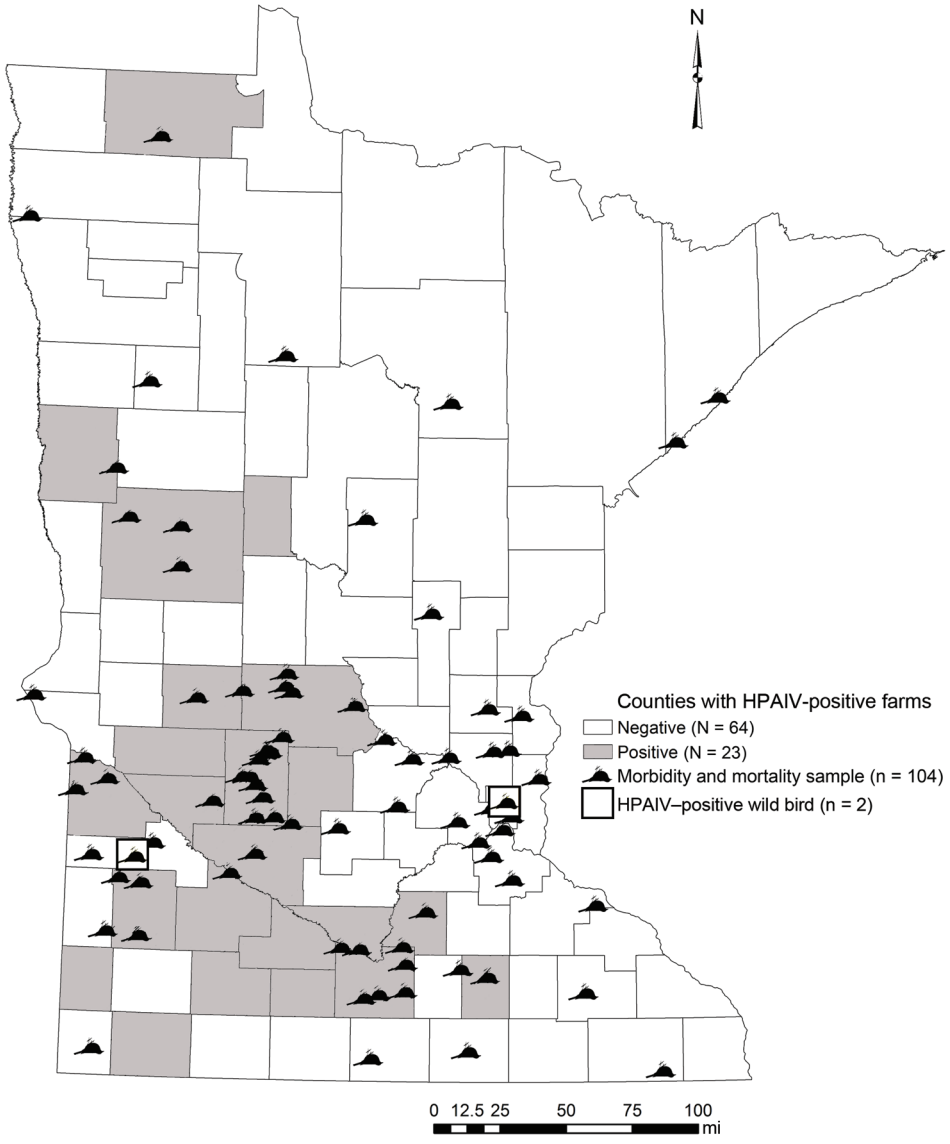

Figure 2. Wild bird morbidity and mortality samples (n = 104) screened for highly pathogenic avian influenza virus (HPAIV) in Minnesota through June 4, 2015. A Cooper's hawk was confirmed to be HPAIV positive in Yellow Medicine County on April 29, 2015, whereas weak titers of Eurasian H5 RNA were detected in a sampled black-capped chickadee from Ramsey County collected in June 2015.

Acknowledgments

Many people were involved in the coordination of sampling, collection of samples, and logistical support for this study. We regret that we cannot name each person involved in these efforts, but we thank the participants from the Minnesota Department of Natural Resources; US Department of Agriculture–National Veterinary Services Laboratory; US Department of Agriculture–Wildlife Services; US Fish and Wildlife Service, US Geological Survey – National Wildlife Health Center; US Department of Agriculture– National Wildlife Research Center; University of Minnesota College of Veterinary Medicine, Public Health and Preventive Medicine Residents; University of Minnesota Veterinary Diagnostic Laboratory; and citizens of Minnesota who reported morbidity and mortality samples. We also thank Robert Dusek, Susan Shriner, and an anonymous reviewer for helpful comments on earlier drafts of the manuscript.

Dr. Jennelle is a research scientist in the Wildlife Health Program of the Minnesota Department of Natural Resources, Forest Lake, Minnesota, USA. He is interested in the ecology of wildlife diseases, quantitative methods for understanding wildlife disease dynamics, and conservation and management of wildlife populations.

References

1. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev. 1992;56:152–79.
2. Swayne DE, Suarez DL. Highly pathogenic avian influenza. Rev Sci Tech. 2000;19:463–82.
3. Kawaoka Y, Nestorowicz A, Alexander DJ, Webster RG. Molecular analyses of the hemagglutinin genes of H5 influenza viruses: origin of a virulent turkey strain. Virology. 1987;158:218–27. http://dx.doi.org/10.1016/0042-6822(87)90256-X

4. França MS, Brown JD. Influenza pathobiology and pathogenesis in avian species. In: Compans RW, Oldstone MBA, editors. Influenza pathogenesis and control, vol. I. New York: Springer International Publishing; 2014. p. 221–42.
5. Stallknecht DE, Brown JD. Tenacity of avian influenza viruses. Rev Sci Tech. 2009;28:59–67.
6. World Organisation for Animal Health. Summary of immediate notifications and follow-ups—2014. Highly pathogenic avian influenza [cited 2015 Oct 14]. http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Immsummary
7. US Department of Agriculture. December 2014–June 2015 wild bird highly pathogenic avian influenza cases in the United States. 2015 Dec [cited 2016 Feb 15]. https://www.aphis.usda.gov/wildlife_damage/downloads/
8. USDA Animal and Plant Health Inspection Service. Update on avian influenza findings: poultry findings confirmed by USDA's National Veterinary Services Laboratory. 2015 [cited 2015 Oct 16]. https://www.aphis.usda.gov/wps/portal/aphis/ourfocus/animalhealth/sa_animal_disease_information/
9. University of Minnesota Extension. Economic impact of the avian flu, updated 7/10/2015. 2015 Jul [cited 2015 Oct 16]. http://www.extension.umn.edu/community/economic-impact-analysis/reports/docs/Avian-flu-update-fact-sheet.pdf
10. Pantin-Jackwood MJ, Swayne DE. Pathobiology of Asian highly pathogenic avian influenza H5N1 virus infections in ducks. Avian Dis. 2007;51(Suppl):250–9. http://dx.doi.org/10.1637/7710-090606R.1
11. Hénaux V, Parmley J, Soos C, Samuel MD. Estimating transmission of avian influenza in wild birds from incomplete epizootic data: implications for surveillance and disease spread. J Appl Ecol. 2013;50:223–31. http://dx.doi.org/10.1111/1365-2664.12031
12. Hoye BJ, Munster VJ, Nishiura H, Klaassen M, Fouchier RAM. Surveillance of wild birds for avian influenza virus. Emerg Infect Dis. 2010;16:1827–34. http://dx.doi.org/10.3201/eid1612.100589
13. Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Copal Murti K. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology. 1978;84:268–78. http://dx.doi.org/10.1016/0042-6822(78)90247-7
14. Sturm-Ramirez KM, Hulse-Post DJ, Govorkova EA, Humberd J, Seiler P, Puthavathana P, et al. Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? J Virol. 2005;79:11269–79. http://dx.doi.org/10.1128/JVI.79.17.11269-11279.2005
15. Breban R, Drake JM, Stallknecht DE, Rohani P. The role of environmental transmission in recurrent avian influenza epidemics. PLOS Comput Biol. 2009;5:e1000346. http://dx.doi.org/10.1371/journal.pcbi.1000346

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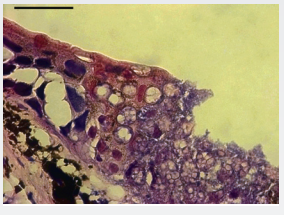
etymologia

Batrachochytrium salamandrivorans [bə-tray'-koh-kit"-ri-um sa"-la-man-dri-vo'rans]

Batrachochytrium salamandrivorans is a recently discovered fungus that kills amphibians. It is related to *B. dendrobatidis*, which also kills amphibians (from the Greek *dendron*, "tree," and *bates*, "one who climbs," referring to a genus of poison dart frogs). *Batrachochytrium* is derived from the Greek words *batrachos*, "frog," and *chytra*, "earthen pot" (describing the structure that contains unreleased zoospores); *salamandrivorans* is from the Greek *salamandra*, "salamander," and Latin *vorans*, "eating," which refers to extensive skin destruction and rapid death in infected salamanders.



Basal infection in skin of a fire salamander (*Salamandra salamandra*) characterized by extensive epidermal necrosis, high numbers of intra-epithelial colonial chytrid thalli, and loss of epithelial integrity. Photo by A. Martel and F. Pasmans, courtesy of Wikipedia.

Sources

1. Longcore JE, Pessier AP, Nichols DK. *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia. 1999;91:219–27. http://dx.doi.org/10.2307/3761366
2. Martel A, Spitzen-van der Sluijs A, Blooi M, Bert W, Ducatelle R, Fisher MC, et al. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proc Natl Acad Sci U S A. 2013;110:15325–9. http://dx.doi.org/10.1073/pnas.1307356110

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Highly Pathogenic Avian Influenza Viruses and Generation of Novel Reassortants, United States, 2014–2015

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Asian highly pathogenic avian influenza A(H5N8) viruses spread into North America in 2014 during autumn bird migration. Complete genome sequencing and phylogenetic analysis of 32 H5 viruses identified novel H5N1, H5N2, and H5N8 viruses that emerged in late 2014 through reassortment with North American low-pathogenicity avian influenza viruses.

Highly pathogenic avian influenza (HPAI) viruses cause systemic infection and high mortality in poultry species and belong to either the H5 or H7 hemagglutinin (HA) subtypes. In particular, the Asian-origin influenza A(H5N1) A/goose/Guangdong/1/1996 (Gs/GD) lineage of HPAI viruses has become widespread across 4 continents, affecting poultry, wild birds, and humans (*1*).

The H5N1 HPAI virus has evolved into 10 genetically distinct virus clades (0–9) and subclades (*2*). During 2005–2006, clade 2.2 viruses spread from Qinghai Lake, China, to countries across Asia, Europe, and Africa (*3*). Since 2008, HPAI viruses bearing the HA gene of the Gs/GD lineage H5 clade 2.3.4 with N2, N5, and N8 neuraminidase (NA) subtypes have been identified in mainland China (*4*,*5*). In early 2014, outbreaks of novel reassortant H5N6 viruses of clade 2.3.4.4 HA were reported in China, Laos, and Vietnam (*6*) and of H5N8 viruses of the same clade in Japan and South Korea (*7*). Subsequently, H5 clade 2.3.4.4 HPAI viruses originating in East Asia were detected in countries of Asia and Europe and, in late 2014, in North America (*8*). Since first being identified in the Pacific Northwest of the United States, HPAI viruses have been detected in 21 states. Approximately 7.5 million turkeys and 42.1 million chickens have died or have been depopulated as a result (https://www.aphis.usda.gov/wps/portal/aphis/ourfocus/animalhealth/sa_animal_disease_information).

In this study, we conducted a comparative phylogenetic analysis of 32 newly sequenced H5 clade 2.3.4.4 HPAI viruses identified in the United States, including 2 H5N1, 12 H5N2, and 18 H5N8 viruses, to estimate the evolutionary history and to elucidate diversification patterns since emergence in North America. The methods used are detailed in online Technical Appendix 1 (http://wwwnc.cdc.gov/EID/article/22/7/16-0048-Techapp1.pdf).

Phylogenetic analyses confirmed the wide geographic dispersion of Gs/GD-lineage H5 clade 2.3.4.4 HPAI viruses since late 2014 and movement of this virus from East Asia to North America, West Asia, and Europe (online Technical Appendix 1 Figure 1). High bootstrap values (>70%) and long branches in the HA phylogeny supported the delineation of these viruses into 4 groups (online Technical Appendix 1 Figure 2). Group intercontinental A (icA) comprises H5N8 viruses identified from China in early 2014 and South Korea, Japan, Taiwan, Canada, the United States, and European countries. The estimated time to most recent common ancestor (tMRCA) was June 2013 (95% Bayesian credible interval [BCI] April–October 2013). Group icA includes reassortant H5N2 and H5N3 viruses from Taiwan and H5N1 and H5N2 viruses from North America. Group B comprises H5N8 viruses identified from China in 2013 and Korea in 2014 (tMRCA April 2013, 95% BCI October 2012–August 2013). Group C comprises H5N6 viruses identified from China and Laos during 2013–2014 and H5N1 viruses identified from China and Vietnam in 2014 (tMRCA November 2012, 95% BCI March 2012–May 2013). Group D comprises H5N6 viruses identified from China and Vietnam during 2013–2014, including isolates from infected humans (A/Sichuan/26221/2014[H5N6] and A/Guangzhou/39715/2014 [H5N6]) (tMRCA September 2012, 95% BCI February 2012–February 2013). These H5 reassortant viruses were descendants of clade 2.3.4 H5N1 viruses identified in 2005 (online Technical Appendix 1 Figure 1).

Previous studies reported novel reassortant H5N1 and H5N2 viruses of group icA (*9*,*10*); the H5N1 and H5N2 viruses we sequenced in this study in had identical genome constellations (Figure; Technical Appendix 1 Figures 3–5). Reassortment events after the initial introduction of a

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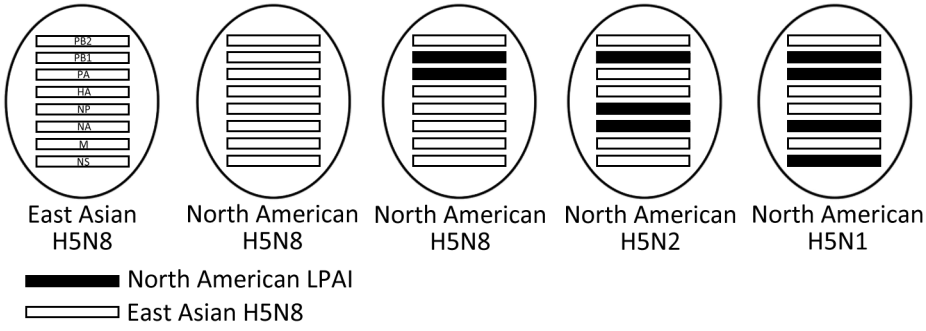

Figure. Schematic diagram of the H5 clade 2.3.4.4 highly pathogenic avian influenza virus genotypes identified in this study, United States, 2014–2015. Reassortant H5N8 comprises Eurasian PB2, PA, HA, NP, M, and NS gene segments, and North American PB1 and PA gene segments; reassortant H5N2 comprises Eurasian PB2, PA, HA, M, and NS gene segments, and North American NA, PB1, and NP gene segments; reassortant H5N1 comprises Eurasian HA, NP, M, and PB2 gene segments and North American NA, NS, PA, and PB1 gene segments. HA, hemagglutinin; LPAI, low-pathogenicity avian influenza; M, matrix; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; PA, polymerase acidic; PB1, polymerase basic 1; PB2, polymerase basic 2.

group icA H5N8 virus to low-pathogenicity avian influenza (LPAI) viruses led to the divergence of H5 viruses into distinct subtypes, including H5N1, H5N2, and reassortant H5N8. Sixteen H5N8 viruses sequenced in this study had identical genome constellations with previously reported H5N8 viruses from East Asia. In addition, 2 H5N8 reassortant isolates identified from Oregon in January 2015 (A/American wigeon/Oregon/AH0012525/2015 and A/Canada goose/Oregon/AH0012452/2015) had polymerase basic 1 and polymerase acidic genes derived from North American lineage LPAI viruses that did not cluster with the H5N1 and H5N2 reassortant viruses (online Technical Appendix 1 Figure 5). Ongoing analysis of existing wild bird surveillance data might aid in filling in the relatively long horizontal branches of the NA and internal genes of H5 reassortant viruses derived from North American LPAI viruses. The occurrence of multiple reassortment events means that group icA H5N8 virus was infecting the same wild birds that were infected with North American LPAI viruses but also that the tissue tropism of Asian H5N8 HPAI and North American LPAI viruses were overlapping, most likely in the cells lining the respiratory and intestinal tract (*11*).

The estimated tMRCA of H5 viruses identified in the United States was October 2014 (95% BCI July–November 2014). The estimated tMRCA of reassortant viruses identified in the United States was December 2014 for H5N1 (95% BCI December 2014–December 2014), November 2014 for H5N2 (95% BCI October 2014–November 2014), and December 2014 for H5N8 (95% BCI November 2014–January 2015) (Table). The tMRCA of H5N8 viruses corresponded to the autumn bird migration season, supporting the hypothesis that Eurasian H5N8 clade 2.3.4.4 virus spread via migratory birds (*8*,*12*,*13*). Subsequently, H5N2 reassortant viruses emerged in November 2014, and H5N1 and H5N8 reassortant viruses emerged in December 2014 (Table; online Technical Appendix 1 Figures 3–5).

Wild bird migration and illegal trade of infected poultry, eggs, and poultry products have caused the spread of HPAI viruses (*14*). The South Korea H5N8 outbreak in January

Table. tMRCA for H5 highly pathogenic avian influenza viruses, by gene, United States, 2014–2015

Gene	tMRCA (95% BCI, posterior probability)			
	H5N8	H5N8 reassortant	H5N1 reassortant	H5N2 reassortant
HA	Oct 2014 (Jul 2014–Nov 2014, 0.81)	Dec 2014 (Nov 2014–Jan 2015, 0.67)	Dec 2014 (Dec 2014–Dec 2014, 1.00)	Nov 2014 (Oct 2014–Nov 2014, 0.99)
NA	Jul 2014 (Feb 2014–Nov 2014, 0.76)	Dec 2014 (Nov 2014–Jan 2015, 1.00)	Mar 2014 (Jul 2013–Oct 2014, 1.00)	Sep 2014 (Jun 2014– Nov 2014, 1.00)
PB2	Oct 2014 (Aug 2014–Nov 2014, 1.00)	Dec 2014 (Oct 2014– Jan 2015, 0.48)	Nov 2014 (Oct 2014–Dec 2014, 0.95)	Nov 2014 (Oct 2014– Nov 2014, 0.99)
PB1	Oct 2014 (Jul 2014–Nov 2014, 1.00)	Dec 2014 (Nov 2014–Jan 2015, 1.00)	Dec 2014 (Nov 2014–Dec 2014, 1.00)	Oct 2014 (Aug 2014– Nov 2014, 1.00)
PA	Sep 2014 (Jul 2014–Nov 2014, 0.38)	Nov 2014 (Sep 2014–Jan 2015, 1.00)	Nov 2014 (Oct 2014–Dec 2014, 0.98)	Oct 2014 (Sep 2014–Nov 2014, 1.00)
NP	Jul 2014 (Mar 2014–Nov 2014, 0.53)	Nov 2014 (Jul 2014–Jan 2015, 0.67)	Nov 2014 (Oct 2014–Dec 2014, 1.00)	Nov 2014 (Jun 2014–Nov 2014, 1.00)
M	Jul 2014 (Jan 2014–Dec 2014, 0.95)	Nov 2014 (Jun 2014–Jan 2015, 0.38)	Dec 2014 (Nov 2014–Dec 2014, 1.00)	Aug 2014 (Mar 2014–Nov 2014, 0.34)
NS	May 2014 (Nov 2013–Nov 2014, 0.08)	Dec 2014 (Nov 2014–Jan 2015, 1.00)	Nov 2014 (Sep 2014–Dec 2014, 1.00)	May 2014 (Oct 2013–Oct 2014, 0.86)

*BCI, Bayesian credible interval; HA, hemagglutinin; M, membrane; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; PA, polymerase acidic; PB1, polymerase basic 1; PB2, polymerase basic 2; tMRCA, time to most recent common ancestor.

2014 was the first H5N8 virus reported outside of China. Wild migratory birds were suspected to play a key role in the introduction of group icA and B viruses from eastern China and in the subsequent spread during the 2014 South Korea outbreak (*15*). Previous studies hypothesized that wild bird migration might play a role in dispersal of these viruses; the H5N8 virus was identified in a long-distance migrant bird (Eurasian wigeon, *Anas penelope*) in eastern Siberia in September 2014 and subsequently in multiple wild bird species in Japan, Europe, and the west coast of North America in November and December 2014 (*8*,*12*). In contrast, group C H5N6 HPAI viruses in Laos were most likely transmitted by live poultry imports from China (*6*).

The continued reassortment of H5 clade 2.3.4.4 HPAI viruses with co-circulating HPAI and LPAI viruses created a diverse genetic pool of H5 clade 2.3.4.4 that has spread to various countries. This contrasts with the expansion of H5N1 clade 2.2 from Asia to Western Europe during 2005–2006, when such frequent reassortment was not recorded. In eastern China, H5N2 HPAI viruses isolated in 2011 were generated from reassortment events in which the neuraminidase and nonstructural gene segments of H5N1 HPAI viruses were replaced with those derived from locally circulating LPAI viruses (*4*). The H5N8 viruses of group B had polymerase basic 2, neuraminidase, and nonstructural genes derived from local LPAI viruses (*5*). The H5N6 viruses of group C identified in Laos were generated through reassortment between H5N1 viruses from clade 2.3.2.1b, clade 2.3.4, and H6N6 LPAI viruses that circulate broadly in duck populations in China (*6*).

H5 clade 2.3.4.4 viruses have spread globally through wild bird migration and the poultry trade (*6*,*8*,*12*,*13*). In addition, these viruses generated a variety of reassortant viruses that shuffled genes with prevailing local viruses. The continued circulation of HPAI viruses in wild and domestic avian populations contributes to the persistence and diversity of circulating avian influenza viruses. Enhanced active surveillance provides the opportunity to monitor the spread and reassortment of clade 2.3.4.4 and to fortify the biosecurity of farms in affected regions.

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References

1. Swayne DE, Suarez DL, Sims LD. Influenza. In: Swayne DE, Glisson JR, McDougald LR, Nair V, Nolan LK, Suarez DL, editors. Diseases of poultry, 13th edition. Ames (Iowa): Wiley-Blackwell; 2013. p. 181–218.
2. WHO/OIE/FAO H5N1 Evolution Working Group. Toward a unified nomenclature system for highly pathogenic avian influenza virus (H5N1). Emerg Infect Dis. 2008;14:e1. http://dx.doi.org/10.3201/eid1407.071681
3. Salzberg SL, Kingsford C, Cattoli G, Spiro DJ, Janies DA, Aly MM, et al. Genome analysis linking recent European and African influenza (H5N1) viruses. Emerg Infect Dis. 2007;13:713–8. http://dx.doi.org/10.3201/eid1305.070013
4. Zhao G, Gu X, Lu X, Pan J, Duan Z, Zhao K, et al. Novel reassortant highly pathogenic H5N2 avian influenza viruses in poultry in China. PLoS One. 2012;7:e46183. http://dx.doi.org/10.1371/journal.pone.0046183
5. Wu H, Peng X, Xu L, Jin C, Cheng L, Lu X, et al. Novel reassortant influenza A(H5N8) viruses in domestic ducks, eastern China. Emerg Infect Dis. 2014;20:1315–8. http://dx.doi.org/10.3201/eid2008.140339
6. Wong FY, Phommachanh P, Kalpravidh W, Chanthavisouk C, Gilbert J, Bingham J, et al. Reassortant highly pathogenic influenza A(H5N6) virus in Laos. Emerg Infect Dis. 2015;21:511–6. http://dx.doi.org/10.3201/eid2103.141488
7. Lee YJ, Kang HM, Lee EK, Song BM, Jeong J, Kwon YK, et al. Novel reassortant influenza A(H5N8) viruses, South Korea, 2014. Emerg Infect Dis. 2014;20:1087–9. http://dx.doi.org/10.3201/eid2006.140233
8. Lee DH, Torchetti MK, Winker K, Ip HS, Song CS, Swayne DE. Intercontinental spread of Asian-origin H5N8 to North America through Beringia by migratory birds. J Virol. 2015;89:6521–4. http://dx.doi.org/10.1128/JVI.00728-15
9. Torchetti MK, Killian ML, Dusek RJ, Pedersen JC, Hines N, Bodenstein B, et al. Novel H5 clade 2.3.4.4 reassortant (H5N1) virus from a green-winged teal in Washington, USA. Genome Announc. 2015;3:e00195–15. http://dx.doi.org/10.1128/genomeA.00195-15
10. Ip HS, Torchetti MK, Crespo R, Kohrs P, DeBruyn P, Mansfield KG, et al. Novel Eurasian highly pathogenic avian influenza A H5 viruses in wild birds, Washington, USA, 2014. Emerg Infect Dis. 2015;21:886–90. http://dx.doi.org/10.3201/eid2105.142020
11. Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology. 1978;84:268–78. http://dx.doi.org/10.1016/0042-6822(78)90247-7
12. Verhagen JH, Herfst S, Fouchier RA. Infectious disease. How a virus travels the world. Science. 2015;347:616–7. http://dx.doi.org/10.1126/science.aaa6724
13. Saito T, Tanikawa T, Uchida Y, Takemae N, Kanehira K, Tsunekuni R. Intracontinental and intercontinental dissemination of Asian H5 highly pathogenic avian influenza virus (clade 2.3.4.4) in the winter of 2014–2015. Rev Med Virol. 2015;25:388–405. http://dx.doi.org/10.1002/rmv.1857
14. Suarez DL. Avian influenza: our current understanding. Anim Health Res Rev. 2010;11:19–33. http://dx.doi.org/10.1017/S1466252310000095
15. Jeong J, Kang HM, Lee EK, Song BM, Kwon YK, Kim HR, et al. Highly pathogenic avian influenza virus (H5N8) in domestic poultry and its relationship with migratory birds in South Korea during 2014. Vet Microbiol. 2014;173:249–57. http://dx.doi.org/10.1016/j.vetmic.2014.08.002

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Expanding Distribution of Lethal Amphibian Fungus *Batrachochytrium salamandrivorans* in Europe

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Emerging fungal diseases can drive amphibian species to local extinction. During 2010–2016, we examined 1,921 urodeles in 3 European countries. Presence of the chytrid fungus *Batrachochytrium salamandrivorans* at new locations and in urodeles of different species expands the known geographic and host range of the fungus and underpins its imminent threat to biodiversity.

Amphibians provide an iconic example of disease-driven global loss in biodiversity. The recently described chytrid fungus *Batrachochytrium salamandrivorans* (*Bsal*) is an emerging pathogen that is driving amphibian populations to local extinction (*1*,*2*). This highly pathogenic fungus causes a lethal skin disease that has so far been restricted to urodele amphibians (newts and salamanders); the fungus was most likely introduced from East Asia into Europe via the pet trade (*2*). In Europe, *Bsal* infection has led to dramatic declines of fire salamander (*Salamandra salamandra*) populations in the Netherlands and Belgium (*2*). Within 7 years after the supposed introduction of the fungus, a population in the Netherlands declined by 99.9% (*3*,*4*). In the United Kingdom and Germany, *Bsal* has been detected in captive salamanders and newts (*5*,*6*). Infection trials suggest that *Bsal* represents an unprecedented threat to diversity of Western Palearctic urodeles (*2*); nevertheless, reports of deaths among salamanders and newts in their naturalized ranges have been restricted to a few populations of a single salamander species in the southern Netherlands and adjacent Belgium (*1*,*3*). Using data from field surveillance, we examined the hosts and the geographic range of *Bsal* in Europe.

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The Study

During 2010–2016, we collected samples of free-living populations of newts and salamanders from 48 sites in the Netherlands, Belgium, and adjacent regions of the Eifel region in Germany (near the border with the Netherlands and Belgium) (Figure; online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/7/16-0109-Techapp1.pdf). Site selection was based on reported amphibian deaths, apparent negative amphibian population trends, preventive *Bsal* surveillance in susceptible populations, or geographic proximity to known outbreak sites. Samples were also collected at 6 additional sites in Germany and 1 in the Netherlands, which were located >100 km from the nearest known outbreak (online Technical Appendix Table 2). Sampling was conducted by swabbing skin (*7*,*8*) of live animals and collecting skin samples from dead animals. All samples were kept frozen at -20°C until they were analyzed for the presence of *Bsal* DNA via real-time PCR, as described (*9*).

Across all 55 sites, we tested 1,019 fire salamanders (43 dead, 976 skin swab samples); at site 14, skin swab samples instead of tissue samples were collected from 16 dead salamanders. We also collected samples from 474 alpine newts (*Ichthyosaura alpestris*; 18 dead, 456 skin swab samples), 239 smooth newts (*Lissotriton vulgaris*; 2 dead, 237 skin swab samples), 80 palmate newts (*Lissotriton helveticus*; all skin swab samples), 79 crested newts (*Triturus cristatus*; all skin swab samples), and

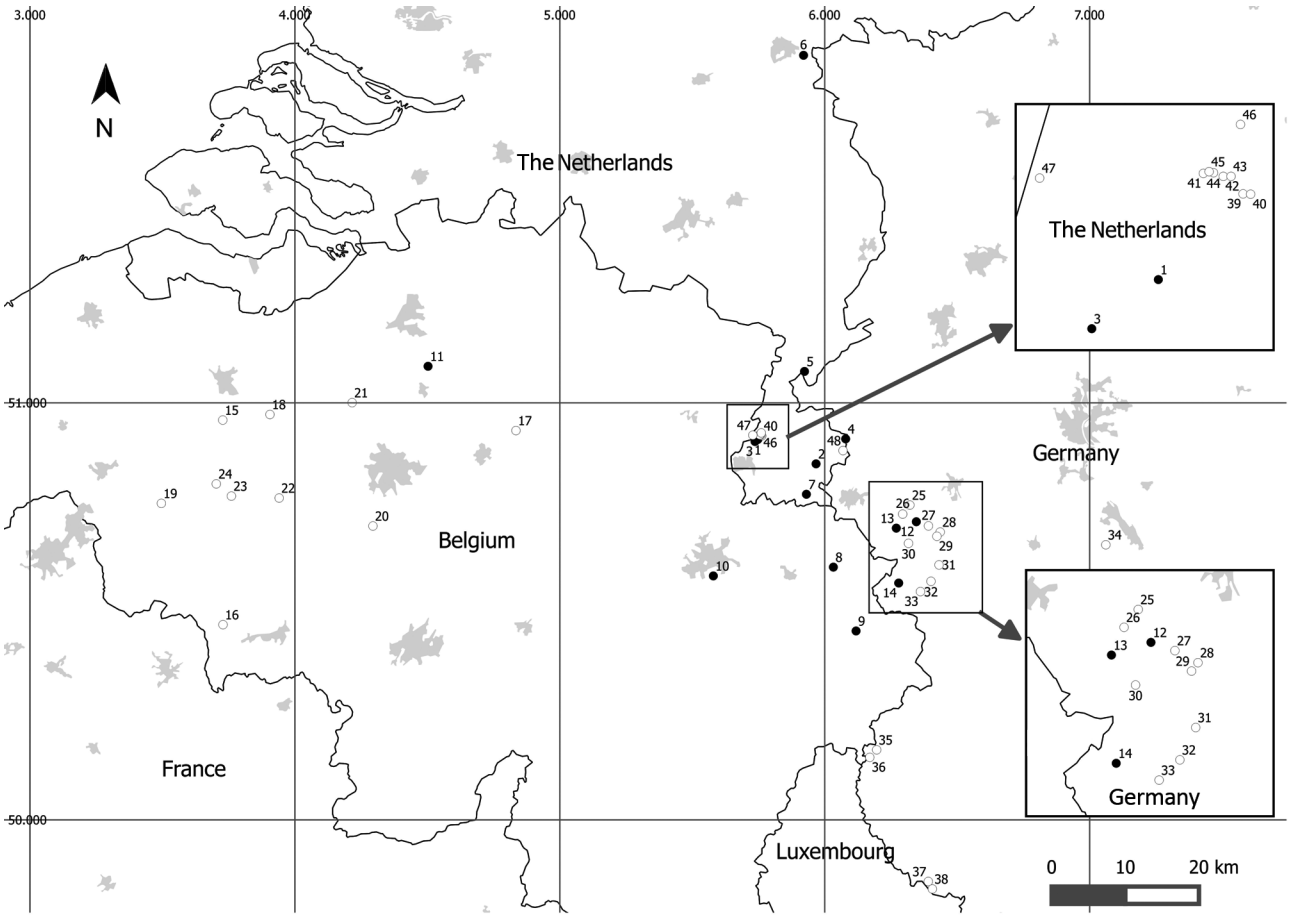

Figure. Study sites for collection of amphibians in Western Europe, 2010–2016. Numbers correspond to field sites at which amphibians were collected and examined for *Batrachochytrium salamandrivorans* (*Bsal*) (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/16-0109-Techapp1.pdf). Solid circles, *Bsal* detected; open circles, *Bsal* not detected. Larger cities are indicated in light gray. Note that there are additional sites where the fungus remained undetected (not shown).

30 Italian crested newts (*Triturus carnifex*; all skin swab samples). To obtain a Bayesian 95% credible interval for prevalence (online Technical Appendix), we used the computational methods of Lötters et al. (*10*). We ran 3 parallel Markov chains with 20,000 iterations each and discarded the first 5,000 iterations as burn-in; chains were not thinned.

Bsal was found at 14 of the 55 sites; infected amphibians were fire salamanders, alpine newts, and smooth newts. Our results demonstrate that the range of *Bsal* distribution may be up to ≈10,000 km^2 (measured as the surface of a minimum convex polygon encompassing the outermost points) across Germany, Belgium, and the Netherlands (Figure). The presence of *Bsal* in wild alpine newts and smooth newts shows distinct expansion of the known host range in the wild (online Technical Appendix Table 1). Furthermore, we document that *Bsal* is present in natural fire salamander populations in Germany (confined to the Eifel region). At some sites, because of our sample sizes, the upper limit of the 95% credible interval for *Bsal* prevalence was as high as 0.7; therefore, we may have failed to detect *Bsal* at these sites (online Technical Appendix Table 1). In addition, the fungus may have been present at several sites before first detection. For example, *Bsal* was detected at site 4, where population-monitoring efforts in the years before detection (2000–2013) showed declines in 4 newt species (http://www.ravon.nl/EID_SI_Spitzen_et_al_2016). However, because no samples were collected before 2015, we have no evidence for a causal relationship between the presence of *Bsal* and the declines. We have also recorded the presence of *Bsal* in populations with no evidence of population change so far, such as the incidental findings of dead *Bsal*-positive newts in fyke nets at sites 5 and 11, and the incidental findings of dead *Bsal*-positive fire salamanders at sites 12 and 14. Clinical signs of mycosis, such as lethargy and skin shedding (*1*), were observed at some *Bsal*-positive sites (1, 2, 7, 8, 14) but not at others.

Conclusions

Our study provides evidence that *Bsal* among wild amphibians in Europe is more widely distributed and affects a wider host range than previously known, which can either indicate recent spread of the fungus or point to historically infected sites that hitherto remained undetected. The presence of *Bsal* in wild populations can easily remain unnoticed because the lesions develop only near the final stage of the disease (*1*). This information is crucial for the design of field surveys for *Bsal* surveillance. Our data might be used to inform a management strategy and to implement the recommendation of the Bern Convention (*11*) to halt the spread of *Bsal* in Europe. Research to search for molecular evidence that the outbreak locations are connected is under way. Chytrid disease dynamics are affected by multiple factors (e.g., temperature regimes [*1*]), and yet undetermined environmental determinants might be essential for disease outbreaks (*12*). Untangling these factors, as well as the modes of *Bsal* spread and its geographic distribution, are points for further research to fully map the problem and identify populations and species at risk.

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References

1. Martel A, Spitzen-van der Sluijs A, Blooi M, Bert W, Ducatelle R, Fisher MC, et al. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proc Natl Acad Sci U S A. 2013;110:15325–9. http://dx.doi.org/10.1073/pnas.1307356110
2. Martel A, Blooi M, Adriaensen C, Van Rooij P, Beukema WM, Fisher MC, et al. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science. 2014;346:630–1. http://dx.doi.org/10.1126/science.1258268
3. Spitzen-van der Sluijs A, Spikmans F, Bosman W, De Zeeuw M, Van der Meij T, Goverse E, et al. Rapid enigmatic decline drives the fire salamander (*Salamandra salamandra*) to the edge of extinction in the Netherlands. Amphibia-Reptilia. 2013;34:233–9. http://dx.doi.org/10.1163/15685381-00002891
4. Goverse E, De Zeeuw M. Schubben & slijm: resultatan meetnet amfibieën 2014 [cited 2016 Mar 21]. http://www.ravon.nl/Portals/0/PDF3/schubbenslijm26.pdf
5. Cunningham AA, Beckmann K, Perkins M, Fitzpatrick L, Cromie R, Redbond J, et al. Emerging disease in UK amphibians. Vet Rec. 2015;176:468. http://dx.doi.org/10.1136/vr.h2264
6. Sabino-Pinto J, Bletz M, Hendrix R, Perl RGB, Martel A, Pasmans F, et al. First evidence of *Batrachochytrium salamandrivorans* from a captive salamander population in Germany. Amphibia-Reptilia. 2015;36:411–6. http://dx.doi.org/10.1163/15685381-00003008
7. Hyatt AD, Boyle DG, Olsen V, Boyle DB, Berger L, Obendorf D, et al. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis.* Dis Aquat Organ. 2007;73:175–92. http://dx.doi.org/10.3354/dao073175
8. Van Rooij P, Martel A, Nerz J, Voitel S, Van Immerseel F, Haesebrouck F, et al. Detection of *Batrachochytrium dendrobatidis* in Mexican bolitoglossine salamanders using an optimal sampling protocol. EcoHealth. 2011;8:237–43. http://dx.doi.org/10.1007/s10393-011-0704-z
9. Blooi M, Pasmans F, Longcore JE, Spitzen-van der Sluijy A, Vercammen F, Martel A. Duplex real-time PCR for rapid simultaneous detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in amphibian samples. J Clin Microbiol. 2013;51:4173–7. http://dx.doi.org/10.1128/JCM.02313-13
10. Lötters S, Kielgast J, Sztatecsny M, Wagner N, Schulte U, Werner P, et al. Absence of infection with the amphibian chytrid fungus in the terrestrial alpine salamander, *Salamandra atra.* Salamandra. 2012;48:58–62.
11. Council of Europe. Convention on the Conservation of European Wildlife and Natural Habitats, 35th meeting; 2015 Dec 1–4; Strasbourg, France. Recommendation no. 176 (2015) on the prevention and control of the Batrachochytrium salamandrivorans chytrid fungus [cited 2016 Mar 21]. https://wcd.coe.int/com.instranet.InstraServlet?command=com.instranet.CmdBlobGet&InstranetImage=2859613&SecMode=1&DocId=2339082&Usage=2
12. Van Rooij P, Martel A, Haesenbrouck F, Pasmans F. Amphibian chytridiomycosis: a review with focus on fungus-host interactions. Vet Res. 2015;46:137. http://dx.doi.org/10.1186/s13567-015-0266-0

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Two Related Occupational Cases of *Legionella longbeachae* Infection, Quebec, Canada

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Two patients with no exposure to gardening compost had related *Legionella longbeachae* infections in Quebec, Canada. Epidemiologic investigation and laboratory results from patient and soil samples identified the patients' workplace, a metal recycling plant, as the likely source of infection, indicating a need to suspect occupational exposure for *L. longbeachae* infections.

Several *Legionella* species can cause legionellosis, which results in influenza-like illness (Pontiac fever) or pneumonia (Legionnaires' disease) (*1,2*). *L. pneumophila*, which is mainly transmitted from aerosolized water, has been the principal *Legionella* species reported from Canada (*3*). Unlike *L. pneumophila*, *L. longbeachae* is highly adapted to the soil environment and primarily transmitted from potting soils and compost (*2*).

During summer 2015, a regional public health authority in Quebec, Canada, received reports of 2 cases of pneumonia attributable to *L. longbeachae* infection. These cases occurred 1 month apart in persons who shared the same workplace. We conducted epidemiologic and environmental investigations to identify the source of infection and propose appropriate control measures.

The Study

On July 3, 2015, the provincial public health laboratory (Laboratoire de santé publique du Québec [LSPQ], Sainte-Anne-de-Bellevue, Quebec, Canada), informed the regional public health authority (Centre intégré de santé et de services sociaux de la Montérégie-Centre, Longueuil, Quebec) about a case of *L. longbeachae* serogroup 1 infection. The investigation team included members with expertise in infectious diseases and in occupational and environmental health. Public health experts from the Institut national de santé publique du Québec (Quebec City, Quebec), the Institut de recherche Robert-Sauvé en santé et en sécurité du travail (Montreal, Quebec), and the LSPQ joined the investigation team of the regional public health authority. The investigators questioned the patient by using a standardized epidemiologic questionnaire and explored potential relationships between the patient's illness (i.e., clinical manifestations, laboratory results, and diagnosis) and personal factors (i.e., demographic, behavioral, and medical risk factors) and possible exposure sources. During the investigation, another worker from the same workplace was hospitalized with severe pneumonia, and the public health team recommended testing for *L. longbeachae*. On July 20, 2015, the LSPQ confirmed *L. longbeachae* serogroup 1 infection for the second patient, and the investigation team questioned this patient by using the same standardized epidemiologic questionnaire answered by the first patient. No other causal organism was identified for either patient.

A lag of 1 month separated onset of symptoms in the 2 patients. Both had severe pneumonia that required admission to intensive care. They recovered and returned to work a few months later. Both had personal risk factors for Legionnaires' diseases. However, neither had a history of travel, gardening, visits to gardening centers, or exposure to hanging plant pots or compost. Both worked at the same metal recycling plant for many years and shared no nonprofessional activities. One was a shredder operator at a fixed work station; the other was responsible for machinery maintenance throughout the plant (3,750 m^2 in size); the only shared spaces were the locker and lunch rooms. Their work shifts overlapped for a few hours. The company, which employed ≈25 workers, has been in operation for >40 years and had no prior case of legionellosis.

On July 8, 2015, the regional public health authority investigated the workplace and assessed the industrial processes. Trucks containing cars and other bulk metal materials unload at the site. An industrial grapple clamps the materials and feeds them to a shredder. Any overload is stacked until it can be processed. Diverse metals are then sorted out and sold. The business operates during April–December.

The investigation identified different sources of soil exposure. First, most of the site lies on bare ground. A tanker truck regularly sprinkles water to control dust. Second, a

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conveyor belt with foam residue and other debris generates aerosols that contain soil particles. However, employees are not allowed near the conveyor belt when it is operating. Third, some cars are reportedly filled with soil by suppliers to increase weight and raise selling value.

On July 31, 2015, multiple soil samples were taken from a workplace area where the soil could have been at higher risk for *L. longbeachae* contamination (i.e., because of greater-than-usual humidity, less exposure to wind, and less ultraviolet exposure from the sun). A control sample was taken from an area of undisturbed soil in this workplace. Proper sterilization of equipment was ensured between collections of samples. Sixteen randomly located sites (each 4 m^2) were sampled, including the control site.

The Institut de recherche Robert-Sauvé en santé et en sécurité du travail obtained an isolate from 1 soil sample and used PCR for identification. The LSPQ obtained isolates from bronchoalveolar lavages and, in collaboration with Canada's National Microbiology Laboratory, confirmed their identity by using the 16S sequencing method. Pulsed-field gel electrophoresis was used to investigate concordance of the outbreak strains and to compare the isolates' patterns with those obtained from previous *L. longbeachae* isolates from Quebec. An adaptation of the full pulsed-field gel electrophoresis *Sfi*I protocol developed for *L. pneumophila* (*4*) was also conducted by using *Asc*I for *L. longbeachae* isolates.

All soil samples were positive for *Legionella* spp., an expected outcome because these bacteria are ubiquitous in the environment. PCR and cultures conducted on the soil sample taken near the truck-unloading station were positive for *L. longbeachae*. By using the 2 enzymes (*Asc*I and *Sfi*I) protocol, laboratory findings showed that the strains from the 2 patients and from the positive soil sample were concordant (Figure), except for 1 difference, and were closely related, according to Tenover's criteria (*5*).

Conclusions

L. longbeachae infections are rarely reported in Quebec. During 2003–2014, the LSPQ identified only 7 sporadic cases and no geographic clustering. In 2015, 2 severe *L. longbeachae* pneumonia cases occurred 1 month apart. The determination that the only common temporospatial exposure for the 2 patients was the workplace constitutes a strong epidemiologic link. Furthermore, *L. longbeachae* of the same genotype was isolated in the workplace soil samples. Although the diversity and distribution of *L. longbeachae* strains in Quebec soils are unknown, finding the same *L. longbeachae* genotype in the workplace soil suggests a causal link between the 2 case-patients and their workplace.

Unlike clusters of *L. longbeachae* described in the literature (*6,7*), these 2 patients did not come into contact with potting soils or compost during the exposure period. However, several sources of soil were found in their work environment. Although *L. longbeachae* is usually found in highly organic soil (*2*), the positive soil sample in this investigation came from poor soil. Until now, no Legionnaires' disease case has been linked to *L. longbeachae* in this type of soil. Possibly, *L. longbeachae* traveled from the environment surrounding the plant or from soil trapped in trunks of wrecked cars. Also, soil analysis results might not reflect the conditions that prevailed during the exposure period.

This outbreak resolved spontaneously. The regional public health authority recommended preventive measures, such as handwashing, reinforced personal hygiene, avoidance of soil dumping from car trunks, and dust control.

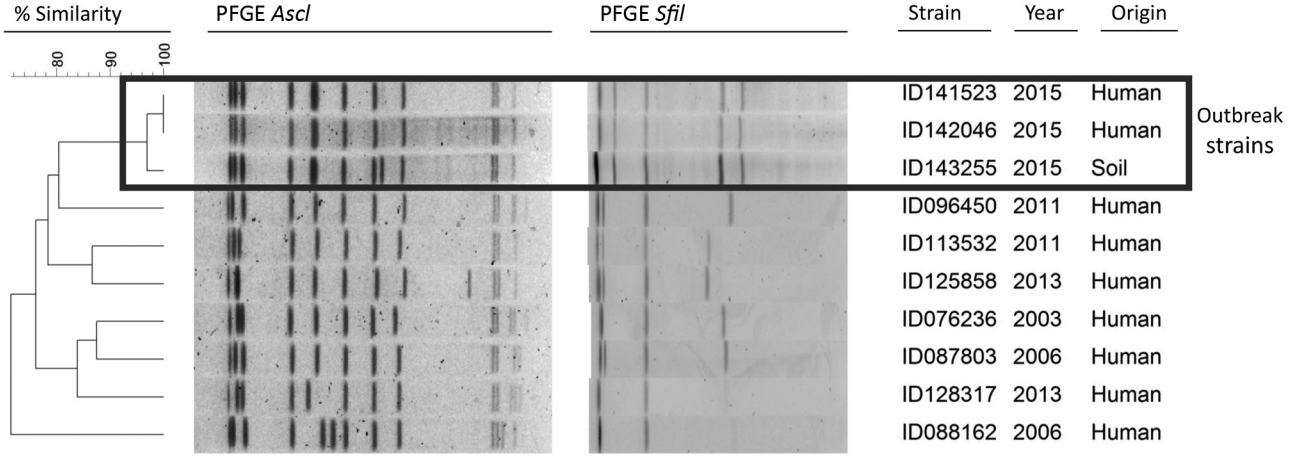

Figure. Patterns of pulsed-field gel electrophoresis (PFGE) using *Asc*I and *Sfi*I enzymes for specimens from 2 occupational cases of *Legionella longbeachae* infection, a positive soil sample, and various other *L. longbeachae* strains analyzed during 2003–2015 at the Laboratoire de Santé Publique du Québec, Quebec, Canada.

The small number of cases in this outbreak and a general paucity of knowledge about *L. longbeachae* limited this investigation. The precise mechanism leading to infection has not yet been identified. Whereas hand-to-mouth contamination followed by microaspiration (*8*) seems the most probable route of exposure, dust inhalation cannot be ruled out. Early mobilization of experts and good collaboration with the implicated company facilitated the outbreak investigation.

These cases highlight the need to search for *L. longbeachae* in cases of severe pneumonia by performing appropriate cultures and to consider the risk for occupational exposure when soil is present. Environmental investigation appears useful to understand *L. longbeachae* transmission and ecology in Canada's soils. Additional research is needed to improve understanding of sources of exposure, the pathogenesis of this species, and appropriate control measures.

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References

1. Steinert M, Hentschel U, Hacker J. *Legionella pneumophila*: an aquatic microbe goes astray. FEMS Microbiol Rev. 2002;26:149–62. http://dx.doi.org/10.1111/j.1574-6976.2002.tb00607.x
2. Whiley H, Bentham R. *Legionella longbeachae* and legionellosis. Emerg Infect Dis. 2011;17:579–83. http://dx.doi.org/10.3201/eid1704.100446
3. Agence de la santé publique du Canada. *Legionella pneumophila* [in French]. Modified 2011 Apr 19 [cited 2015 Dec 1]. http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/legionella-fra.php
4. Lévesque S, Plante PL, Mendis N, Cantin P, Marchand G, Charest H, et al. Genomic characterization of a large outbreak of *Legionella pneumophila* serogroup 1 strains in Quebec City, 2012. PLoS One. 2014;9:e103852. http://dx.doi.org/10.1371/journal.pone.0103852
5. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995;33:2233–9.
6. Potts A, Donaghy M, Marley M, Othieno R, Stevenson J, Hyland J, et al. Cluster of Legionnaires' disease cases caused by *Legionella longbeachae* serogroup 1, Scotland, August to September 2013. Euro Surveill. 2013;18:20656 http://dx.doi.org/10.2807/1560-7917.ES2013.18.50.20656. PMID 2432515
7. Pravinkumar SJ, Edwards G, Lindsay D, Redmond S, Stirling J, House R, et al. A cluster of Legionnaires' disease caused by *Legionella longbeachae* linked to potting compost in Scotland, 2008–2009. Euro Surveill. 2010;15:19496.
8. Steele TW. The ecology of *Legionella longbeachae* in Australia. Med J Aust. 1996;164:703–4.

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Effective Chemical Inactivation of Ebola Virus

Elaine Haddock, Friederike Feldmann, Heinz Feldmann

Reliable inactivation of specimens before removal from high-level biocontainment is crucial for safe operation. To evaluate efficacy of methods of chemical inactivation, we compared in vitro and in vivo approaches using Ebola virus as a surrogate pathogen. Consequently, we have established parameters and protocols leading to reliable and effective inactivation.

The safe operation of high-level biocontainment laboratories throughout the world is of highest importance. These laboratories are under stringent national oversight and must adhere to international guidelines. Laboratories in the United States that handle select agents are further regulated by the US Centers for Disease Control and Prevention's Division of Select Agents and Toxins and the US Department of Agriculture's Animal and Plant Health Inspection Service.

Proper and reliable inactivation of specimens destined for removal from high-level biocontainment is a critical aspect for laboratory certification and operation. Standard operating procedures (SOPs) are approved by institutional biosafety committees in most cases and additionally by state and/or national regulatory authorities in other cases. In the past, specimens were commonly inactivated on the basis of operational experiences rather than well-documented protocols (*1*–*3*).

To evaluate the efficacy of chemical inactivation procedures for specimen removal, we used the US prime select agent and Tier-1 pathogen (*4*) *Zaire ebolavirus* (EBOV) as a surrogate model for enveloped high-level containment viruses with single-strand, negative-sense RNA genomes, such as arenaviruses, bunyaviruses, filoviruses, orthomyxoviruses, and paramyxoviruses. These viruses share certain biologic, biochemical, and structural features, making them sensitive to the same chemical inactivation methods. Furthermore, EBOV is currently a prominent example as the causative agent of an unprecedented epidemic in West Africa (*5*,*6*).

The Study

Standard biologic specimens containing infectious EBOV commonly generated in high-level biocontainment operations were inactivated by several methods of chemical treatment (Figure; Table, http://wwwnc.cdc.gov/EID/article/22/7/16-0233-T1.htm; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/16-0233-Techapp1.pdf). For in vitro testing, we used wild-type EBOV expressing enhanced green fluorescent protein (EBOV-eGFP) (*7*), which allows for cytopathic effect (CPE) and fluorescence as simple readout parameters. For in vivo testing, we used mouse-adapted EBOV (MA-EBOV) (*8*) infection of BALB/c mice. Virus stocks were grown in Vero E6 cells and titrated by using a 50% tissue culture infectious dose ($TCID_{50}$) assay (*9*). Infected cells were produced by infecting Vero E6 cells at a multiplicity of infection of 0.01. Cells were harvested at CPE of ≈75%, pelleted, and resuspended in 6 mL Dulbecco's phosphate-buffered saline (DPBS); 1 mL aliquots were stored at −80°C. Samples were chemically treated according to the specific testing parameters and dialyzed or run over detergent-removal columns to remove inactivating reagents. In brief, samples were dialyzed by using a 10-kDa molecular weight cutoff (Spectrum Laboratories, Lawrenceville, GA, USA, or Fisher Scientific, Pittsburgh, PA, USA) and using DPBS over a stir plate at 4°C (>500-fold exchange volumes, 5 changes over 32–48 h); detergent was removed by using DetergentOUT GBS10–5000 columns (G-Biosciences, St. Louis, MO, USA).

Negative control samples included DPBS and noninfected Vero E6 cells and tissue homogenates (mouse); positive control samples included untreated virus stocks and infected Vero E6 cells and mouse tissues. For in vitro testing, all samples were increased in volume to 3 mL and equally divided to infect Vero E6 cells (80% confluency) in triplicates. Cells were incubated at 37°C for 14 days and monitored regularly for CPE or fluorescence. For in vivo testing, samples were increased in volume to 1 mL and equally divided to infect 5 mice intraperitoneally. BALB/c mice (female, 6–8 weeks old; Charles River Laboratories, Wilmington, MA, USA) were housed in microisolator cages and were monitored daily for 28 days. Because in vitro and in vivo safety testing correlated well, we discontinued mouse infections for ethical reasons.

Nucleic acid extraction is often carried out with commercial guanidinium isothiocyanate buffers. We used Buffer AVL and Buffer RLT (QIAGEN, Valencia, CA, USA) and TRIzol (Life Technologies, Grand Island, NY, USA) according to manufacturers' recommendations. AVL was mixed with stock virus at different ratios, and infected cells were resuspended in RLT (Table). Samples were either

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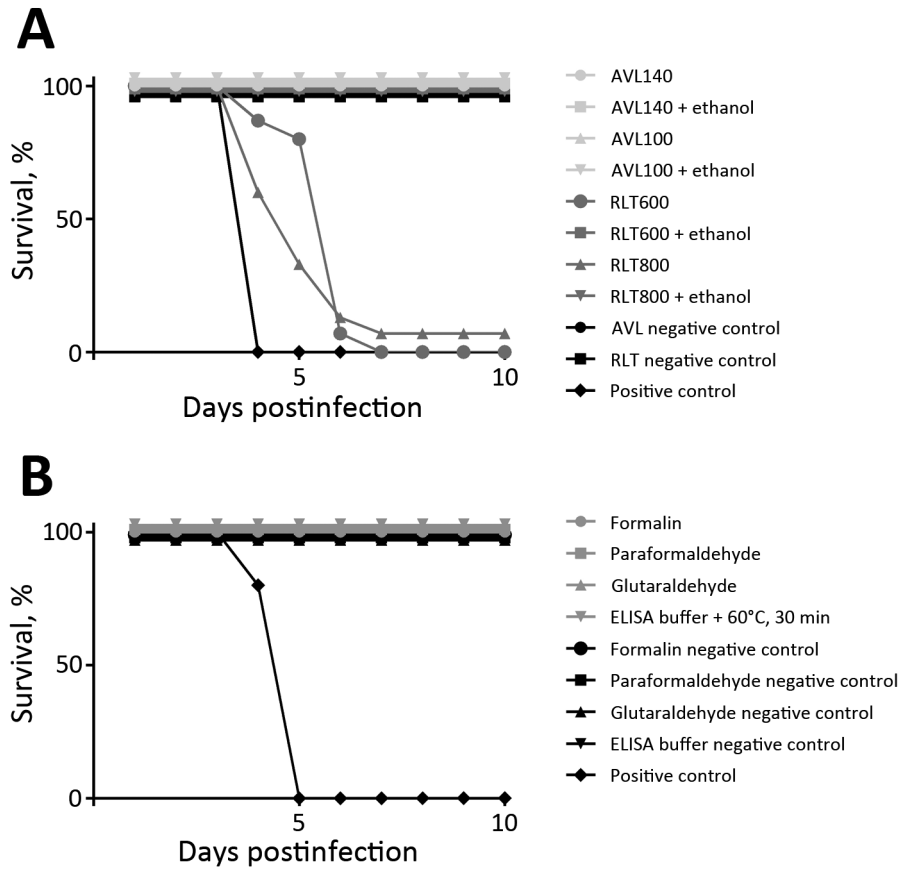

Figure. Ebola virus inactivation results as tested in BALB/c mouse model. A) Survival in animal groups tested with samples inactivated by guanidinium isothiocyanate buffers. AVL140, 140 µL Buffer AVL (QIAGEN, Valencia, CA, USA) + 560 µL sample; AVL100, 100 µL Buffer AVL + 600 µL sample; RLT600, 600 µL Buffer RLT (QIAGEN) treatment of cells; RLT800, 800 µL Buffer RLT treatment of cells; + ethanol, after a Buffer AVL or Buffer RLT inactivation contact time of 10 min, addition of 100% or 70% ethanol, respectively, for an additional 20 min of contact time. B) Survival in animal groups tested with samples inactivated by fixative or detergent buffers. For all test groups, n = 15; for all control groups, n = 5.

immediately dialyzed or treated with ethanol (AVL, 100% ethanol, 560 µL; RLT, 70% ethanol, 600 µL). Infected liver tissue was homogenized in RLT with a stainless steel bead (10 min at 30 Hz). A soluble aliquot (≈30 mg) was transferred to a new tube, and fresh RLT was added, followed by 70% ethanol (600 µL). After dialysis, samples were used to infect Vero E6 cells and mice. Similar to a results in a previous study (*10*), AVL and RLT treatment alone for 10 minutes at either ratio did not fully inactivate EBOV; however, the addition of ethanol (the next step of the manufacturer's protocol) rendered all samples completely noninfectious. AVL alone resulted in complete inactivation with longer contact times (i.e., refrigerated overnight or frozen for 7 days) (Table; Figure).

Infected cells were resuspended and treated with TRIzol (1:4 vol/vol). Infected liver samples were homogenized in 1 mL TRIzol as described in the previous paragraph. After centrifugation, an aliquot of tissue homogenate (≈50 mg) was transferred to a new tube, and fresh TRIzol was added. Additionally, blood from infected animals was mixed (1:4 vol/vol) with TRIzol. After dialysis, Vero E6 cells were inoculated and monitored for CPE or fluorescence. In all cases, virus growth was not detected (Table), indicating complete inactivation.

Formalin, paraformaldehyde, and glutaraldehyde can be used to fix cells or tissues for histologic or microscopic studies. Infected cells were diluted 1:4 in 10% neutral-buffered formalin (7.5% fixative) or 1:5 in either 2.5% glutaraldehyde or 2.5% paraformaldehyde (2% fixative). Samples were dialyzed and used to infect Vero E6 cells or mice. Monitoring of cell culture and animals resulted in the absence of CPE or fluorescence and clinical signs, respectively, indicating complete inactivation of EBOV (Table; Figure).

Infected liver segments were incubated in 10% neutral-buffered formalin, 2% glutaraldehyde, or 2% paraformaldehyde (10 mL) for a period of 7 days (<1-cm^3 piece) or 30 days (>1-cm^3 piece) at 4°C. Subsequently, a small section of tissue (≈150 mg) was dissected, homogenized in DPBS with a stainless steel bead (10 min at 30 Hz), and then dialyzed. After dialysis, samples were used to infect Vero E6 cells. All samples were completely inactivated (Table).

Samples for protein assays are often inactivated by a combination of detergent and heat. We tested the parameters of 60°C for 30 min, 65°C for 15 or 30 min, and 70°C for 15 min in conjunction with a buffer containing 0.5% Triton X-100 and 0.5% Tween-20 (both from Sigma-Aldrich,

St. Louis, MO, USA); this mixture is commonly used for ELISA. Stock virus was diluted 1:25 in this buffer and heated for the appropriate times before samples were clarified of detergent and used to infect Vero E6 cells or mice. All samples were completely inactivated as indicated by lack of CPE or fluorescence in cells and clinical signs in mice (Table; Figure).

Boiling (at 100°C for 10 min or 120°C for 5 min) might be sufficient to inactivate EBOV (Table) (*11*) but is often used in conjunction with sodium dodecyl sulfate (SDS)–containing buffers for protein analysis. Aliquots of infected cells were diluted in DPBS and 4× loading buffer (1% SDS final). Infected liver tissue (≈150 mg) were placed in DPBS and 4× loading buffer (1% SDS final). The samples were then homogenized with a stainless steel bead (10 min at 30 Hz). After detergent removal, samples were used to infect Vero E6 cells; all treated cells and tissue homogenates were negative for infectious EBOV (Table).

Conclusions

Our study establishes inactivation procedures for EBOV that can be safely applied to distinct specimen types and research purposes and might also apply to other enveloped, single-strand, negative-sense RNA viruses. Our findings should help to improve and approve SOPs for inactivation without the need for safety testing each individual sample, an unfeasible and unwarranted task in current diagnostic and research operations in high-level biocontainment settings. However, any changes to inactivation SOPs make further safety testing essential. Safety testing for inactivation, at least for EBOV, can rely on cell culture only because this seems to be as sensitive as in vivo testing.

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References

1. Mitchell SW, McCormick JB. Physicochemical inactivation of Lassa, Ebola, and Marburg viruses and effect on clinical laboratory analyses. J Clin Microbiol. 1984;20:486–9.
2. Elliott LH, McCormick JB, Johnson KM. Inactivation of Lassa, Marburg, and Ebola viruses by gamma irradiation. J Clin Microbiol. 1982;16:704–8.
3. Lupton HW. Inactivation of Ebola virus with 60Co irradiation. J Infect Dis. 1981;143:291. http://dx.doi.org/10.1093/infdis/143.2.291
4. US Centers for Disease Control and Prevention, US Department of Agriculture. Select agents and toxins list [cited 2016 Mar 29]. http://www.selectagents.gov/SelectAgentsandToxinsList.html
5. World Health Organization. Ebola virus disease outbreak: Ebola is no longer a public health emergency of international concern [cited 2016 Mar 29]. http://www.who.int/csr/disease/ebola/en
6. US Centers for Disease Control and Prevention. 2014 Ebola outbreak in West Africa [cited 2016 Mar 29]. http://www.cdc.gov/vhf/ebola/outbreaks/2014-west-africa/index.html
7. Ebihara H, Theriault S, Neumann G, Alimonti JB, Geisbert JB, Hensley LE, et al. In vitro and in vivo characterization of recombinant Ebola viruses expressing enhanced green fluorescent protein. J Infect Dis. 2007;196(Suppl 2):S313–22. http://dx.doi.org/10.1086/520590
8. Bray M, Davis K, Geisbert T, Schmaljohn C, Huggins J. A mouse model for evaluation of prophylaxis and therapy of Ebola hemorrhagic fever. J Infect Dis. 1998;178:651–61. http://dx.doi.org/10.1086/515386
9. Marzi A, Feldmann F, Hanley PW, Scott DP, Günther S, Feldmann H. Delayed disease progression in Cynomolgus macaques infected with Ebola virus Makona strain. Emerg Infect Dis. 2015;21:1777–83. http://dx.doi.org/10.3201/eid2110.150259
10. Smither SJ, Weller SA, Phelps A, Eastaugh L, Ngugi S, O'Brien LM, et al. Buffer AVL alone does not inactivate Ebola virus in a representative clinical sample type. J Clin Microbiol. 2015;53:3148–54. http://dx.doi.org/10.1128/JCM.01449-15
11. Chong YK, Ng WY, Chen SP, Mak CM. Effects of a plasma heating procedure for inactivating Ebola virus on common chemical pathology tests. Hong Kong Med J. 2015;21:201–7. http://dx.doi.org/10.12809/hkmj144373

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Single-Reaction Multiplex Reverse Transcription PCR for Detection of Zika, Chikungunya, and Dengue Viruses

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Clinical manifestations of Zika virus, chikungunya virus, and dengue virus infections can be similar. To improve virus detection, streamline molecular workflow, and decrease test costs, we developed and evaluated a multiplex real-time reverse transcription PCR for these viruses.

Zika virus is a mosquitoborne flavivirus that, in 2015, spread throughout the tropical and subtropical regions of the Western Hemisphere. In January 2016, the first autochthonous cases of Zika fever were confirmed in Nicaragua (*1*). The diagnosis of human Zika virus infections is confounded by a nonspecific clinical presentation, which overlaps substantially with that of dengue virus (DENV) and chikungunya virus (CHIKV) (*2*,*3*) and by cross-reaction with DENV IgM and DENV nonstructural protein 1 in assays for Zika virus (*4*–*7*).

Molecular assays can detect and differentiate these 3 pathogens during the acute phase of illness. Although a number of molecular tests have been published for detecting DENV and CHIKV, only 2 Zika virus real-time reverse transcription PCRs (rRT-PCRs) have been reported and were characterized by using human specimens (*6*–*8*). These assays are run as individual reactions, and molecular testing for all 3 viruses, using established protocols, requires multiple reactions for a single patient sample (*6*,*9*,*10*). We describe a Zika virus rRT-PCR that was designed to be run in multiplex with published assays for pan-DENV and CHIKV detection (*11*,*12*). We then evaluated the single-reaction multiplex rRT-PCR for Zika virus, CHIKV, and DENV (referred to as the ZCD assay) by testing clinical samples from persons with suspected cases in Nicaragua.

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The Study

The Zika virus primers and probe (online Technical Appendix Table 1 (http://wwwnc.cdc.gov/EID/article/22/7/16-0326-Techapp1.pdf) were designed by using all complete or nearly complete (≥10,000 kb) Zika virus genome sequences available in GenBank (n = 21) accessed March 28, 2014). Target sequences were subsequently confirmed to match strains from the Americas. All rRT-PCR reactions were performed on an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA) by using 25-mL reactions of the SuperScript III Platinum One-Step qRT-PCR kit (Life Technologies, Carlsbad, CA, USA) and 5 mL of RNA template. Cycling conditions for the ZCD assay were as follows: 52°C for 15 min; 94°C for 2 min; 45 cycles at 94°C for 15 sec, 55°C for 20 sec (acquisition), and 68°C for 20 sec. Each run included a no-template control and positive controls for Zika virus, CHIKV, and DENV.

Linear range and lower limit of 95% detection (95% LLOD) for each target were determined as recommended. We determined linear range and 95% LLOD for each target as recommended (*13*; online Technical Appendix). The linear range of the ZCD assay extended from 10^8 to 10 copies/mL for Zika virus and DENV-3 and from 10^8 to 100 copies/mL for DENV-1, -2, –4, and CHIKV. The 95% LLOD for each target, in copies/mL of eluate (5 mL added to each ZCD reaction), was as follows: Zika virus, 7.8; CHIKV, 13.2; DENV-1, 11.7; DENV-2, 13.5; DENV-3, 4.1; DENV-4, 10.5.

Assay exclusivity was established by testing genomic RNA from the following viruses: West Nile, Japanese encephalitis, tickborne encephalitis, yellow fever, Saint Louis encephalitis, o'nyong-nyong, Semliki Forest, Mayaro, Ross River, Getah, Barmah Fores, and Unas (*12*,*14*). No amplification was detected for any of these viruses.

De-identified serum samples, collected from Nicaraguan patients with suspected Zika virus, CHIKV, and/or DENV infections, were tested (online Technical Appendix). We tested 216 samples by using the ZCD assay and the pan–DENV-CHIKV rRT-PCR, which is a validated duplex assay containing the DENV and CHIKV primers and probes used in the ZCD assay (*12*). Both assays were performed on an ABI7500 (Applied Biosystems) (Table 1). A total of 173 samples were positive for DENV alone

Table 1. Comparison of DENV and CHIKV detection in the ZCD assay and pan–DENV-CHIKV rRT-PCR*

ZCD assay	pan–DENV-CHIKV rRT-PCR					
	DENV Detection			CHIKV Detection		
	Pos	Neg	Total	Pos	Neg	Total
Pos	55	5	60	113	23	136
Neg	3	153	156	12	68	80
Total	58	158	216	125	91	216

*CHIKV, chikungunya virus; DENV, dengue virus; neg, negative; pos, positive; rRT-PCR, real-time reverse transcription PCR; ZCD assay, single-reaction multiplex rRT-PCR for Zika virus, CHIKV, and DENV.

(n = 25), CHIKV alone (n = 110), or both (n = 38). The ZCD assay and pan–DENV-CHIKV rRT-PCR showed very good agreement for DENV detection (k = 0.907). Six of 8 discrepant samples were co-infected with DENV and CHIKV, and the 2 discrepant samples with DENV mono-infections had cycle threshold (C_t) values of 41.34 and 42.25. The 2 assays demonstrated good agreement for CHIKV detection (k = 0.662). C_t for the 35 CHIKV discrepant samples were reached significantly later (mean 39.8, SD ±1.5) than the 113 concordant samples (28.7, ±SD 9.7; p<0.0001).

The first case of Zika virus infection in Nicaragua was detected with the ZCD assay during the assay comparison described above. After Zika virus identification, 133 consecutive samples were tested by using both the ZCD assay and a comparator Zika virus rRT-PCR targeting the capsid gene (*6*) (Table 2). When the comparator rRT-PCR was analyzed according to the published validation (C_t ≤38.5 defining a positive result), these assays demonstrated only moderate agreement (k = 0.47), and Zika virus was detected in significantly more samples by using the ZCD assay (p<0.001). Of the 31 samples positive only for Zika virus in the ZCD assay, 22 (71%) produced a C_t (>38.5) that was reached later in the comparator Zika virus rRT-PCR. These 22 samples had mean C_t of 32.06 (SD ±2.45) in the ZCD assay and 42.09 (SD ±1.41) in the comparator Zika virus rRT-PCR. If all samples in the comparator rRT-PCR with C_t >38.5 were considered positive, the assays demonstrated very good agreement (k = 0.81; online Technical Appendix Table 2). Of the 56 Zika virus–positive samples in the ZCD assay, 39 were positive only for Zika virus, and 17 showed evidence of mixed infection: Zika virus–DENV (n = 3); Zika virus–CHIKV (n = 10), or Zika virus–CHIKV–DENV (n = 4).

Table 2. Comparison of Zika virus detection in the ZCD assay and the Zika virus comparator rRT-PCR*

ZCD assay	Zika virus rRT-PCR		
	Pos	Neg	Total
Pos	25	31†	56
Neg	1‡	76	77
Total	26	107	133

*CHIKV, chikungunya virus; C_t, cycle threshold; DENV, dengue virus; neg, negative; pos, positive; rRT-PCR, real-time reverse transcription PCR; ZCD assay, single-reaction multiplex rRT-PCR for Zika virus, CHIKV, and DENV.
†22 samples produced a late C_t in the comparator Zika virus rRT-PCR (C_t >38.5)
‡Sample was also pos for a DENV-CHIKV co-infection and tested pos for Zika virus when repeated in the ZCD assay.

Conclusions

The ZCD assay improved detection of Zika virus relative to the comparator rRT-PCR, and 31 samples were positive only for Zika virus by the ZCD assay when the comparator was interpreted as published (*6*). Notably, 22 (71%) of these 31 samples produced a late signal in the comparator Zika virus rRT-PCR (C_t >38.5), indicating that these most likely are true, late-positive results. Improved sensitivity for Zika virus is needed given the low viremia detected in clinical samples and the current lack of accurate alternative diagnostics, such as serology (*6*,*7*,*15*). Additionally, the ZCD assay identified 17 co-infections in Zika virus–positive patients. Although preliminary, these data provide evidence for the utility of a multiplex diagnostic test for these pathogens.

The performance of the ZCD assay for DENV detection was similar to that of the pan–DENV-CHIKV rRT-PCR, and the analytical sensitivity for CHIKV was similar in both assays (*12*). CHIKV detection in clinical samples in the ZCD assay and pan–DENV-CHIKV rRT-PCR demonstrated good agreement, although both assays contain the same CHIKV primers and probes. Discrepant samples all had C_t of ≥37.36, which correspond to 10 copies/mL of eluate and fall below the 95% LLOD. Although ZCD assay results for these CHIKV-positive samples were reproducible, the clinical significance of such low-level viremia in patients with suspected chikungunya fever is unclear and warrants further study.

A limitation to our study is the use of a single comparator Zika virus rRT-PCR. This assay was 1 of 2 rRT-PCRs developed for the 2007 Yap Island Zika virus strain (*6*). The second rRT-PCR, targeting the membrane gene, was evaluated for the current study but proved consistently less analytically sensitive. Therefore, performance of this second Zika virus assay most likely would not have affected result interpretation.

In conclusion, the single-reaction multiplex ZCD assay detected and differentiated Zika virus, CHIKV, and DENV. This assay should streamline molecular workflow and decrease test costs while improving detection of these 3 human arboviruses.

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Ministry of Health, and the Sustainable Sciences Institute in Managua, Nicaragua.

Patent applications or provisional patent applications that cover the ZCD multiplex assay and the primers and probes described in this report have been filed (J.J.W. and B.A.P.).

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References

1. El Nuevo Diario. Confirman dos casos positivos de Zika en Nicaragua [cited 2016 Apr 13]. http://www.elnuevodiario.com.ni/nacionales/383401-nicaragua-confirma-dos-casos-positivos-zika/
2. Duffy MR, Chen TH, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009;360:2536–43. http://dx.doi.org/10.1056/NEJMoa0805715
3. Zanluca C, Melo VC, Mosimann AL, Santos GI, Santos CN, Luz K. First report of autochthonous transmission of Zika virus in Brazil. Mem Inst Oswaldo Cruz. 2015;110:569–72. http://dx.doi.org/10.1590/0074-02760150192
4. Fonseca K, Meatherall B, Zarra D, Drebot M, MacDonald J, Pabbaraju K, et al. First case of Zika virus infection in a returning Canadian traveler. Am J Trop Med Hyg. 2014;91:1035–8. http://dx.doi.org/10.4269/ajtmh.14-0151
5. Gyurech D, Schilling J, Schmidt-Chanasit J, Cassinotti P, Kaeppeli F, Dobec M. False positive dengue NS1 antigen test in a traveller with an acute Zika virus infection imported into Switzerland. Swiss Med Wkly. 2016;146:w14296.
6. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis. 2008;14:1232–9. http://dx.doi.org/10.3201/eid1408.080287
7. Waggoner JJ, Pinsky BA. Zika virus: diagnostics for an emerging pandemic threat. J Clin Microbiol. 2016;54:860–7.;54:860–7. http://dx.doi.org/10.1128/JCM.00279-16
8. Faye O, Faye O, Dupressoir A, Weidmann M, Ndiaye M, Alpha Sall A. One-step RT-PCR for detection of Zika virus. J Clin Virol. 2008;43:96–101. http://dx.doi.org/10.1016/j.jcv.2008.05.005
9. Lanciotti RS, Kosoy OL, Laven JJ, Panella AJ, Velez JO, Lambert AJ, et al. Chikungunya virus in US travelers returning from India, 2006. Emerg Infect Dis. 2007;13:764–7. http://dx.doi.org/10.3201/eid1305.070015
10. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase–polymerase chain reaction. J Clin Microbiol. 1992;30:545–51.
11. Waggoner JJ, Abeynayake J, Sahoo MK, Gresh L, Tellez Y, Gonzalez K, et al. Development of an internally controlled real-time reverse transcriptase PCR assay for pan-dengue virus detection and comparison of four molecular dengue virus detection assays. J Clin Microbiol. 2013;51:2172–81. http://dx.doi.org/10.1128/JCM.00548-13
12. Waggoner JJ, Ballesteros G, Gresh L, Mohamed-Hadley A, Tellez Y, Sahoo MK, et al. Clinical evaluation of a single-reaction real-time RT-PCR for pan-dengue and chikungunya virus detection. J Clin Virol. 2016;78:57–61. http://dx.doi.org/10.1016/j.jcv.2016.01.007
13. Burd EM. Validation of laboratory-developed molecular assays for infectious diseases. Clin Microbiol Rev. 2010;23:550–76. http://dx.doi.org/10.1128/CMR.00074-09
14. Waggoner JJ, Abeynayake J, Sahoo MK, Gresh L, Tellez Y, Gonzalez K, et al. Single-reaction, multiplex, real-time RT-PCR for the detection, quantitation, and serotyping of dengue viruses. PLoS Negl Trop Dis. 2013;7:e2116. http://dx.doi.org/10.1371/journal.pntd.0002116
15. Musso D, Roche C, Nhan TX, Robin E, Teissier A, Cao-Lormeau VM. Detection of Zika virus in saliva. J Clin Virol. 2015;68:53–5. http://dx.doi.org/10.1016/j.jcv.2015.04.021

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Letters

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

Around the World in 1,475 *Salmonella* Geo-serotypes

Céline M. Gossner,[1] Simon Le Hello,[1] Birgitta de Jong, Per Rolfhamre, Daniel Faensen, François-Xavier Weill, Johan Giesecke

It's easy to remember *Salmonella* serotypes names, isn't it? Surely, this is because the naming system of *Salmonella* serotypes is by far the most scientist friendly. Traditionally, most *Salmonella* serotypes have been named after geographic locations. We decided to explore the geographic locations to which *Salmonella* serotypes refer and describe some unexpected twists in the naming scheme. We found that 93% (n = 1,475) of the 1,585 serotypes could be categorized as geo-serotypes; that is, the name refers to a geographic location. The 3 countries with the most geo-serotypes are Germany, the United Kingdom, and the United States. Other serotype names refer to the name of a person, animal, tribe, or food item or are a composite of symptoms and host. The *Salmonella* serotypes naming scheme has had a valuable effect on public health microbiology, and in the current era of fast development of whole-genome sequencing, it should remain a reference.

What do the cities of Paris, Pisa, and Toronto have in common? Yes, all 3 are famous for their towers but what else? You don't know? Let's see if this will help you: what do the states of Colorado, Florida, and Ohio in the United States have in common with the 3 cities above? No idea? If we tell you *Salmonella* serotypes…. If you still don't know, by the end of this essay you will, without a doubt, be able to answer these questions.

Salmonella was first isolated from a human sample in 1884 by bacteriologist Georg Gaffky and later identified as *Salmonella enterica* subspecies *enterica* serotype Typhi. The following year, the veterinary surgeon Daniel Elmer Salmon (whose name was later given to the *Salmonella* genus) and microbiologist Theobald Smith isolated *S. enterica* ser. Choleraesuis from a swine sample, while searching for the agent causing cholera in hogs (*1*). Since then, a plethora of *Salmonella* names was given to strains with new serotypes; that is, new combinations of flagellar (H) and/or somatic (O) antigens. In 1934, a first list of 44 validated *Salmonella* serotypes, called the Kauffmann-White scheme, was published (*2*).

The naming scheme of serotypes (also called serovars) evolved over time. At the beginning of the 20th century, serotype names referred to clinical syndromes either in humans (e.g., *enteritidis*, *typhi*, *paratyphi*) or in animals (e.g., *abortus-ovis, abortus-equi, typhi-murium, cholerae-suis*). The host specificity was correct for some serotypes (e.g., *abortus-ovis, abortus-equi*) but proved to be wrong for many others (e.g., *typhi-murium, cholerae-suis*) (*2*).

By the mid-1930s, Fritz Kauffmann was heading the World Health Organization Collaborating Centre for Reference and Research on *Salmonella* at the Statens Serum Institut, Copenhagen, Denmark. While there, he began to name new serotypes according to the geographic origin of the isolated strain. After Kauffmann's retirement in 1965, Léon Le Minor became director of the World Health Organization Collaborating Centre at the Institut Pasteur, Paris, France (*3*), and he perpetuated the serotype naming scheme established by Kauffmann.

Kauffmann considered each serotype as a species and, consequently, in the old literature, the serotype names were italicized (e.g., *typhi)*. DNA-DNA hybridization, which arrived in the 1980s, showed otherwise: only 2 species (*S. enterica* and *S. bongori*) were found to be in the genus *Salmonella*. This discovery led to a long-standing debate until, in 2005, the Judicial Commission of the International Committee for Systematics of Prokaryotes made the decision to recognize the new nomenclature (*4*). Consequently, the serotype names must no longer be italicized and the first letter must be capitalized (e.g., Typhi). Names are only given to subspecies *enterica* serotypes, which represent 99.5% of all *Salmonella* strains. The remaining *Salmonella* strains are named after their antigenic formula (*2*).

Currently, >2,500 *Salmonella* serotypes have been described and listed in the "bible" of *Salmonella* serovars: the White-Kauffmann-Le Minor (WKL) scheme (*2*). Last revised in January 2007, WKL has since been completed, with 1 supplement published in 2010 (*5*) and another in 2014 (*6*). Listed in the WKL scheme are 1,585 serotypes of *S. enterica* subsp. *enterica.*

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We decided to assess the geographic locations for which subspecies *enterica* serotypes are named and describe some unexpected twists in the naming scheme. First, we searched for published articles and books that recorded the first isolation of specific *Salmonella* serotypes (*7*–*12*). A large part of this exploration relied on the extensive work of the microbiologist Eckehart Kelterborn, who cataloged the history of *Salmonella* serotypes first isolations in 2 books: *Salmonella*-species: First Isolations, Names and Occurrence (*7*) and Catalogue of *Salmonella* First Isolations 1965–1984 (*8*). Then, we used the open GeoNames database (*13*) and Google Maps (*14*) to find the geographic locations corresponding with the serotype names.

Of the 1,585 serotypes of *S. enterica* subsp. *enterica* that we considered, 1,475 (93%) are geo-serotypes (i.e.,the name is associated with a geographic location); 95 (6%) have names related to a nongeographic origin (e.g., person, animal); and 15 (1%) have names of unknown origin. Geo-serotypes include serotypes for which there is a clear reference in the literature of the first isolation and link to a geographic location and for which there is no clear reference in the literature but the name is most likely associated with a geographic location with the same name. For instance, a serotype that was first described in a patient returning from France and to which was given the name of a French city was considered as a possible geo-serotype (unless contradictory information was found). The geo-serotypes were named after continents, countries, regions, islands, cities, neighborhoods, streets, gardens, rivers, lakes, and hills but also after university auditoriums, laboratories, hospitals, kibbutzim, markets, and mines.

Four geo-serotypes are linked to a broad region or continent: Africana, Antarctica, Orientalis, and Westafrica. Remarkably, serotype Antarctica was first isolated from an Emperor penguin in 1977 in the South Pole continent. The remaining 1,471 geo-serotypes can be directly associated with 1 country. The 10 countries with the most geo-serotypes are Germany (n = 181; e.g., Berlin, Brandenburg, Heidelberg); the United Kingdom (n = 167; e.g., Chester, Derby, Stanley); the United States (n = 148; e.g., Brooklyn, Chicago, Saintpaul); Nigeria (n = 74; e.g., Abuja, Ibadan, Lagos, Nigeria); France (n = 70; e.g., Avignon, Lyon, Marseille); Togo (n = 58; e.g., Adime, Lome, Djame); the Democratic Republic of the Congo (n = 58; e.g., Leopoldville, Mbandaka, Zaire); Senegal (n = 55; e.g., Dakar, Kedougou, Saboya); Sweden (n = 39; e.g., Goeteborg, Lund, Stockholm); and Ghana (n = 39; e.g., Accra, Ashanti, Victoriaborg, Goldcoast) (Figures 1, 2).

Among the 1,474 *Salmonella* geo-serotypes that could be attached to a continent (Orientalis was excluded), the names of 43% are related to Europe and the names of 34% are related to Africa (Figure 2). A total of 41 geo-serotypes (3%) were named after a country, which includes current and former names of countries. Among the geo-serotypes with country names are Australia, Brazil, Bulgaria, Malaysia, and Tanzania. Singapore is represented twice, with Singapore and Sinchew, the Chinese name for Singapore. Cubana and Papuana also count as country names because they derive from Cuba and Papua New Guinea.

Fifty geo-serotypes (3%) were named after a capital city (current capital names, former capital names, and former capitals). Let's revise our knowledge of capitals! Bangkok, Thailand; Brazzaville, Republic of Congo; Caracas, Venezuela; and Stockholm, Sweden, are current capitals. Bonn was the capital of West Germany from 1949 to 1990; Berlin is the current capital of Germany. In addition, Tananarive is the previous name of Antananarivo, the capital of Madagascar. The capital of France is named in different ways: Paris, Lutetia (the Latin name of Paris), Picpus, Vaugirard, Miromesnil, and Portedeslilas (4 metro stations), and Morillons (a street where the food safety laboratory was located). The serotype London was isolated in the city of Reading in the United Kingdom from a patient whose last name began with the letter "L." Because the serotype Reading already existed, this serotype was named London by extension of the patient's name.

Twenty-four states of the United States gave their names to serotypes, among which are Alabama, California, Colorado, Florida, Kentucky, Michigan, Ohio, Texas, and Utah. The states/regions of Ontario and Quebec in Canada, Nordrhein in Germany, Ashanti in Ghana, and Demerara-Mahaica in Guiana also gave their names to serotypes.

Through the years, ≈300 serotypes have been removed from the WKL scheme because they were shown to belong to other subspecies or the variant was no longer recognized. Among them, 11 referred to names of capital cities (Bern, Cairo, Buenosaires, Helsinki, Khartoum, Nairobi, Sofia, Windhoek, Zagreb, Manila, Kinshasa); 4 referred to names of countries (Angola, Argentina, Congo, Rhodesiense); and 3 referred to states of the United States (Oregon, Arkansas, Illinois). Although the serotype Buenosaires was removed from the WKL scheme, Bonariensis, the Latin name of Buenos Aires, was entered (*2*,*15*).

Instead of a location, some serotypes take their name from the name of the patient (e.g., Agbeni, Ayinde); a laboratory employee (e.g., Bamboye, Souza); an animal owner (e.g., Sarajane); the patient's tribe (e.g., Azteca, Lokomo, Yoruba); a ship (e.g., Maron); the animal type or the food item in which the strain was isolated (e.g., Agama [lizards], Epicrates [boa], Djinten [cumin spice], Egusi [seeds]); a combination of symptoms and host (e.g., Abortusovis, Typhimurium, Typhisuis); and the Latin name of the vehicle (e.g., Aqua [water], Carno [meat], Os [bone]) . Would you think that the serotype Heron is called after the bird? That would be too easy. The strain was isolated in 1962 from a turtle by a biologist called Madam Heron (*7*).

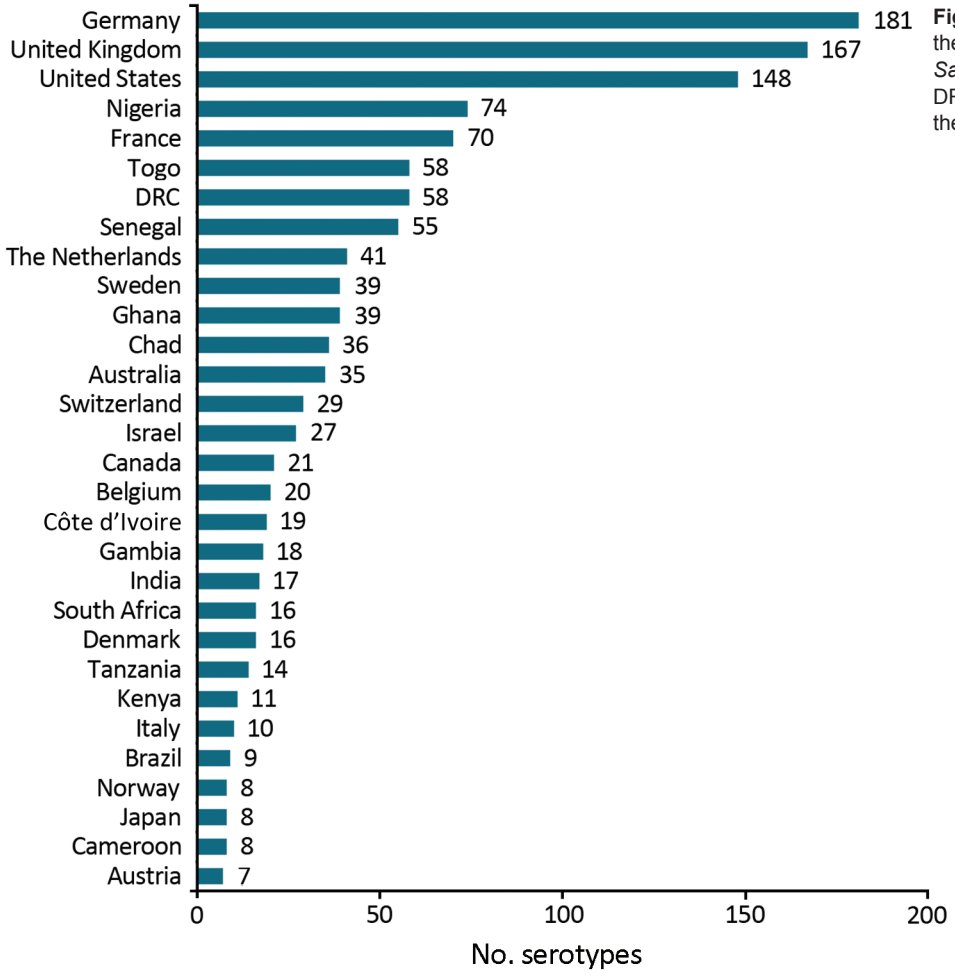

Figure 1. Top 30 countries with the highest number of associated *Salmonella* geo-serotypes (n = 1,259). DRC, Democratic Republic of the Congo.

Who says that biologists have no sense of humor? The serotype Hiduddify is named after a fictional island (*8*). The story goes as follows: In 1941, a Swede named Einar Pettersson-Skämtkvist escaped from a Japanese prisoner of war camp to arrive to the yet undiscovered island of Hiduddify, which was home of a unique ecosystem. The island was inhabited by the Rhinogradentia, mammals of a new order that were using their nose as mean of locomotion (*16*). This unique discovery was described in 1961 in a book by German zoologist Gerolf Steiner under the pseudonym Harald Stümpke. The entire story remains today a major hoax in the field of biology (*17*).

Serotype Grumpensis refers to grumpy, the name given to the owner of the guinea pig from which the strain was isolated (*7*). Ironically, the serotype Fortune refers to luck (*7*), which is certainly not the emotion felt by the person with a diagnosis of *Salmonella* infection!

In 1961, the laboratory of Colindale in the United Kingdom isolated, for the first time, serotype Egusi in egusi seeds. The same year, Colindale identified another new serotype in egusi seeds and, consequently, it was named Egusitoo (*7*). Serotype Jukestown was named by a doctor who was passionate about the juke box who lived in Georgetown, Guiana (*7*). Isolated in Chicago, the serotype Mjordan refers to the famous basketball player of the Chicago Bulls, Michael Jordan (unpub. data). Finally, other serotypes are portmanteaus or acronyms: Anfo (animal food), Ank (address not known), Ceyco (Ceylonese coconut), Chincol (Chinese egg, Colindale), Echa (egron and chamoiseau [family names of scientists who discovered this serotype]), and Inpraw (Indian prawns) (*8*).

Most of the 1,585 *Salmonella* serotypes are named after a geographic location. The list of countries that have named the most geo-serotypes correlates well with countries with strong laboratory capacities in Europe and the Americas and with countries in Africa (generally former European colonies) where some laboratory capacities (e.g., an Institut Pasteur) or close links with a laboratory in Europe had been established.

A naming scheme based on tangible names (e.g., cities, countries) has obvious advantages, such as making it easier to communicate about and pinpoint outbreaks. It is

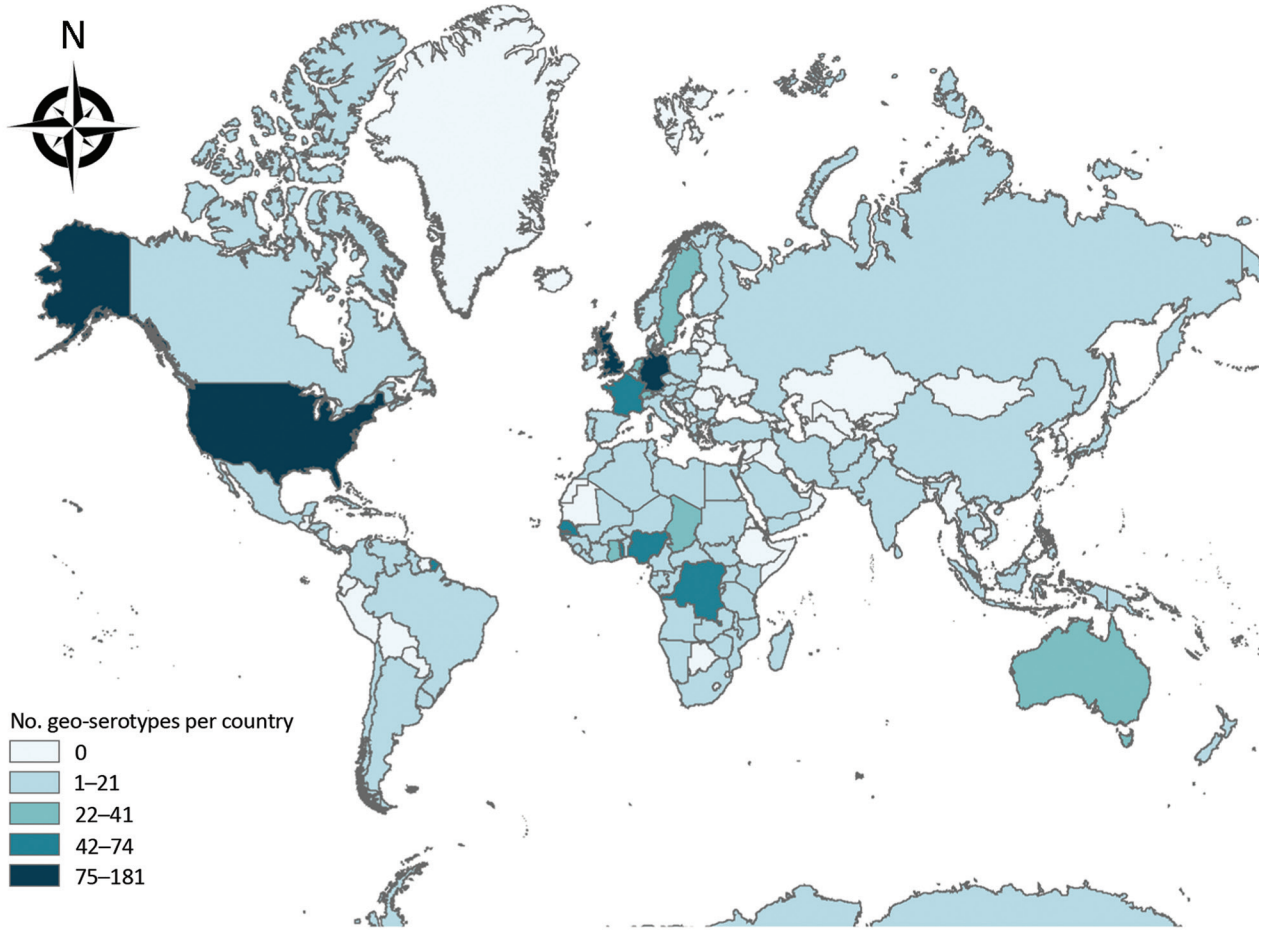

Figure 2. Worldwide geographic distribution of *Salmonella* geo-serotypes (n = 1,472). The geo-serotypes Africana, Orientalis, and Westafrica were excluded. Administrative boundaries copyright by Eurographics and the United Nations Food and Agricultural Organization.

much easier to remember a label like "Agona" than the formula 1,4,[5],12:f,g,s:[1,2]. Using a naming system based on locations may, however, raise some sensitivity. National or local authorities may not appreciate the association of their area with a pathogen, especially when large foodborne outbreaks are highly publicized by the media. The same applies for serotype names based on the name of a food product. For instance, outbreaks of *S. enterica* ser. Djinten (cumin spice) are certainly not a good selling pitch for cumin producers/distributors. Therefore, serotype names should be interpreted with caution, and consumers should be reminded that no direct relationship exists between the serotype name and the prevalence of cases in the specific location or by the consumption of a specific product. The likelihood of acquiring *S. enterica* ser. Heidelberg infection in the city of Heidelberg, Germany, is probably no higher than the chance of acquiring the same infection in Miami, Florida, USA. Studying the correlation between serotypes' names and places of infection could be intriguing.

The affiliation of a new variant to a previously recognized serotype may have more implications than a simple name attribution. Although the monophasic variant 1,4,[5],12:i:- emerged in the 2000s, only in 2010 was it officially recognized as part of serotype Typhimurium by the European Union (*18*). Because of its atypical antigenic formula, this variant avoided for years all European Union laws applying to *S. enterica* ser. Typhimurium. It is certainly a proof of natural selection against European Union legislation.

The introduction of DNA-based methods targeting neutral markers such as multilocus sequence typing demonstrated that most of *Salmonella* serotypes span multiple, genetically unrelated clusters (*19*). Therefore, as multilocus sequence typing and, ultimately, sequence-based typing methods based on entire genomes are more discriminatory than serotyping, the serotype-based nomenclature will ideally be complemented by a genome sequence-based typing scheme (*19*). A genome type/serotype dictionary should be

developed to maintain the link with the serotyping nomenclature, to continue building on >80 years of accumulated data, and to ensure a smooth transition for countries or regions in the world that will not switch to whole-genome sequencing as fast as others.

To answer the question posed at the beginning of this article—indeed, Paris, Pisa, Toronto, Colorado, Florida, and Ohio have all given their name to *Salmonella* serotypes. As promised, the material provided in this short review on the *Salmonella* naming scheme will help you interpret and decipher *Salmonella* names.

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References

1. Mastroeni P, Maskell D, editors. *Salmonella* infections: clinical, immunological and molecular aspects. Cambridge (UK): Cambridge University Press; 2006.
2. Grimont PAD, Weill FX. Antigenic formulae of the *Salmonella* serovars, 9th ed. Paris: World Health Organization Collaborating Center for Reference and Research on *Salmonella*, Institut Pasteur; 2007.
3. Institut Pasteur. *Salmonella*. Centre Collaborateur de l'OMS (CCOMS) de Référence et de Recherche pour les *Salmonella* [cited 2016 Feb 17]. http://www.pasteur.fr/fr/sante/centres-collaborateurs-l-oms-ccoms/salmonella
4. De Vos P, Truper HG, Tindall BJ. Judicial Commission of the International Committee on Systematics of Prokaryotes Xth International (IUMS) Congress of Bacteriology and Applied Microbiology. Minutes of the meetings, 28, 29 and 31 July and 1 August 2002, Paris, France. Int J Syst Evol Microbiol. 2005;55:525–32. http://dx.doi.org/10.1099/ijs.0.63585-0
5. Guibourdenche M, Roggentin P, Mikoleit M, Fields PI, Bockemühl J, Grimont PA, et al. Supplement 2003–2007 (no. 47) to the White-Kauffmann-Le Minor scheme. Res Microbiol. 2010;161:26–9. http://dx.doi.org/10.1016/j.resmic.2009.10.002
6. Issenhuth-Jeanjean S, Roggentin P, Mikoleit M, Guibourdenche M, de Pinna E, Nair S, et al. Supplement 2008–2010 (no. 48) to the White-Kauffmann-Le Minor scheme. Res Microbiol. 2014;165:526–30. http://dx.doi.org/10.1016/j.resmic.2014.07.004
7. Kelterborn E. *Salmonella*-species. First isolations, names and occurrence [in German]. Leipzig (Germany): Hirzel; 1967.
8. Kelterborn E. Catalogue of *Salmonella* first isolations, 1965–1984. Boston, Dordrecht (the Netherlands): Nijhoff; 1987.
9. Lapage SP, Taylor J, Nicewonger CR, Phillips AG. New serotypes of *Salmonella* identified before 1964 at the Salmonella reference laboratory, Colindale. Int J Syst Bacteriol. 1966;16:253–98. http://dx.doi.org/10.1099/00207713-16-3-253
10. Ikumapayi UN, Antonio M, Sonne-Hansen J, Biney E, Enwere G, Okoko B, et al. Molecular epidemiology of community-acquired invasive non-typhoidal *Salmonella* among children aged 2 29 months in rural Gambia and discovery of a new serovar, *Salmonella enterica* Dingiri. J Med Microbiol. 2007;56:1479–84. http://dx.doi.org/10.1099/jmm.0.47416-0
11. McDougal DL, Treleaven BE, Renshaw EC Jr. New *Salmonella* serotype: *Salmonella* enteritidis serotype Grandhaven (30(1):r:1,2). J Clin Microbiol. 1982;16:197–9.
12. Monzón C, O'Shanahan L, Echeita MA, Usera MA, Popoff MY. [A new serotype of Salmonella: Grancanaria]. Enferm Infecc Microbiol Clin. 1993;11:228–9.
13. GeoNames. 2014 [cited 2 Oct 2014]. http://www.geonames.org/
14. Google Maps. 2014 [cited 1 Oct 2014]. https://www.google.se/maps/preview?source=newuser-ws
15. Kauffmann F. Entero-bacteriaceae, 2nd ed. Copenhagen: E. Munksgaard; 1954.
16. Stümpke H. Bau und Leben der Rhinogradentia. Stuttgart (Germany): Fischer Verlag; 1961.
17. Lewin RA. Humor in the scientific literature. Bioscience. 1983; 33:266–8. http://dx.doi.org/10.2307/1309040
18. EFSA Panel on Biological Hazards (BIOHAZ). Scientific opinion on monitoring and assessment of the public health risk of "Salmonella Typhimurium-like" strains. EFSA. 2010;8:1826 [cited 2016 May 11]. http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/1826.pdf
19. Achtman M, Wain J, Weill FX, Nair S, Zhou Z, Sangal V, et al. Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica.* PLoS Pathog. 2012;8:e1002776. http://dx.doi.org/10.1371/journal.ppat.1002776

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Confirming Legionnaires' Disease Outbreak by Genome-Based Method, Germany, 2012

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To the Editor: We report an outbreak of Legionnaires' disease in southwestern Germany. On July 31, 2012, the State Health Agency of Rhineland-Palatinate was informed by the local health department of the city of Zweibrücken that 10 patients tested positive for *Legionella pneumophila*, the bacterium that causes Legionnaires' disease. The onset of disease for all case-patients was from June 26 through July 25, which exceeded the yearly average of 1–4 patients a month. By August 23, we had received notifications of 19 patients with pneumonia and notification of 1 patient who did not exhibit pneumonia. We set 3 parameters for reporting a patient as a Legionnaires' disease case-patient. First, the patient had to either live in or have been visiting the city of Zweibrücken in June 2012 before onset of disease. Second, the respiratory samples from the patient had to contain *L. pneumophila* or the results of patient's serogroup 1 urinary antigen test had to be positive for the bacterium (*1*). Finally, clinical or radiologic confirmation of the disease was required. Of 20 patients who fit the case definition, 14 were male and 6 were female. Nine smoked and 2 were immunocompromised; none died.

All case-patients were positive for *L. pneumophila* serogroup 1 urinary antigen. From clinical samples of 2 patients, legionellae were cultured, and the infecting strain was confirmed as *L. pneumophila* serogroup 1, monoclonal subgroup Allentown-France, sequence type (ST) 82 (*2*,*3*). Currently, 118 strains of this ST are found in the European database for sequence-based typing of *L. pneumophila* (*2*). Most ST82 strains were isolated from clinical samples; thus, this ST appears more likely than other strains to infect humans. Further, 3 respiratory samples from case-patients were positive in a PCR for *L. pneumophila* serogroup 1 (*4*) but were negative by culture. These samples were investigated with the nested sequence-based typing protocol, which allows typing data to be obtained directly from clinical samples (*2*). Of the 3 samples, 2 were confirmed as ST82.

The local health authority did not initially identify likely sources of transmission such as cooling towers, public spas, or warm water supply systems in the vicinity of the patients (*5*). Environmental samples were taken from the homes of 15 of the 20 patients; all samples tested negative for *Legionella* (*6*).

To find the source of the outbreak, we plotted 20 home and 7 work addresses of patients using Quantum-GIS software (*7*) and found that 18 addresses were within a 2-km radius of each other, including 2 patients who had limited mobility and had not left their homes during their incubation period (Figure). We conducted a site visit on August 22 to inspect a sewage plant and 2 large manufacturing plants (A and B) that were within the same 2-km radius. Neither the sewage plant nor plant A had a potential *Legionella* source. Plant B had a cooling tower mounted on a rooftop that was described by the company as a closed circuit cooling system, indicating that no aerosols would be released, and thus was missed by the initial local health department inquiry. However, closed circuit referred only

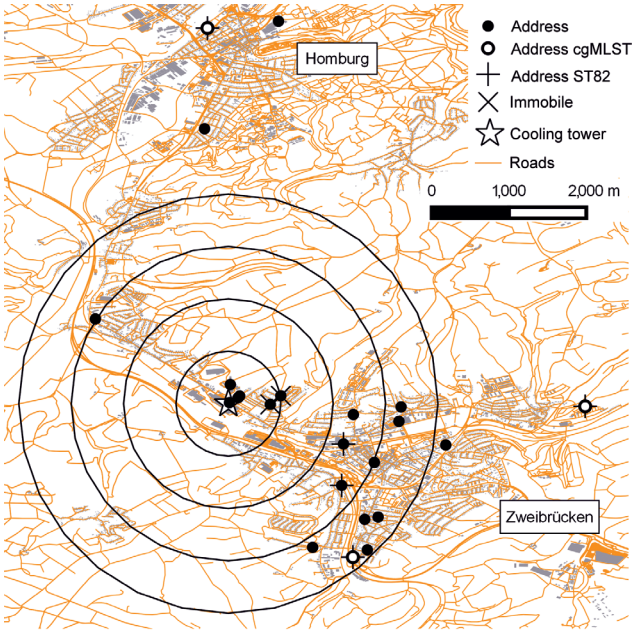

Figure. Geographic distribution of cooling tower and home and work addresses (n = 23) of patients; 1 patient may be represented twice with home and work address, because place of infection is unknown. The addresses marked "immobile" belong to 2 patients who had not left their homes. Two samples had undergone core genome multilocus sequence typing (cgMLST), and sequence type (ST) 82 was typed (represented by 2 home addresses and 1 work address). For 2 samples, only ST82 was typed. Two dots in the 1-km radius are overlapping each other. Four addresses (9 km, 10 km, 19 km, and 26 km from the cooling tower) are outside the scale of the map. Circle radii are from 1 km to 4 km, centered on the cooling tower. Shapefiles for mapping by OpenStreetMap contributors.

to the primary cooling circuitry, whereas excess heat was exchanged through wet surface cooling, allowing release of aerosols into the atmosphere. The local health department immediately shut down the cooling tower, and plant B used shot-dose chlorine to disinfect it. Before disinfection, we obtained 3 swab specimens and 250-mL samples of water from the reservoir and plated them in dilutions with and without acid wash (*6*,*8*). Samples without acid wash were completely overgrown, whereas a single 1-mL sample with acid wash showed 20 *Legionella* colonies after 7 days. Three colonies were typed and found to belong to the epidemic strain. Of the 27 work and home addresses, 6 were within a 1-km radius of the cooling tower, and 18 were within a 4-km radius (Figure). No further cases occurred within the incubation period (up to 14 days after closure of the cooling tower).

To further confirm this cooling tower as the source of the outbreak, we applied core genome multilocus sequence typing (cgMLST) (*3*). We analyzed allelic differences of 1,521 gene targets of the core genome of *L. pneumophila* using the pairwise ignore missing values option in SeqSphere+ software (Ridom GmbH, Münster, Germany). Results showed that the strains from 2 patients with culture-positive test results and the 3 environmental ST82 strains were identical in their cgMLST profile, which covers 47% of the Philadelphia-1 reference genome.

Currently, no German law requires a registry for cooling towers; such a registry would accelerate identification of potential *L. pneumophila* emission during outbreaks (*9*). In January 2015, a code of conduct for maintenance of cooling towers went into effect (*10*). Modern typing methods such as cgMLST can serve as supporting tools in confirming infection origin. However, this method must be validated on a larger scale, and its discriminatory power compared with that of current typing methods. Further cgMLST studies with other ST82 strains are underway.

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References

1. European Center for Disease Control, Stockholm, Sweden. Case definitions for Legionnaires' disease: 2014 [cited 2016 Apr 29]. http://www.ecdc.europa.eu/en/activities/surveillance/ELDSNet/Pages/EU%20case%20definition.aspx
2. European Study Group for *Legionella* Infections (ESGLI). The EWGLI SBT database for the typing of *Legionella pneumophila*: 2015 [cited 2016 Apr 29]. http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php
3. Moran-Gilad J, Prior K, Yakunin E, Harrison TG, Underwood A, Lazarovitch T, et al. Design and application of a core genome multilocus sequence typing scheme for investigation of Legionnaires' disease incidents. Euro Surveill. 2015;20: pii=21186. http://dx.doi.org/10.2807/1560-7917.ES2015.20.28.21186
4. Mérault N, Rusniok C, Jarraud S, Gomez-Valero L, Cazalet C, Marin M, et al. Specific real-time PCR for simultaneous detection and identification of *Legionella pneumophila* serogroup 1 in water and clinical samples. Appl Environ Microbiol. 2011;77:1708–17. http://dx.doi.org/10.1128/AEM.02261-10
5. Mercante JW, Winchell JM. Current and emerging *Legionella* diagnostics for laboratory and outbreak investigations. Clin Microbiol Rev. 2015;28:95–133. http://dx.doi.org/10.1128/CMR.00029-14
6. International Standardisation Organisation. ISO 11731. Water quality—detection and enumeration of *Legionella*. International Standardisation Organisation; 1998 [cited 2016 Apr 29]. http://www.iso.org/iso/catalogue_detail?csnumber=19653
7. Quantum Geographic Information System Team (QGDT). Open Source Geospatial Foundation Project, 2012 [cited 2016 Mar 27]. http://qgis.osgeo.org
8. Deutsche Industrie Norm. EN ISO 11731–2. Water quality—detection and enumeration of *Legionella*. Part 2: direct membrane filtration method for waters with low bacterial counts. Deutsche Industrie Norm, Berlin; 2008 [cited 2016 Apr 29]. http://www.beuth.de/de/norm/din-en-iso-11731-2/107294968
9. Maisa A, Brockmann A, Renken F, Luck C, Pleischl S, Exner M, et al. Epidemiological investigation and case-control study: a Legionnaires' disease outbreak associated with cooling towers in Warstein, Germany, August–September 2013. Euro Surveill. 2015;20:30064. http://dx.doi.org/10.2807/1560-7917.ES.2015.20.46.30064
10. Verein Deutscher Ingenieure. Securing hygienically sound operation of evaporative cooling systems (VDI Cooling Tower Code of Practice). Verein Deutscher Ingenieure, Berlin; 2015 [cited 2016 Apr 29]. http://www.beuth.de/de/technische-regel/vdi-2047-blatt-2/224206933

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Crimean-Congo Hemorrhagic Fever with Acute Subdural Hematoma, Mauritania, 2012

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To the Editor: Crimean-Congo hemorrhagic fever (CCHF) was first described in Crimea in 1944 and in the Congo in 1969. Since then, many cases in humans have been reported from different regions (*1*–*3*). The disease is transmitted to humans through the bite of an infected tick or by direct contact with blood or tissue from infected humans and livestock. We report an unusual case of acute subdural hematoma secondary to CCHF.

A 58-year-old man, a shepherd, was admitted to Centre Hospitalier National (Nouakchott, Mauritania) on July 2, 2012, with fever and epistaxis. One week earlier, he had fever, nausea, and vomiting. Without biologic confirmation of the infection, his doctors treated him for malaria. His leukocyte count was 3,200 cells/mm^3 (reference range [RR] 4,000–10,000 cells/mm^3), hemoglobin level was 10.6 g/dL (RR 14.0–17.5 g/dL), and platelet count was 22,000/mm^3 (RR 200,000–400,000 cells/mm^3). His aspartate aminotransferase level was elevated to 162 IU/L (RR 8–30 IU/L), and his alanine aminotransferase level was elevated to 200 IU/L (RR 8–35 IU/L). Glasgow Coma Scale score was 15. Results were positive from tests for CCHF virus-specific IgM by ELISA and CCHF virus by real-time reverse transcription PCR.

Treatment with platelet transfusions and supportive therapy was initiated. Fever and epistaxis improved on the third day of admission. On hospitalization day 6, headache and acute encephalopathy developed in the patient. Glasgow Coma Scale score was 13 (Figure, panel A). A computed tomography (CT) scan of his head without contrast showed acute subdural hematoma on the left side. On day 16 of admission, the patient's general condition worsened; he became more obtunded (experienced reduced consciousness), and right-sided upper limb hemiparesis developed. A repeat CT scan of his head showed a subdural hematoma with surrounding edema and midline shift (Figure, panel B).

Our care team considered a conservative management approach. We gave the patient corticosteroids and saline. After 4 weeks, his symptoms had improved markedly and he was discharged in stable condition. A 1-month follow-up CT scan of his head without contrast showed complete resolution of the subdural hematoma (Figure, panel C). Thrombocytopenia could be considered a risk factor for the development of a spontaneous acute subdural hematoma of arterial origin with more rapid and aggressive evolution (*4*).

The main vector for CCHF virus transmission appears to be ticks from the genus *Hyalomma* (*2*). CCHF that affects multiple organs is characterized by fever, myalgia, headache, shock, disseminated intravascular coagulation, recurrent extensive bleeding, and thrombocytopenia. After 5–6 days of illness, petechial rash, signs of bleeding (e.g., hematemesis and melena), and liver failure occur. CCHF can be diagnosed by using serologic tests to detect IgM and IgG against the virus and by using molecular-based techniques, such as conventional and real-time reverse transcription PCRs, to detect the genome of the virus (*5*,*6*).

Brain hemorrhage in persons with CCHF is rare. We report a case of acute subdural hematoma secondary to CCHF, where thrombocytopenia was the main cause of cerebral hemorrhage. Management of this case was challenging due to the underlying bleeding tendency of the patient and risk for nosocomial infection. We provided conservative treatment and the patient showed total remission. The patient improved due to the use of corticosteroids and the natural progressive resorption of blood.

Alavi-Naini et al. reported a case of CCHF in a person with a bilateral frontal parasagittal hematoma that was managed with oral ribavirin and intravenous ceftriaxone, platelet transfusions, and supportive therapy (*5*). The patient recovered. Kumar et al. reported 5 case-patients with dengue hemorrhagic fever and intracranial bleeding. Two of these patients underwent surgery after platelet transfusion and recovered (*7*). A high case-fatality rate has been reported in many countries among persons who became infected with CCHF after having contact with a hospitalized CCHF patient (*2*). Swanepoel et al. reported a case of

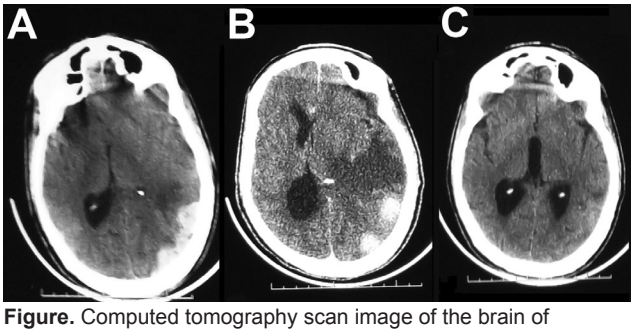

Figure. Computed tomography scan image of the brain of a 58-year-old man with Crimean-Congo hemorrhagic fever, Mauritania, 2012. A) Acute subdural hematoma, on the left side. B) Subdural hematoma with perihematomal edema and midline shift. C) Complete resorption of the subdural hematoma with residual edema, 1 month later.

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CCHF in which the patient died of complications following surgical intervention for cerebral hemorrhage (*8*).

Death from CCHF usually occurs after 5–14 days of illness (*1*,*8*,*9*). The basic pathogenesis of CCHF virus at the molecular level is complex and not well defined. Endothelial cells, immune response, virus load, and coagulation cascade play major roles in the disease pathogenesis. Blood and endothelium appear to be the target tissues of the disease (*9*). The coagulation cascade becomes activated over 24–48 hours; however, thrombin becomes activated and promotes edema formation and further disruption of the integrity of the blood–brain barrier. The edema formation starts when erythrocytes in the hematoma begin to lyse and its degradation products are deposited into the brain parenchyma, initiating a potent inflammatory reaction (*10*).

Although surgery remains the first choice for the treatment of acute subdural hematoma, some patients may benefit from conservative management with careful monitoring. This report highlights the value of an early diagnosis of CCHF and neuroimaging for severe cases when brain hemorrhage is suspected.

References

1. Karti SS, Odabasi Z, Korten V, Yilmaz M, Sonmez M, Caylan R, et al. Crimean-Congo hemorrhagic fever in Turkey. Emerg Infect Dis. 2004;10:1379–84. http://dx.doi.org/10.3201/eid1008.030928
2. Nabeth P, Cheikh DO, Lo B, Faye O, Vall IO, Niang M, et al. Crimean–Congo hemorrhagic fever, Mauritania. Emerg Infect Dis. 2004;10:2143–9. http://dx.doi.org/10.3201/eid1012.040535
3. Nabeth P, Thior M, Faye O, Simon F. Human Crimean–Congo hemorrhagic fever, Senegal. Emerg Infect Dis. 2004;10:1881–2. http://dx.doi.org/10.3201/eid1010.040586
4. Depreitere B, Van Calenbergh F, van Loon J. A clinical comparison of non-traumatic acute subdural haematomas either related to coagulopathy or of arterial origin without coagulopathy. Acta Neurochir (Wien). 2003;145:541–6. http://dx.doi.org/10.1007/s00701-003-0020-7
5. Alavi-Naini R, Moghtaderi A, Metanat M. An unusual intracerebral hemorrhage. Can J Infect Dis Med Microbiol. 2004;15:175–7.
6. Elata AT, Karsany MS, Elageb RM, Hussain MA, Eltom KH, Elbashir MI, et al. A nosocomial transmission of Crimean-Congo hemorrhagic fever to an attending physician in North Kordufan, Sudan.Virol J. 2011;8:303. http://dx.doi.org/10.1186/1743-422X-8-303
7. Kumar R, Prakash O, Sharma BS. Intracranial hemorrhage in dengue fever: management and outcome: a series of 5 cases and review of literature. Surg Neurol. 2009;72:429–33. http://dx.doi.org/10.1016/j.surneu.2009.01.021
8. Swanepoel R, Shepherd AJ, Leman PA, Shepherd SP, Miller GB. A common-source outbreak of Crimean-Congo haemorrhagic fever on a dairy farm. S Afr Med J. 1985;68:635–7.
9. Duru F, Fisgin T. Hematological aspects of Crimean-Congo hemorrhagic fever. Turk J Hematol. 2009;26:161–6.
10. Thiex R, Tsirka SE. Brain edema after intracerebral hemorrhage: mechanisms, treatment options, management strategies, and operative indications. Neurosurg Focus. 2007;22:E6. http://dx.doi.org/10.3171/foc.2007.22.5.7

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Use of Plasma Therapy for Severe Fever with Thrombocytopenia Syndrome Encephalopathy

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To the Editor: The central nervous system (CNS) manifestations of severe fever with thrombocytopenia syndrome (SFTS) include apathy, seizure, muscular tremor, and coma (*1*,*2*); however, the mechanism underlying CNS manifestations in SFTS is not clear. Deng et al. reported that illness of 15 (13%) of 115 patients with SFTS met the case definition for suspected encephalitis (*1*). However, they did not present any straightforward evidence of CNS invasion by STFS virus (SFTSV). Cui et al. similarly reported that encephalitis developed in one fifth of 538 patients with SFTS (*2*). They found evidence of SFTSV by isolating the virus from the cerebrospinal fluid (CSF) in 1 of 2 patients with SFTS whose CSF was obtained, but they did not mention CSF pleocytosis (*2*). We report a case of SFTS-associated encephalopathy, without pleocytosis and with normal CSF protein and glucose levels, that was confirmed by real-time reverse transcription PCR of the CSF. The patient was treated with experimental plasma exchange followed by convalescent plasma therapy.

During 2015, a 62-year-old woman who had a history of treated tuberculous meningitis 10 years earlier was admitted to a tertiary hospital in Seoul, South Korea (Republic of Korea), with a 5-day fever, myalgia, and headache. On hospital day (HD) 2, CSF examination revealed 1 leukocyte/mm^3, protein 35 mg/dL (reference 9–58 mg/dL), glucose 74 mg/dL (reference 45–80 mg/dL), and CSF/blood glucose ratio 0.66 (reference 0.50–0.80). No bacteria or fungi were isolated from CSF. On HD 4, her headache worsened, and she displayed confused verbal responses and lacked orientation of time and place. No focal neurologic signs were observed. On HD 5, magnetic resonance imaging of the brain indicated no additional abnormalities of the parenchyma and extra-axial structures except for a focal parenchymal defect in the right midbrain that had been discovered as a

[1]These authors contributed equally to this article.

sequelae of tuberculous meningitis 10 years earlier. On HD 7, follow-up CSF examination revealed no leukocytes, protein 57 mg/dL, glucose 209 mg/dL, and CSF/blood glucose ratio 0.62. SFTSV was detected by real-time reverse transcription PCR (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/15-1791-Techapp1.pdf) in plasma and CSF (Figure). On HD 8, the patient became comatose and had no eye, verbal, and motor responses to noxious stimuli (Glasgow coma scale 3). Bilateral exotropia was noted with spared light and corneal reflexes and oculocephalic responses. Experimental plasma exchange was performed, and her viral load declined slightly; however, consciousness and platelet count did not change. An ABO-identical nurse who had recovered from SFTS in September 2014 agreed to donate plasma; her indirect immunofluorescence antibody assay (IFA) for SFTSV IgG had been 1:1,024 in October 2014. On HD 17, the patient's titer of SFTSV IgG was 1:64 before the plasma therapy. We obtained ≈400 mL of convalescent plasma (IFA assay for SFTSV IgG 1:256 at the time of donation) from the donor and transfused it into the patient on HD 17. The viral load in the blood decreased steeply by a factor of 10 (6×10^2 to 6×10^1 copies/mL) during the first 7 hours (4–11 PM on HD 17); it then gradually decreased from 3×10^1 at 7 AM on HD 18 to 6×10^0 copies/mL on HD 20, by which time the patient's mental status had fully recovered (Figure).

This case is unique in that SFTS was detected in CSF in the absence of pleocytosis and with normal CSF protein and glucose levels, as in previous reports on influenza-associated acute encephalopathy (*3*). Although headache and encephalitis can occur in patients with SFTS (*1*,*2*), the pathophysiology of CNS manifestations in SFTS is unknown. As with influenza-associated acute encephalopathy, a possible hypothesis is direct invasion of SFTSV into the CNS; another hypothesis is that elevated cytokine levels or renal and hepatic dysfunction are associated with SFTS encephalopathy.

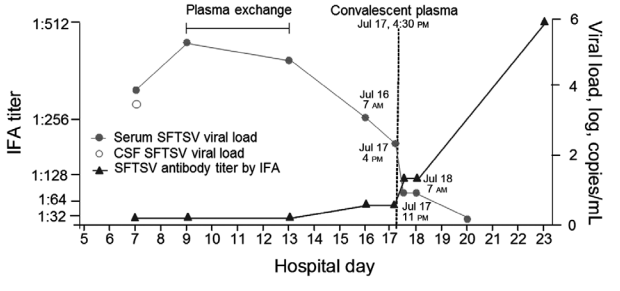

Figure. Changes in viral RNA load and immunofluorescence antibody titer and timing of therapies for a 62-year-old woman with SFTSV-associated encephalopathy in response to plasma exchange followed by convalescent plasma therapy, South Korea, 2015. CSF, cerebrospinal fluid; IFA, indirect immunofluorescence antibody assay; SFTSV, severe fever with thrombocytopenia syndrome virus.

We are aware of 1 report of a favorable outcome of plasma exchange and ribavirin in 2 patients with SFTS and multiorgan failure in South Korea (*4*). However, the patients' clinical condition did not substantially improve despite the 5-day plasma exchange therapy and viral load only slightly decreased. Use of convalescent plasma therapy in severe acute respiratory syndrome, influenza A(H1N1) and A(H5N1), and Ebola virus disease has been reported (*5*–*7*), but little evidence exists to support its use. However, given the lack of conclusive data, these potential experimental treatments for emerging infectious diseases warrant further study in a clinical trial. Response was favorable in a mouse model of SFTS treated postexposure with antiserum from a patient who had recovered from SFTS (*8*).

We do not know whether the convalescent plasma therapy given to the patient described here actually had a positive effect because her IFA titer was already increasing around the time she received the plasma therapy. At the time of this writing, 2 patients with SFTS who were treated with intravenous immunoglobulin and corticosteroid had been reported (*9*). Cautious interpretation of these experimental therapies is necessary because these therapies may not have had anything to do with the patients' recovery.

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References

1. Deng B, Zhou B, Zhang S, Zhu Y, Han L, Geng Y, et al. Clinical features and factors associated with severity and fatality among patients with severe fever with thrombocytopenia syndrome bunyavirus infection in northeast China. PLoS One. 2013;8:e80802. http://dx.doi.org/10.1371/journal.pone.0080802
2. Cui N, Liu R, Lu QB, Wang LY, Qin SL, Yang ZD, et al. Severe fever with thrombocytopenia syndrome bunyavirus–related human encephalitis. J Infect. 2015;70:52–9. http://dx.doi.org/10.1016/j.jinf.2014.08.001
3. Moon SM, Kim SH, Jeong MH, Lee EH, Ko TS. Acute encephalopathy and pandemic (H1N1) 2009. Emerg Infect Dis. 2010;16:1811–3. http://dx.doi.org/10.3201/eid1611.100682
4. Oh WS, Heo ST, Kim SH, Choi WJ, Han MG, Kim JY. Plasma exchange and ribavirin for rapidly progressive severe fever with thrombocytopenia syndrome. Int J Infect Dis. 2014;18:84–6. http://dx.doi.org/10.1016/j.ijid.2013.08.011
5. Mair-Jenkins J, Saavedra-Campos M, Baillie JK, Cleary P, Khaw FM, Lim WS, et al. The effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology: a systematic

review and exploratory meta-analysis. J Infect Dis. 2015; 211:80–90. http://dx.doi.org/10.1093/infdis/jiu396
6. Zhou B, Zhong N, Guan Y. Treatment with convalescent plasma for influenza A (H5N1) infection. N Engl J Med. 2007;357:1450–1. http://dx.doi.org/10.1056/NEJMc070359
7. van Griensven J, Edwards T, de Lamballerie X, Semple MG, Gallian P, Baize S, et al. Evaluation of convalescent plasma for Ebola virus disease in Guinea. N Engl J Med. 2016;374:33–42. http://dx.doi.org/10.1056/NEJMoa1511812
8. Shimada S, Posadas-Herrera G, Aoki K, Morita K, Hayasaka D. Therapeutic effect of post-exposure treatment with antiserum on severe fever with thrombocytopenia syndrome (SFTS) in a mouse model of SFTS virus infection. Virology. 2015;482:19–27. http://dx.doi.org/10.1016/j.virol.2015.03.010
9. Kim UJ, Kim DM, Ahn JH, Kang SJ, Jang HC, Park KH, et al. Successful treatment of rapidly progressing severe fever with thrombocytopenia syndrome with neurological complications using intravenous immunoglobulin and corticosteroid. Antivir Ther. 2016 Feb 17. Epub ahead of print. http://dx.doi.org/10.3851/IMP3036

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Naturally Circulating Hepatitis A Virus in Olive Baboons, Uganda

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To the Editor: Hepatitis A (HAV; family *Picornaviridae*; genus *Hepatovirus*) is an ≈7.5-kb single-stranded positive-sense RNA virus that causes acute inflammation of the liver in humans and nonhuman primates. Although HAV is most commonly transmitted by food and water contaminated with feces, humans have acquired HAV from handling infected nonhuman primates in captivity (*1*).

HAV has been detected in recently imported captive primates after spontaneous outbreaks of acute hepatitis in animal facilities, but the definitive hosts of this virus have remained obscure (*2*,*3*). We identified by next-generation sequencing HAV in the blood of a free-living olive baboon (*Papio anubis*) from Kibale National Park, Uganda, sampled in September 2010. Subsequent testing of a separate Kibale olive baboon troop in 2014 indicated the virus was prevalent and shed in feces.

As part of a long-term study of nonhuman primate health and ecology, 23 animals were immobilized and sampled in 2010 as previously described (*4*). All animal protocols received prior approval from the Uganda National Council for Science and Technology (Kampala, Uganda), the Uganda Wildlife Authority (Kampala, Uganda), and the University of Wisconsin–Madison Animal Care and Use Committee (Madison, WI, USA). All samples were shipped in accordance with international laws under Convention on International Trade in Endangered Species of Wild Fauna and Flora Ugandan permit no. 002290.

During May 2012, we subjected total RNA from 1 mL of blood plasma of each animal to next-generation sequencing as previously described (*4*); results showed HAV-like sequences in 1 of 23 baboons. De novo assembly of these reads yielded a nearly complete HAV genome, which we term KibOB-1. KibOB-1 is most similar (94.2% nt identity; Figure) to AGM-27, an HAV originally detected in an African green monkey (*Chlorocebus aethiops*) imported to a Russian primate facility from Kenya (*3*).

For 11 baboons, we also collected a paired fecal sample, which we analyzed for evidence of viral shedding. Samples were preserved in RNAlater (Ambion Inc., Austin, TX, USA) at –20°C, and viral RNA was isolated by using the ZR Soil/Fecal RNA Microprep kit (Zymo Research, Irvine, CA, USA) following manufacturer's protocols. Reverse transcription PCR (RT-PCR) of RNA was primed with random hexamers by using the RNA to cDNA Ecodry Premix (Random Hexamers) (Clontech Laboratories, Inc., Mountain View, CA, USA), and diagnostic PCR was conducted with primers flanking the C-terminal extension of the HAV viral protein (VP) 1 gene (*pX*) by using the High Fidelity PCR Master Mix-Ecodry Premix (Clontech Laboratories, Inc.). Five of 11 paired fecal samples tested positive for HAV by RT-PCR, indicating a higher prevalence of the virus in feces than in blood.

We then surveyed a second troop of habituated olive baboons at the same field site during February–April 2014 (*5*). From these baboons, 7 of 19 fecal samples tested positive by RT-PCR. Confirmatory Sanger sequencing of RT-PCR amplicons was successful for 3 of these 7 animals (GenBank accession nos. KT819576–KT819578). Phylogenetic analyses of these sequences demonstrate monophyly and a low degree of interhost variability (≥94% nt identity).

The risk to humans posed by KibOb-1 remains unknown. Although human infection with HAV genotype V has not been reported, evidence suggests that HAV variants might be capable of infecting a diversity of primate hosts (*6*). Although it is not known whether the closely related AGM-27 strain was discovered infecting its natural host, the similarity of KibOB-1 and AGM-27 raises the possibility of a recent host transfer. Major host shifts characterize the evolutionary histories of recently discovered bat and rodent hepatoviruses (*7*). Host fidelity of KibOB-1 is similarly unknown, but experimental infection of several nonhuman primate species with the similar AGM-27 virus found varying pathogenicity in different species (*6*). In particular, the AGM-27 caused productive infection in chimpanzees, with stimulation of a broadly reactive HAV immunoglobulin response (*6*).

Human and simian HAVs are considered a single serotype (*6*); thus, serosurveillance for HAV in humans might be unable to distinguish between human and zoonotic simian HAV infection, enabling the possibility of cryptic zoonotic transmission. Similarly, detection of HAV antibodies in wild primates, such as in a recent study of baboons in South Africa living in close proximity to humans (*8*), might not indicate anthroponotic transmission of human viruses but rather infection with an endemic HAV.

Prior studies have documented cross-species transmission between the primates of Kibale National Park and neighboring human populations, especially of gastrointestinal pathogens (*9*). A study tracking food-crop–raiding events on 97 farms within 0.5 km of Kibale's forest edge found that 72% of households faced baboon raids over a 23-month period, including 228 discrete baboon raids (*10*). This finding suggests that a major portion of the local community remains at risk for exposure to potentially infectious baboon excreta. Such exposure, in addition to the evidence presented here that HAV is prevalent in wild baboons of Uganda and is shed into the environment, merits increased attention to the zoonotic risk for simian hepatoviruses.

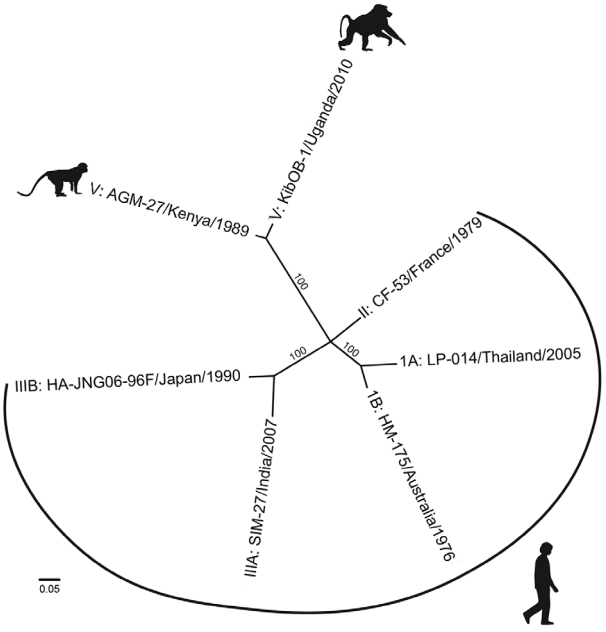

Figure. Whole-genome phylogenetic reconstruction of representative HAVs. HAVs are grouped into 6 genotypes based on 168 bp of the C-terminal extension of the viral protein 1 gene. Baboon HAV detected in Kibale National Park, Uganda, in 2010 and 2014 (GenBank accession number KT819575) clusters with AGM-27 (*3*), previously the sole member of genotype V. jModeltest 2 (http://jmodeltest.org) was used to find the best-fit evolutionary model for the data, after which the maximum-likelihood tree was estimated using the heuristic search method in PAUP* (http://paup.csit.fsu.edu), with starting trees obtained by neighbor-joining, random stepwise addition, and branch swapping by tree-bisection reconnection and starting branch lengths obtained using Rogers-Swofford approximation. Bootstrap values were derived from 1,000 replicates of the heuristic search; only values ≥50% are shown. GenBank accession nos.: IA, EF207320; IB, M14707; II, AY644676; IIIA, FJ227135; IIIB, AB258387; V, D00924). HAV, hepatitis A virus. Scale bar indicates substitutions per site.

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References

1. Dienstag JL, Davenport FM, McCollum RW, Hennessy AV, Klatskin G, Purcell RH. Nonhuman primate–associated viral hepatitis type A. Serologic evidence of hepatitis A virus infection. JAMA. 1976;236:462–4. http://dx.doi.org/10.1001/jama.1976.03270050018021
2. Robertson BH, Jansen RW, Khanna B, Totsuka A, Nainan OV, Siegl G, et al. Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. J Gen Virol. 1992;73:1365–77. http://dx.doi.org/10.1099/0022-1317-73-6-1365
3. Tsarev SA, Emerson SU, Balayan MS, Ticehurst J, Purcell RH. Simian hepatitis A virus (HAV) strain AGM-27: comparison of genome structure and growth in cell culture with other HAV strains. J Gen Virol. 1991;72:1677–83. http://dx.doi.org/10.1099/0022-1317-72-7-1677
4. Lauck M, Hyeroba D, Tumukunde A, Weny G, Lank SM, Chapman CA, et al. Novel, divergent simian hemorrhagic fever viruses in a wild Ugandan red colobus monkey discovered using direct pyrosequencing. PLoS One. 2011;6:e19056. http://dx.doi.org/10.1371/journal.pone.0019056
5. Johnson CA, Swedell L, Rothman JM. Feeding ecology of olive baboons (*Papio anubis*) in Kibale National Park, Uganda: preliminary results on diet and food selection. Afr J Ecol. 2012;50:367–70.
6. Emerson SU, Tsarev SA, Govindarajan S, Shapiro M, Purcell RH. A simian strain of hepatitis A virus, AGM-27, functions as an

attenuated vaccine for chimpanzees. J Infect Dis. 1996;173:592–7. http://dx.doi.org/10.1093/infdis/173.3.592
7. Drexler JF, Corman VM, Lukashev AN, Brand JMA, van den Gmyl AP, Brünink S, et al. Evolutionary origins of hepatitis A virus in small mammals. Proc Natl Acad Sci U S A. 2015;112:15190–5. http://dx.doi.org/10.1073/pnas.1516992112
8. Drewe JA, O'Riain MJ, Beamish E, Currie H, Parsons S. Survey of infections transmissible between baboons and humans, Cape Town, South Africa. Emerg Infect Dis. 2012;18:298–301. http://dx.doi.org/10.3201/eid1802.111309
9. Goldberg TL, Gillespie TR, Rwego IB, Estoff EL, Chapman CA. Forest fragmentation as cause of bacterial transmission among nonhuman primates, humans, and livestock, Uganda. Emerg Infect Dis. 2008;14:1375–82. http://dx.doi.org/10.3201/eid1409.071196
10. Naughton-Treves L. Predicting patterns of crop damage by wildlife around Kibale National Park, Uganda. Conserv Biol. 1998; 12:156–68. http://dx.doi.org/10.1046/j.1523-1739.1998.96346.x

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Porcine Bocavirus Infection Associated with Encephalomyelitis in a Pig, Germany[1]

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To the Editor: In 2013, a 6-week-old female piglet kept in a flatdeck cage had coughing, growth retardation, and diarrhea and was taken to a local veterinarian in Hannover, Germany; the piglet was euthanized. After necropsy at the University of Veterinary Medicine in Hannover, histologic investigation found interstitial pneumonia; a mild, multifocal, lymphohistiocytic panencephalitis that affected the cerebrum and cerebellum, including brain stem and medulla oblongata; and a mild, multifocal, lymphohistiocytic panmyelitis. Results from screening for typical neurotropic viruses (classical swine fever virus, suid herpesvirus 1, rabies virus, teschovirus, porcine enterovirus 8, 9, and 10) were negative; *Mycoplasma hyorhinis* was detected by multiplex PCR (Institute of Virology, University of Veterinary Medicine Hannover) within the lung and pulmonary lymph nodes. Cerebral tissue from the pig was processed for viral metagenomics by random RNA and DNA virus screening and next-generation sequencing (NGS) with the 454 sequencing platform (GS Junior; Roche, Basel, Switzerland), as described (*1*), and 21,359 reads were obtained. Analysis by using blastn and blastx (*2*) showed 10 reads had ≥97% nt identity with porcine bocavirus (PBoV) KU14. No other viral sequences were detected.

By using primers based on sequence data of the PBoV, partially overlapping PCR amplicons were obtained to confirm and extend the NGS data of the isolate, which was named PBoV S1142/13 (*1*; GenBank accession no. KU311698). A total of 2,176 nt of PBoV S1142/13 were obtained, consisting of the partial nucleoprotein (NP) 1 and the nearly complete viral protein (VP) 1 gene. By using MAFFT version 7 (http://mafft.cbrc.jp/alignment/server/), we aligned the nearly complete VP1 gene of PBoV S1142/13 with various closely related members of the genus *Bocaparvovirus* and built a maximum-likelihood tree by using the general time reversible plus invariable sites plus gamma distribution method, as determined by jModelTest 2.0 (*3*) and default parameters in MEGA6.06 (*4*). Results confirmed that PBoV S1142/13 was most closely related to PBoV KU14 (Figure, panel A). The partial genome of PBoV S1142/13 differed at 8 nt positions from PBoV KU14, resulting in 99.6% nt identity. Of these nucleotide differences, 4 resulted in an amino acid difference, including position 2733 (T→C on the basis of PBoV KU14 as a reference genome), which is part of the NP1 stopcodon of PBoV KU14. These results indicate that the stopcodon was located 39 nt farther downstream than for PBoV KU14. The other 3 aa differences were present in the VP1 protein; each of these differences was within the same group of amino acids as those detected in PBoV KU14.

For further substantiation of a potential cause-effect relationship of histologic (Figure, panel B) and NGS results, we performed fluorescent in situ hybridization (FISH) on formalin-fixed, paraffin-embedded central nervous system (CNS) sections of the diseased animal and of a control pig with no CNS lesions. We used an RNA probe specific for the obtained NP1 and VP1 sequences covering 1,153 nt (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's protocol, with minor variations (ViewRNA ISH Tissue 1-Plex Assay Kit and ViewRNA Chromogenic Signal Amplification Kit, Affymetrix). A probe specific for porcine ubiquitin (*Sus scrofa* ubiquitin

[1]Preliminary results from this study were presented at the 3rd International One Health Congress, March 15–18, 2015, Amsterdam, the Netherlands; and at the Conference of the German Veterinary Medical Association, March 8–10, 2015, Fulda, Germany.

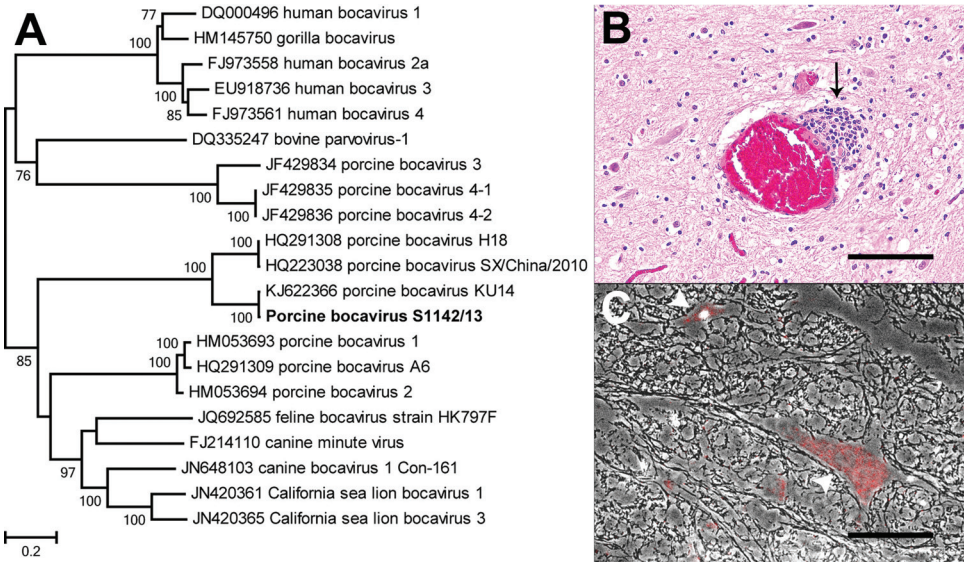

Figure. Phylogenetic analysis and staining of porcine bocavirus (PBoV) from the spinal cord of a diseased pig, Hannover, Germany. A) Phylogenetic relationship of PBoV isolate S1142/13 (bold) with other bocaviruses. The nucleotide sequence of the nearly complete viral protein 1 of PBoV S1142/13 was aligned with other members of the genus *Bocaparvovirus*, and a maximum-likelihood phylogenetic tree was prepared by using the general time reversible plus invariable sites plus gamma distribution model and 500 bootstrap replicates. Only bootstrap values >70 are shown. Scale bar indicates nucleotide substitutions per site. B) Spinal cord of the diseased pig showing perivascular accentuated mild, focal, nonsuppurative inflammation (arrow). Hematoxylin and eosin stain. Scale bar indicates 100 µm. C) Intracytoplasmic and intranuclear PBoV-specific positive signals in neurons of the spinal cord of the diseased pig (arrowheads), determined by using fluorescent in situ hybridization (Fast Red; ViewRNA Chromogenic Signal Amplification Kit; Affymetrix, Santa Clara, CA, USA). Phase contrast and fluorescent microscopy. Scale bar indicates 100 µm.

C; GenBank accession no. XM_005657305; nt 2–890) served as a positive control.

The spinal cord of the diseased pig showed diffuse intracytoplasmic and intranuclear PBoV-specific signals within scattered neurons adjacent to the histologically detected inflammatory lesions (Figure, panel C). The negative control and the nonprobe incubation lacked PBoV-specific signals. The porcine ubiquitin probe provided a strong intracellular and extracellular staining within the CNS of both pigs.

PBoV (genus *Bocaparvovirus*, family *Parvoviridae*) was first described in 2009 as porcine boca-like virus in pigs in Sweden with postweaning multisystemic wasting syndrome (*5*). PBoV is usually involved in respiratory and intestinal diseases in pigs (*5*) but has not been detected in the CNS. In the pig in our study, the lack of detection of other viral sequences by using NGS indicates the potential role of PBoV as a pathogen that triggers encephalomyelitis. FISH substantiated the NGS results and revealed neuronal intracytoplasmic and intranuclear PBoV-specific signals adjacent to the lesion, indicating intraneuronal transcription and replication (*6*). Nevertheless, a potential synergistic effect of *M. hyorhinis* on the PBoV pathogenesis cannot be ruled out. Similarly, co-infection of *M. hyorhinis* and porcine circovirus type 2 has been associated with enhanced inflammatory lesions in the lungs of pigs (*7*).

The CNS tropism of PBoV S1142/13 could result from various factors, including specific amino acid changes that enable the virus to pass the blood–brain barrier and infect neurons. Additional studies are necessary to elucidate a possible role of the amino acid differences between PBoV S1142/13 and PBoV KU14 in the tropism of these viruses.

Human bocavirus has recently been found in the cerebrospinal fluid of patients having encephalitis (*8*), and related human parvovirus 4 (*9*) and human parvovirus B19 (*10*) have been reported in human encephalitis. The correlation of PBoV-specific signals by using FISH for histologic detection of encephalomyelitis assigns PBoV a potential role in provoking CNS lesions. PBoV should be considered as a cause of encephalomyelitis but needs further investigation.

Acknowledgments

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References

1. van Leeuwen M, Williams MM, Koraka P, Simon JH, Smits SL, Osterhaus AD. Human picobirnaviruses identified by molecular screening of diarrhea samples. J Clin Microbiol. 2010;48:1787–94. http://dx.doi.org/10.1128/JCM.02452-09
2. Schürch AC, Schipper D, Bijl MA, Dau J, Beckmen KB, Schapendonk CM, et al. Metagenomic survey for viruses in Western Arctic caribou, Alaska, through iterative assembly of taxonomic units. PLoS One. 2014;9:e105227. http://dx.doi.org/10.1371/journal.pone.0105227
3. Darriba D, Taboada GL, Doallo R, Posada D. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 2012;9:772. http://dx.doi.org/10.1038/nmeth.2109
4. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30:2725–9. http://dx.doi.org/10.1093/molbev/mst197
5. Zhou F, Sun H, Wang Y. Porcine bocavirus: achievements in the past five years. Viruses. 2014;6:4946–60. http://dx.doi.org/10.3390/v6124946
6. Bodewes R, Lapp S, Hahn K, Habierski A, Förster C, König M, et al. Novel canine bocavirus strain associated with severe enteritis in a dog litter. Vet Microbiol. 2014;174:1–8. http://dx.doi.org/10.1016/j.vetmic.2014.08.025
7. Chen D, Wei Y, Huang L, Wang Y, Sun J, Du W, et al. Synergistic pathogenicity in sequential coinfection with *Mycoplasma hyorhinis* and porcine circovirus type 2. Vet Microbiol. 2016;182:123–30. http://dx.doi.org/10.1016/j.vetmic.2015.11.003
8. Mori D, Ranawaka U, Yamada K, Rajindrajith S, Miya K, Perera HK, et al. Human bocavirus in patients with encephalitis, Sri Lanka, 2009–2010. Emerg Infect Dis. 2013;19:1859–62. http://dx.doi.org/10.3201/eid1911.121548
9. Benjamin LA, Lewthwaite P, Vasanthapuram R, Zhao G, Sharp C, Simmonds P, et al. Human parvovirus 4 as potential cause of encephalitis in children, India. Emerg Infect Dis. 2011;17:1484–7.
10. Barah F, Whiteside S, Batista S, Morris J. Neurological aspects of human parvovirus B19 infection: a systematic review. Rev Med Virol. 2014;24:154–68. http://dx.doi.org/10.1002/rmv.1782

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Pegivirus Infection in Domestic Pigs, Germany

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[1]These authors contributed equally to this article.

To the Editor: The family *Flaviviridae* includes many human and animal virus pathogens. Recently, in addition to the genera *Flavivirus*, *Hepacivirus*, and *Pestivirus*, a fourth genus, *Pegivirus*, has been identified (*1*). In addition to human pegiviruses, a range of phylogenetic, highly divergent pegiviral sequences have been identified in various animal species, including primates, bats, rodents, and horses (*2*). We report the detection of a porcine pegivirus (PPgV) in serum samples from pigs.

Initially, we investigated pooled serum samples by using high-throughput sequencing methods and isolated RNA from individual porcine serum samples by using the QIAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). We prepared libraries compatible with Illumina (San Diego, CA, USA) sequencing from pooled samples and individual serum samples by using the ScriptSeq version 2 RNA-Seq Library Preparation Kit (Epicenter, Madison, WI, USA) and sequenced them by using a HiSeq 2500 (2 × 150 cycles paired-end; Illumina) for pooled samples and MiSeq (2 × 250 cycles paired-end; Illumina) for individual samples (*3*).

We conducted quantitative reverse transcription PCR (RT-PCR) by using a Quantitect-SYBR Green Assay (QIAGEN) and primers PPgV_fwd: 5′-CTGTCTATGCTGGTCACGGA-3′ and PPgV_rev: 5′-GCCATAGAACGGGAAGTCGC-3′. By using high-throughput sequencing of the pooled serum sample library (23,167,090 reads), we identified 1 contig (4,582 bp) that had distant nucleotide sequence similarity to bat pegivirus (69% and 4% sequence coverage) and 2 contigs (2,683 bp and 665 bp) that had 73% sequence coverage, thereby covering 8% and 37% of the identified sequence. RT-PCR with primers designed on basis of recovered sequences identified the sample containing pegivirus sequences. Subsequent MiSeq analysis (7,085,595 reads) of an RNA library prepared from a sample from 1 animal identified 1 contig (9,145 nt) with sequence similarity to pegivirus sequences.

We performed 3′ end completion of the viral genome by rapid amplification of cDNA ends and identified the entire open reading frame of PPgV_903 encoding 2,972 aa (GenBank accession no. KU351669). Analysis of the pegivirus 5′ untranslated region identified a highly structured internal ribosome entry site motif (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/16-0024- Techapp1.pdf), which was similar in structure to previously described 5′ untranslated region structures of other pegiviruses (*4*,*5*).

Pegiviruses do not encode a protein homologous to the capsid protein of other viruses of the family *Flaviviridae*, another common feature of pegiviruses (*6*). The presence of cleavage sites for cellular signal peptidases and viral proteases indicates that, similar to polyproteins of other pegiviruses and members of the genus *Hepacivirus*, the pegivirus polyprotein NH_2-E1- E2-Px-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (E [envelope], NS [nonstructural],

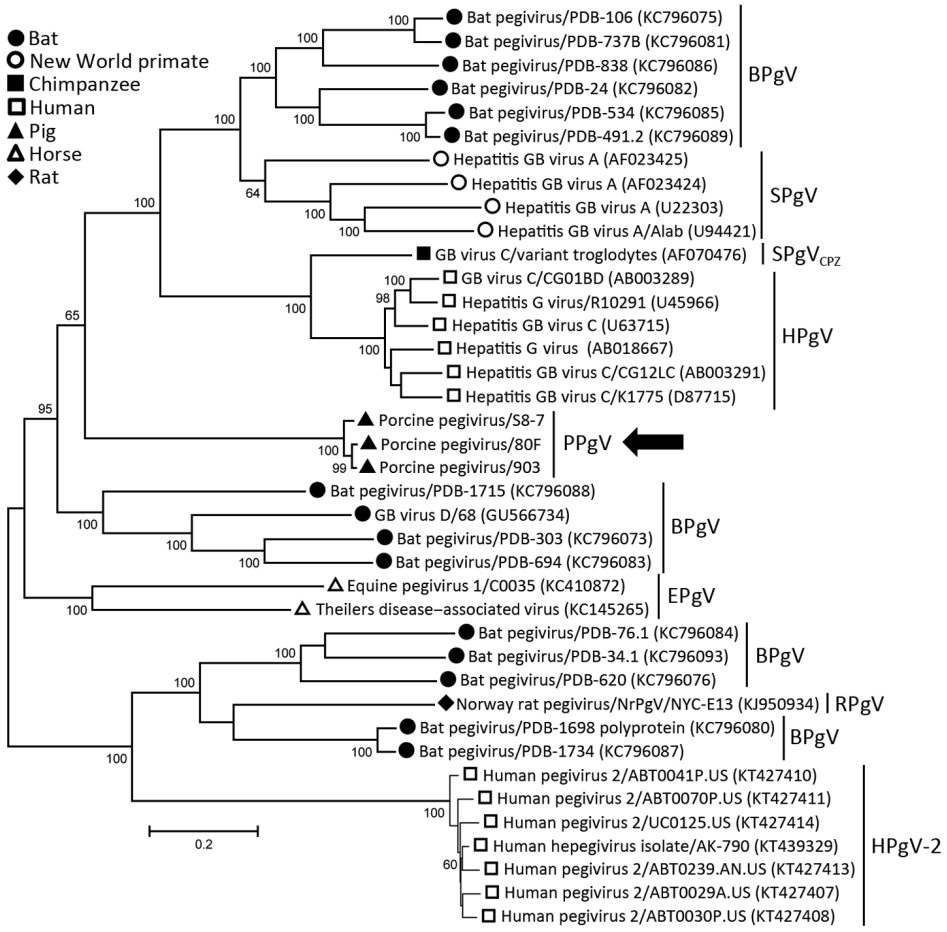

Figure. Phylogenetic analysis of human and animal pegiviruses. We constructed a maximum-likelihood tree on the basis of the complete coding region and used the general time reversible model for modeling of substitutions. Bootstrap analysis was performed with 200 replicates. Numbers along branches are percentage bootstrap values. GenBank accession numbers are in parentheses. Arrow indicates viruses isolated in this study. Scale bar indicates nucleotide substitutions per site. BPgV, bat pegivirus, SPgV, simian pegivirus; SPgV$_{CPZ}$, simian pegivirus (chimpanzee); HPgV, human pegivirus; PPgV, porcine pegivirus; EPgV, equine pegivirus; RPgV, rodent pegivirus. GB viruses have recently been reclassified as pegiviruses.

and Px [protein X]) is cleaved co-translationally and posttranslationally.

We tested 3 additional animals from the same breeding cohort for virus RNA at irregular intervals for 22 months. One animal was positive for pegivirus RNA for 7 months, and the other 2 animals had pegivirus RNA in serum for 16 and 22 months. None of these animals showed obvious clinical signs attributable to virus infection. Follow-up investigation of 455 serum samples from 37 swine holdings from Germany identified 10 (2.2%) samples from 6 pig holdings that contained pegivirus RNA. We obtained 2 additional near full-length genomic sequences (PPgV_80F and PPgV_S8-7) from 2 animals in different herds by high-throughput sequencing, RT-PCR, and Sanger sequencing (GenBank accession nos. KU351670 and KU351671).

Phylogenetic analyses of complete coding regions showed the close relationship of the 3 pegivirus sequences from Germany. These 3 sequences formed a separate clade within the genus *Pegivirus* (Figure). Pairwise comparison between PPgV_903 and the other 2 pegivirus sequences showed strong nucleotide identities (96.0%–98.4%). A distance scan over the entire polyprotein showed genetic distance to other pegiviruses and demonstrated that NS3 and NS5B contain the most conserved regions among pegivirus polyproteins (online Technical Appendix).

In horses, 2 distinct pegiviruses that had different potentials to cause clinical disease in infected animals have been described (*4*,*7*). No obvious clinical effects were observed in pegivirus-infected animals during our study. However, potential consequences of viral infection for animal health and food production need to be explored more closely under field and experimental conditions. Pegiviruses can interact with the immune system of the host. Co-infection with human pegivirus and HIV can have beneficial effects, which result in decreased retroviral loads and delayed disease progression (*8*).

It will be useful to investigate whether co-infections with pegiviruses can influence clinical manifestations of infectious diseases of swine, including multifactorial diseases such as postweaning multisystemic wasting syndrome, in which unknown immune modulating virus infections have been suggested to influence the degree of clinical illness (*9*). RNA viruses have considerable potential to adapt to new environmental conditions and to overcome host restrictions (*10*). Until now, the host tropism of PPgV has not been investigated in detail. Therefore,

additional studies will be required to elucidate whether the spectrum of potential hosts might include other farm or companion animals, and whether the virus might be able to infect humans.

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References

1. Stapleton JT, Bukh J, Muerhoff AS, Foung S, Simmonds P. Assignment of human, simian and bat pegiviruses (previously described as GBV-A, GBV-C, and GBV-D) as members of a new genus (*Pegivirus*) within the *Flaviviridae* [cited 2015 Oct 21]. http://www.ictvonline.org/proposals/2012.011a-dV.A.v2.Pegivirus.pdf
2. Thézé J, Lowes S, Parker J, Pybus OG. Evolutionary and phylogenetic analysis of the hepaciviruses and pegiviruses. Genome Biol Evol. 2015;7:2996–3008. http://dx.doi.org/10.1093/gbe/evv202
3. Baechlein C, Fischer N, Grundhoff A, Alawi M, Indenbirken D, Postel A, et al. Identification of a novel hepacivirus in domestic cattle from Germany. J Virol. 2015;89:7007–15. http://dx.doi.org/10.1128/JVI.00534-15
4. Kapoor A, Simmonds P, Cullen JM, Scheel TK, Medina JL, Giannitti F, et al. Identification of a pegivirus (GB virus-like virus) that infects horses. J Virol. 2013;87:7185–90. http://dx.doi.org/10.1128/JVI.00324-13
5. Simons JN, Desai SM, Schultz DE, Lemon SM, Mushahwar IK. Translation initiation in GB viruses A and C: evidence for internal ribosome entry and implications for genome organization. J Virol. 1996;70:6126–35.
6. Stapleton JT, Foung S, Muerhoff AS, Bukh J, Simmonds P. The GB viruses: a review and proposed classification of GBV-A, GBV-C (HGV), and GBV-D in genus *Pegivirus* within the family *Flaviviridae*. J Gen Virol. 2011;92:233–46. http://dx.doi.org/10.1099/vir.0.027490-0
7. Chandriani S, Skewes-Cox P, Zhong W, Ganem DE, Divers TJ, Blaricum AJ, et al. Identification of a previously undescribed divergent virus from the *Flaviviridae* family in an outbreak of equine serum hepatitis. Proc Natl Acad Sci U S A. 2013;110:E1407–15. http://dx.doi.org/10.1073/pnas.1219217110
8. Schwarze-Zander C, Blackard JT, Rockstroh JK. Role of GB virus C in modulating HIV disease. Expert Rev Anti Infect Ther. 2012;10:563–72. http://dx.doi.org/10.1586/eri.12.37
9. Grau-Roma L, Fraile L, Segalés J. Recent advances in the epidemiology, diagnosis and control of diseases caused by porcine circovirus type 2. Vet J. 2011;187:23–32. http://dx.doi.org/10.1016/j.tvjl.2010.01.018
10. Rosenberg R. Detecting the emergence of novel, zoonotic viruses pathogenic to humans. Cell Mol Life Sci. 2015;72:1115–25. http://dx.doi.org/10.1007/s00018-014-1785-y

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New Chimeric Porcine Coronavirus in Swine Feces, Germany, 2012

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To the Editor: Porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus (TGEV) can cause severe enteritis in pigs accompanied by diarrhea, vomiting, and dehydration. Clinical signs are most prominent in young suckling pigs, in which high mortality rates are common. As seen in recent porcine epidemic diarrhea outbreaks in the United States and Asia, the effect on the pig industry can be tremendous.

Recently, Boniotti et al. (*1*) reported detection and genetic characterization of swine enteric coronaviruses (CoVs) circulating in Italy during 2007–2014. Characterization was based on sequencing and phylogenetic analyses of spike genes of TGEV and PEDV isolates. This study also reported a new recombinant CoV strain with a TGEV backbone and a PEDV spike gene (SeCoV/Italy/213306/2009; KR061459), which was identified as a swine enteric CoV (SeCoV). This chimeric virus presumably resulted from a recombination event.

Accompanying a study of recent porcine epidemic diarrhea cases in Germany caused by a new PEDV Indel strain (*2*), we retrospectively analyzed fecal samples from pigs that showed typical clinical symptoms of a PEDV infection. The sample set included fecal material collected from a farm in southern Germany on which an episode of diarrhea among pigs occurred in 2012. This material was shown by electron microscopy to contain CoV-like particles (Figure), but showed negative results by reverse transcription PCRs specific for the PEDV nucleocapsid gene.

Subsequent metagenomic analyses resulted in the full-genome sequence of a swine enteric CoV (SeCoV/GER/L00930/2012). We found a sequence showing high similarity (99.5% identity) with the TGEV/PEDV recombinant reported by Boniotti et al. (*1*). Network analysis of complete genome sequences of similar CoVs underline the chimeric nature of the genome between TGEV and PEDV genome sequences (online Technical Appendix Figure,

[1]All authors contributed equally to this article.

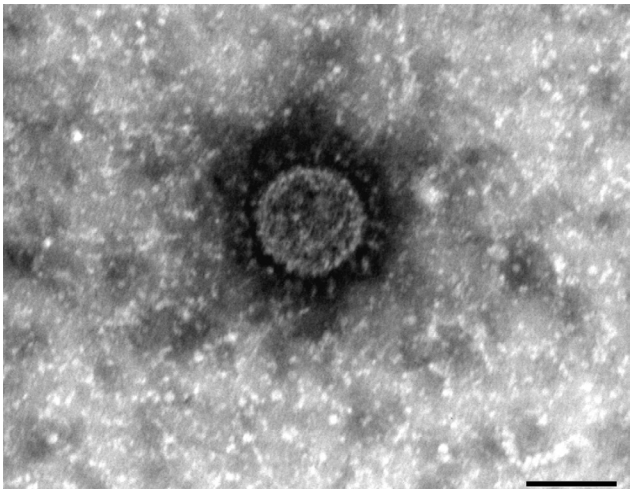

Figure. Electron micrograph of a new chimeric swine enteric coronavirus (SeCoV/GER/L00930/2012), Germany, 2012. Scale bar indicates 100 nm.

panel A, http://wwwnc.cdc.gov/EID/article/22/7/16-0179-Techapp1.pdf). The chimeric nature of the virus strain was confirmed by RT-PCR with primers spanning possible recombination sites and analysis of overlapping reads from next-generation sequencing.

Annotation of the sequence of SeCoV/GER/L00930/2012 performed on the basis of SeCoV/Italy/213306/2009 identified a similar putative coding sequence with a TGEV backbone and a spike coding sequence similar to that for PEDV (online Technical Appendix panel B). Downstream of the spike protein–coding open reading frame (ORF), an additional hypothetical ORF was identified in both SeCoV sequences. The coded amino acid sequences (27 aa in the virus from Germany and 30 aa in the virus from Italy) resembled an N- and C-terminally truncated TGEV nonstructural protein 3a. The difference of 3 aa between the 2 strains is the result of a 10-bp deletion at the 3′-end of the hypothetical ORF, which shifted the stop 3 codons to the 5′- end (online Technical Appendix Figure, panel B) in SeCoV/GER/L00930/2012. This deletion is apparently located within the potential 3′ recombination site (online Technical Appendix Figure, panel B).

It is tempting to speculate that SeCoV/Italy/213306/2009 is a precursor of SeCoV/GER/L00930/2012, and that other members of this novel genotype are still undetected. These viruses might be targets of secondary mutation and recombination events. Therefore, more chimeric CoVs should be identified to determine the potential origin of the recombination event.

In conclusion, we detected an enteric CoV that resembled the TGEV/PEDV chimeric virus reported by Boniotti et al. (*1*). Although these findings support the notion that CoV genomes are subject to mutations and recombination events, problems in disease diagnosis can be foreseen. In countries where porcine epidemic diarrhea, transmissible gastroenteritis, or both of these diseases are reportable, correct diagnosis and reporting might be difficult. Thus, diagnosticians should be aware of possible recombinants of swine CoVs. Diagnostic problems can be prevented by use of a double-check strategy with techniques specific for different genome regions. Apart from diagnostic obstacles, the effect of virus recombinations in terms of virulence and organ tropism is unknown and needs further investigations.

References

1. Boniotti MB, Papetti A, Lavazza A, Alborali G, Sozzi E, Chiapponi C, et al. Porcine epidemic diarrhea virus and discovery of a recombinant swine enteric coronavirus, Italy. Emerg Infect Dis. 2016;22:83–7. http://dx.doi.org/10.3201/eid2201.150544
2. Hanke D, Jenckel M, Petrov A, Ritzmann M, Stadler J, Akimkin V, et al. Comparison of porcine epidemic diarrhea viruses from Germany and the United States, 2014. Emerg Infect Dis. 2015;21:493–6.

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Colistin-Resistant *mcr-1*–Positive Pathogenic *Escherichia coli* in Swine, Japan, 2007–2014

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To the Editor: Colistin is an old-generation antimicrobial agent; however, because it is one of the few agents that remain effective against multidrug-resistant gram-negative bacteria (e.g., carbapenem-resistant *Pseudomonas aeruginosa* and *Enterobacteriaceae*), its clinical usefulness is being increasingly recognized (*1*). Previous reports have described the mechanisms of colistin resistance (*2*) as being chromosomally mediated and not associated with horizontal gene transfer. However, from 2011 through 2014, a plasmid-encoded colistin-resistance gene, *mcr-1*, was identified in colistin-resistant *Escherichia coli* isolated in

China, particularly from animals. Specifically, *mcr-1*–positive isolates were found in 21% of healthy swine at slaughter, 15% of marketed pork and chicken meat, and 1% of hospitalized human patients (*3*). A study of *E. coli* isolated from healthy cattle, swine, and chickens in Japan during 2000–2014 found only 2 (0.02%) of 9,308 isolates positive for *mcr-1* (*4*). We report the rates at which *mcr-1* was detected in our stored collection of *E. coli* isolates from diseased swine (swine with diarrhea or edema disease), hereafter referred to as swine-pathogenic *E. coli*.

We recently analyzed swine-pathogenic *E. coli* strains isolated from diseased swine throughout Japan during 1991–2014 (*5*). We analyzed all swine disease-associated *E. coli* strains isolated from the 23 Livestock Hygiene Service Centers in Japan (including prefectures that covered 75% of total swine production in Japan in 2014) and sent to the National Institute of Animal Health for diagnostic purposes during 1991–2014. Among the 967 strains examined, 684 (71%) belonged to *E. coli* serogroup O139, O149, O116, or OSB9.

In the study reported here, we investigated these 684 strains for susceptibility to colistin and for *mcr-1* carriage. The strains from the 4 predominant serogroups (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/22/7/16-0234-Techapp1.pdf) can be considered representative of swine-pathogenic *E. coli* strains isolated from farm animals, but not food products, in Japan. MICs were determined by using the agar dilution method according to the recommendations of the Clinical and Laboratory Standards Institute (*6*). The presence of *mcr-1* was detected by PCR (*3*).

Among the 684 strains examined, colistin MICs exhibited a bimodal distribution of 0.25–128 μg/mL and peaked at 0.5 and 16 μg/mL (online Technical Appendix Figure). According to the European Committee on Antimicrobial Susceptibility Testing criterion (*7*), in which isolates with an MIC of ≥4 μg/mL are considered colistin resistant, 309 (45%) of the 684 strains were classified as colistin resistant. The gene *mcr-1* was detected in 90 (13%) strains, and the MICs for these *mcr-1*–positive strains ranged from 8 to 128 μg/mL (online Technical Appendix Figure). Among the 309 colistin-resistant strains, *mcr-1*–positive and *mcr-1*–negative isolates had the same 50% and 90% MICs, 16 and 32 μg/mL, respectively. These results indicate that a high proportion of swine-pathogenic *E. coli* in Japan are resistant to colistin, that *mcr-1* has already been widely disseminated among these strains, and that the level of colistin resistance mediated by *mcr-1* is similar to that mediated by *mcr-1*–independent mechanisms.

In 2004, colistin-resistant *E. coli* already represented 77% of the isolates, and the positivity rates varied from year to year (26%–82%) (Figure). First detection of *mcr-1*–positive strains was in 2007, and the proportion of *mcr-1*–positive strains has risen, especially since 2009 (Figure). During 2013–2014, approximately half of the strains isolated were *mcr-1* positive (Figure), and most colistin-resistant strains isolated during these 2 years carried *mcr-1* (85% and 62% in 2013 and 2014, respectively). Of note, the rates of *mcr-1*–positive strains among the 4 serogroups isolated from 2010 through 2014 did not differ significantly (χ^2 test): 22 (20%) of 110 in O139, 38 (38%) of 100 in O149, 19 (26%) of 73 in O116, and 6 (32%) of 19 in OSB9. This finding suggests that the sharp rise in the proportion of *mcr-1*–positive strains has been driven by plasmid-mediated horizontal gene transfer, not by the expansion of a specific clone.

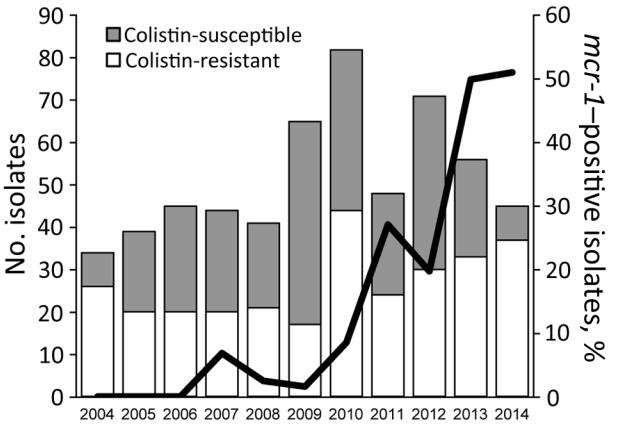

Figure. Changes in the numbers of colistin-susceptible and colistin-resistant *Escherichia coli* isolated from swine with diarrhea or edema disease, Japan, 2004–2014. The line shows the changes in proportion of *mcr-1*–positive isolates among the total isolates for each year.

In Japan, rates of isolation of colistin-resistant and *mcr-1*–positive *E. coli* strains from healthy animals are low, 1.00% and 0.02% of 9,308 strains examined, respectively (*4*). These low rates may be the result of the prudent use of colistin in Japan. During 2000–2007 in Japan, colistin use in swine did not increase significantly (*8*). However, our data show that *mcr-1* has recently been disseminated among swine-pathogenic *E. coli* in Japan, which might be associated with the use of colistin to treat disease in swine. Although *mcr-1*–positive bacteria have not yet been isolated from humans in Japan (*4*), the sharp increase in swine-pathogenic *E. coli* in animal strains implies a risk for transmission of *mcr-1* from these strains to human-pathogenic bacteria, a serious concern for human medicine. More active surveillance of *mcr-1*–positive colistin-resistant bacteria in human and animal environments is needed.

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References

1. Ruppé E, Woerther PL, Barbier F. Mechanisms of antimicrobial resistance in gram-negative bacilli. Ann Intensive Care. 2015;5:61. http://dx.doi.org/10.1186/s13613-015-0061-0
2. Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol. 2014;5:643. http://dx.doi.org/10.3389/fmicb.2014.00643
3. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis. 2016;16:161–8. http://dx.doi.org/10.1016/S1473-3099(15)00424-7
4. Suzuki S, Ohnishi M, Kawanishi M, Akiba M, Kuroda M. Investigation of a plasmid genome database for colistin-resistance gene mcr-1. Lancet Infect Dis. 2016;16:284–5. http://dx.doi.org/10.1016/S1473-3099(16)00008-6
5. Kusumoto M, Hikoda Y, Fujii Y, Murata M, Miyoshi H, Ogura Y, et al. Emergence of a multidrug-resistant Shiga toxin-producing enterotoxigenic *Escherichia coli* lineage in diseased swine in Japan. J Clin Microbiol. 2016;54:1074–81. http://dx.doi.org/10.1128/JCM.03141-15
6. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: 24th informational supplement. Document M100–S24. Wayne (PA): The Institute; 2014.
7. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 60 [cited 2016 Feb 6]. http://www.eucast.org/clinical_breakpoints/
8. Koike R, Asai T, Ozawa M, Ishikawa H. The use of therapeutic antimicrobials for food-producing animals [in Japanese]. Ann Rep Natl Vet Assay Lab. 2009;45:30–3. Summary in English [cited 2016 Apr 6]. http://www.maff.go.jp/nval/kouhou/nenpo/no45/pdf/no45-040-043-p030-033-gaku-gi-3.pdf

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Yellow Fever in a Worker Returning to China from Angola, March 2016

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To the Editor: Yellow fever is disease caused by a flavivirus that is transmitted to humans and nonhuman primates through the bites of infected mosquitoes. In 2013, an estimated 130,000 persons in Africa experienced fever with jaundice or hemorrhage associated with yellow fever; ≈78,000 of these infections were fatal (*1*).

Recently, an outbreak of yellow fever was reported in Angola (*2*). This serious viral disease affects persons living in and visiting tropical regions of Africa and Central and South America (*3*). No case of yellow fever had been confirmed in China until this year (*3*). With the increased population movement between Africa and China, the risk for yellow fever in China is increasing.

In March 2016, a 34-year-old man who had recently returned to China from Angola sought medical treatment at the Shanghai Public Health Clinical Center in Shanghai, China. He reported a 4-day history of malaise, myalgia, weakness, nausea, vomiting, and fever reaching 38.8°C. The patient had been treated with several antimicrobial drugs when he was in Angola, but symptoms did not resolve. He had no history of immunodeficiency or immune-inhibitory drug use. No endocrine, metabolic, or autoimmune abnormalities were found.

Nine years earlier, the patient had undergone cardiac valve replacement for rheumatoid heart disease and was currently receiving warfarin therapy. Because his treating physicians were concerned about the potential effect of yellow fever vaccine on the patient's international normalized ratio (ratio of reference to measured prothrombin times), the patient traveled to Africa for work without receiving vaccination for yellow fever.

Physical examination revealed a temperature of 37°C. Neither rash nor jaundice were evident. Blood examination revealed a low leukocyte count (1.66×10^9 cells/L [reference range $3.50–9.50 \times 10^9$ cells/L]), a low absolute lymphocyte count (0.92×10^9 cells/L [$1.1–3.2 \times 10^9$ cells/L), a normal erythrocyte count (4.60×10^{12} cells/L [$4.30–5.80 \times 10^{12}$ cells/L]), and a low platelet count (43×10^9 platelets/L [$125–350 \times 10^9$ platelets/L). The patient had low levels of circulating CD3+ cells (540/mL [690–2,540/mL) and CD8+ cells (97/mL [190–1,140/mL]) and normal levels of CD4+ T-cells.

C-reactive protein level was 4.31 mg/L (reference range 0–3.0 mg/L), lactate dehydrogenase was 1,086 U/L (109–245 U/L), alanine aminotransferase was 882 U/L (7–40 U/L), total bilirubin was 13.5 μmol/L (0–17 μmol/L), and direct bilirubin was 7.4 μmol/L (0–5.4 μmol/L). The patient had normal levels of thyroid-stimulating hormone, and no DNA, nuclear, or thyroglobulin antibodies were detected.

Test results for HIV, malaria, and dengue virus infection were negative. Serum and urine samples were positive for yellow fever virus and negative for dengue and

Zika viruses by PCR. These results were confirmed by the Shanghai Center for Disease Control and Prevention and the China Center for Disease Control and Prevention. Yellow fever virus RNA remained detectable 9 days after symptom onset in serum and for an additional 3 days in urine and feces.

A person from China traveling to a yellow fever–endemic area would usually receive vaccination against yellow fever (*4*). Persons such as our patient, who cannot or should not receive vaccination for yellow fever, should be monitored closely. As of April 2, 2016, a total of 9 imported cases of yellow fever were reported in China: 4 cases in Fujian Province, 4 cases in Beijing, and 1 case in Shanghai. All 9 cases occurred in travelers returning to China from Angola; no local cases have been reported.

The mosquito density is low in Shanghai, and the temperature typically is low in March, suggesting that the imported case we describe will probably not result in mosquito-borne transmission. However, in the upcoming summer, the risk for onward transmission of travel-associated yellow fever in China will warrant increased vigilance. To help prevent the importation and potential spread of yellow fever in China, the Chinese government now requests proof of vaccination for yellow fever from persons traveling to China from Angola.

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References

1. Garske T, Van Kerkhove MD, Yactayo S, Ronveaux O, Lewis RF, Staples JE, et al. Yellow fever in Africa: estimating the burden of disease and impact of mass vaccination from outbreak and serological data. PLoS Med. 2014;11:e1001638. http://dx.doi.org/10.1371/journal.pmed.1001638
2. National Health and Family Planning Commission of China. China confirms an imported yellow fever case [in Chinese] [cited 2016 Mar 18]. http://www.nhfpc.gov.cn/yjb/s7860/201603/67202ceff58b44e3ba63db1e2bbfc1ab.shtml
3. World Health Organization. Yellow fever—Angola [cited 2016 Mar 18]. http://who.int/csr/don/12-february-2016-yellow-fever-angola/en
4. Centers for Disease Control and Prevention. Yellow fever vaccine recommendations [cited 2016 Mar 18]. http://www.cdc.gov/yellowfever/vaccine

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Clinical Manifestations of Zika Virus Infection, Rio de Janeiro, Brazil, 2015

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To the Editor: Zika virus infection, which has been associated with microcephaly and other neurologic disorders, has reached the level of public health emergency of international concern (*1*). Zika virus (family *Flaviviridae*, genus *Flavivirus*) is transmitted by mosquitos of the genus *Aedes* (*2*). The virus was first isolated from a serum specimen from a rhesus monkey in the Zika Forest of Uganda in 1947 (*3*). After 2007, a rapid geographic expansion of the virus was observed, including outbreaks in the Pacific region (*4*) and, more recently, in South America. Brazil reported the first autochthonous case of Zika virus disease in April 2015 (*5*), and subsequently, increasing numbers of cases have been reported, especially in northeastern Brazil (*6*).

Studies on the natural history of Zika virus infection are scarce. Previous research defined Zika virus infection as a dengue-like illness, typically characterized by fever, maculopapular rash, arthralgia, and conjunctivitis (*4*). Although some patients have all of these symptoms during early onset, fever is not an early symptom for all. Here we describe the frequency of signs and symptoms from a sample of clinic patients in Rio de Janeiro, Brazil, who were later confirmed to have Zika virus disease by using real-time reverse transcription PCR (rRT-PCR).

We retrospectively collected clinical data on a convenience sample of 57 patients found to be Zika virus–positive by rRT-PCR who had medical attention at the 24-hour acute care clinic of Manguinhos in Rio de Janeiro during April 28–June 8, 2015. Data were collected from electronic medical records and surveillance reports. Data were anonymized and included age, sex, and signs and symptoms documented on the first clinic visit of patients who reported acute rash, dengue-like illness, or both. Fever

was documented either through direct measurement in the clinic or by patient self-report. Pregnancy status was not assessed. We collected blood samples for serum sample testing during each patient's initial visit to the clinic and tested for Zika virus using rRT-PCR as described by Lanciotti et al. (*7*); all samples were collected within 7 days of illness onset. Patients were not tested for dengue or chikungunya viruses. We did not measure the duration of any sign or symptom.

Of the 57 Zika virus disease case-patients, median age was 34 years; 63% were women (Table). The most common sign or symptom was exanthema (98%), followed by headache (67%), fever (67%), arthralgias (58%), myalgias (49%), and joint swelling (23%) (Table). Conjunctivitis was observed in 39% case-patients and retro-orbital eye pain was reported by 40%. Among 30 patients who had fever assessed by clinic staff, median temperature was 38°C (range 37.5°C –38.5°C). One patient had no rash or joint swelling but did have all other symptoms. One patient's sole symptom was rash. No patients were referred for hospitalization.

Our clinic-based study of 57 rRT-PCR–confirmed cases of Zika virus disease found rash to be the most common symptom for which patients sought care (98%); fever, generally low-grade, was reported or observed in 67%. Because our study design was retrospective in nature, wherein we reviewed records for selected patients in whom Zika was subsequently found to be laboratory-confirmed by using rRT-PCR, we may have introduced selection bias to our sample, limiting the generalizability and comparability of our results. For example, clinic staff may have seen patients with mild symptoms but decided not to test for the virus, leading to a bias toward testing patients with more severe rash. It is also possible, considering the retrospective nature of our data collection, that some data points were not accurately recorded and could not be validated. Despite these limitations, our data suggest the term "Zika fever" is not a helpful substitute term for Zika virus disease. Furthermore, referring to the illness caused by this virus as "Zika fever" (*8*) may be misleading and should probably be avoided until further more systematic studies clarify the frequency of fever as a symptom.

Although patient sampling and laboratory testing methods are not directly comparable to our study, a 2015–2016 assessment in Puerto Rico detected Zika virus in 30 of 155 case-patients in whom Zika virus disease was suspected. In that study, laboratory-confirmed disease was defined as detection of Zika virus RNA by using rRT-PCR or IgM by using ELISA. Among the 30 confirmed cases, the most frequently reported signs and symptoms were rash (77%), myalgia (77%), arthralgia (73%), and fever (73%) (*9*). The February 12, 2015, interim case definition published by the World Health Organization describes a suspected case-patient as a person with rash, fever, or both, in addition to 1 of 3 other listed symptoms (*10*). Like the Puerto Rico report, our report supports the established World Health Organization case definition indicating that the presence of rash, fever, or both should be emphasized as primary characteristics of Zika virus disease.

Table. Characteristics of Zika virus disease patients seeking care in an acute care clinic, Rio de Janeiro, Brazil, April 28–June 8, 2015

Characteristic	Value*
Cohort, no. patients	57
Age, y	34 (25–40)
Female sex	36 (63)
Symptoms	
Exanthema	56 (98)
Fever†	38 (67)
Days from symptom onset to exanthema	1 (0–2)
Arthralgia	33 (58)
Itching	32 (56)
Headache	38 (67)
Myalgia	28 (49)
Retro-orbital pain	23 (40)
Conjunctivitis	22 (39)
Joint swelling	13 (23)

*Median (interquartile range) or no. (%) case-patients.
†Measured in medical office (n = 30) or self-reported (n = 8).

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References

1. World Health Organization. Zika situation report. 2016 March 31 [cited 2016 Apr 1]. http://www.who.int/emergencies/zika-virus/situation-report/31-march-2016/en/
2. Grard G, Caron M, Mombo IM, Nkoghe D, Mboui Ondo S, Jiolle D, et al. Zika virus in Gabon (Central Africa)—2007: a new threat from Aedes albopictus? Charrel R, editor. PLoS Negl Trop Dis. 2014;8(2):e2681. http://dx.doi.org/10.1371/journal.pntd.0002681
3. Dick GWA, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. Trans R Soc Trop Med Hyg. 1952;46:509–20. http://dx.doi.org/10.1016/0035-9203(52)90042-4
4. Duffy MR, Chen T-H, Hancock WT, Powers AM, Kool JL, Lanciotti RS, et al. Zika virus outbreak on Yap Island, Federated States of Micronesia. N Engl J Med. 2009;360:2536–43. http://dx.doi.org/10.1056/NEJMoa0805715
5. Zanluca C, de Melo VCA, Mosimann ALP, dos Santos GIV, dos Santos CND, Luz K. First report of autochthonous transmission of Zika virus in Brazil. Mem Inst Oswaldo Cruz. 2015;110:569–72. http://dx.doi.org/10.1590/0074-02760150192
6. Campos GS, Bandeira AC, Sardi SI. Zika virus outbreak, Bahia, Brazil. Emerg Infect Dis. 2015;21:1885–6. http://dx.doi.org/10.3201/eid2110.150847
7. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis. 2008;14:1232–9. http://dx.doi.org/10.3201/eid1408.080287

8. Martínez de Salazar P, Suy A, Sánchez-Montalvá A, Rodó C, Salvador F, Molina I. Zika fever. Enferm Infecc Microbiol Clin. In press 2016. http://dx.doi.org/10.1016/j.eimc.2016.02.016
9. Thomas DL, Sharp TM, Torres J, Armstrong PA, Munoz-Jordan J, Ryff KR, et al. Local transmission of Zika virus—Puerto Rico, November 23, 2015–January 28, 2016. MMWR Morb Mortal Wkly Rep. 2016;65:154–8. http://dx.doi.org/10.15585/mmwr.mm6506e2
10. World Health Organization. Zika virus disease. 2015 Feb 12 [cited 2016 Mar 10]. http://www.who.int/csr/disease/zika/case-definition/en/

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Zika Virus–Related News Coverage and Online Behavior, United States, Guatemala, and Brazil

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To the Editor: News coverage of emerging infectious diseases tends to be episodic and ephemeral rather than thematic, comprehensive, and consistent over time, in part because of newsroom constraints (*1–3*). Public health authority announcements may help drive peaks in coverage and warrant attention, in particular given the importance of trust and credibility for information acceptance (*4,5*). Moreover, online search behavior and social media interaction tend to respond to news coverage, especially for novel health issues (*6,7*).

The nature of Zika virus transmission as a novel phenomenon not completely understood by researchers could encourage anxiety and fear among the public (*8,9*). Patterns of social interaction and search behavior regarding Zika virus can point to opportunities and constraints for education efforts.

To assess relationships between news coverage, social media mentions, and online search behavior regarding Zika virus, we studied data available for January 1–February 29, 2016. Although news outlets occasionally covered Zika virus before 2016, our selected period included prominent announcements. For example, on January 28, the World Health Organization declared that Zika virus was "spreading explosively" (*10*), and the Centers for Disease Control and Prevention issued a travel alert. On February 3, authorities reported the first case that appeared in the United States.

Across 3 data sources, we searched for mentions of "Zika" or "El Zika." We used Google Trends (Google Inc., Mountain View, CA, USA) to assess the number of total searches that originated in the United States, Guatemala, or Brazil for these terms, relative to total Google searches for any topic for the same period. We used a scale of 0–100 (as an indicator of relative volume), with 50 representing half the volume as 100 but not a specific absolute number. Zika virus has been detected in >25 countries since 2015; the countries selected were places where transmission has been relatively widespread or where Zika virus had not yet been but was anticipated to be. We used a monitoring tool, Crimson Hexagon (http://www.crimsonhexagon.com/), to capture the total number of daily Twitter posts (tweets) and focused on tweets geotagged as originating from the United

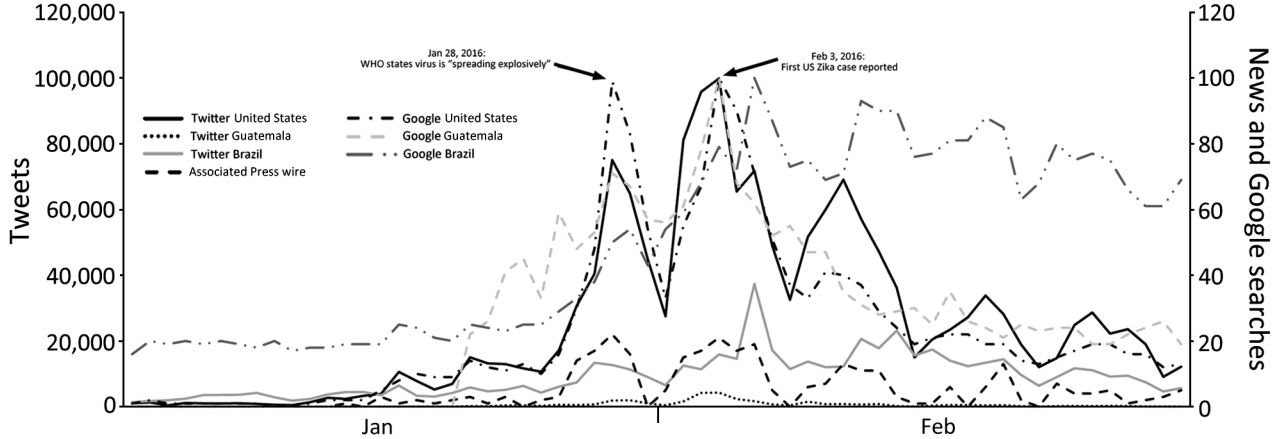

Figure. Comparison of number of tweets by individual persons, Google searches by individual persons, and Associated Press news stories about Zika virus in the United States, Guatemala, and Brazil, January 1–February 29, 2016.

States, Guatemala, or Brazil. Last, we counted Associated Press news wire stories as a proxy for daily volume of Zika news coverage in the Western Hemisphere (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/7/16-0415-Techapp1.pdf).

Using a day as our unit of analysis (i.e., n = 60 in the analysis), we first assessed Pearson product-moment correlations between news coverage, social media mentions, and online search behavior and then fit a time series model. Results suggested prominent but ephemeral peaks in salience and attention, with some variation over time in searches by country (Figure). We found strong positive correlations between news (daily volume) and tweets for all 3 countries (United States, $r = 0.86$, $p<0.001$; Guatemala, $r = 0.78$, $p<0.001$; Brazil, $r = 0.60$, $p<0.001$). We also found strong positive correlations between news and Google searches for all 3 countries (United States, $r = 0.86$, $p<0.001$; Guatemala, $r = 0.74$, $p<0.001$; Brazil, $r = 0.48$, $p<0.001$). Because time series data can reflect autocorrelation that makes observed relationships spurious, interpretation of bivariate correlations alone to link time series data is inadvisable. To assess the relationship between news coverage and online searching related to Zika virus, we used time series analysis to predict US Google searches as a function of other observed trends and date. We fit an autoregressive integrated moving average (0, 1, 3) model to address dependence between residuals, resulting in a Ljung-Box statistic that was not significant ($p>0.05$). This finding indicated that we sufficiently reduced the time series to white noise to assume no autocorrelation in residuals. Our model achieved an R^2 value of 0.90 and stationary R^2 value of 0.53. Associated Press wire stories emerged as a significant and positive predictor (coefficient = 1.52, $t = 3.24$, $p<0.01$). No other predictor predicted variance greater than that of news stories ($p>0.05$). Daily news story volume predicted departures from the expected trend in US search behavior related to Zika virus.

Our results suggest that news coverage of public health authority announcements opens brief windows of information sharing, engagement, and searching that offer opportunities to address perceptions and provide preparation and vector control recommendations through education. Sharing and searching are less apparent outside these windows, especially in contexts in which an emerging infectious disease is not yet prevalent. Our findings may not generalize beyond the initial stages of Zika virus transmission in the United States, and future work could obtain appropriate data for investigating the tone of news coverage and online communication in various countries. Nevertheless, recent trends in online information-seeking about Zika virus has been sensitive to official announcements, suggesting the usefulness of pairing announcements with provision of information resources that can be found through search engines.

References

1. Dudo AD, Dahlstrom MF, Brossard D. Reporting a potential pandemic: a risk-related assessment of avian influenza coverage in U.S. newspapers. Sci Commun. 2007;28:429–54 http://dx.doi.org/10.1177/1075547007302211.
2. Southwell BG, Reynolds BJ, Fowlie K. Communication, media relations and infectious disease surveillance. In: M'ikanatha N, de Valk H, Lynfield R, Van Benden C, editors. Infectious disease surveillance. 2nd ed. Oxford: John Wiley & Sons; 2013. p. 607–17.
3. Southwell BG, Hwang Y, Torres A. Avian influenza and US TV news. Emerg Infect Dis. 2006;12:1797–8. http://dx.doi.org/10.3201/eid1211.060672
4. Kelly B, Squiers L, Bann C, Stine A, Hansen H, Lynch M. Perceptions and plans for prevention of Ebola: results from a national survey. BMC Public Health. 2015;15:1136. http://dx.doi.org/10.1186/s12889-015-2441-7
5. Prue C, Southwell B, Williams P, Alexander J, Zulkiewicz B. Threat, trust, and tools for action: what matters in helping travelers from countries with Ebola outbreaks to follow government monitoring and reporting guidelines? Presented at: National Conference on Health Communication, Marketing, and Media; 2015 Aug 11–13; Atlanta, Georgia, USA.
6. Weeks BE, Friedenberg LM, Southwell BG, Slater JS. Behavioral consequences of conflict-oriented health news coverage: the 2009 mammography guideline controversy and online information seeking. Health Commun. 2012;27:158–66. http://dx.doi.org/10.1080/10410236.2011.571757
7. Towers S, Afzal S, Bernal G, Bliss N, Brown S, Espinoza B, et al. Mass media and the contagion of fear: the case of Ebola in America. PLoS One. 2015;10:e0129179. http://dx.doi.org/10.1371/journal.pone.0129179
8. Rowan KE. Why some health risks upset us and others do not: risk perception and risk communication. Spectra. 2015;51:13–7.
9. Southwell B. Risk communication: coping with imperfection. Minnesota Medicine. 2003;86:14–6.
10. Tavernise S. W.H.O. sounds a global alarm over Zika Virus. New York Times. 2016 Jan 29 [cited 2016 Mar 1]. http://www.nytimes.com/images/2016/01/29/nytfrontpage/scan.pdf

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Detection and Genomic Characterization of Senecavirus A, Ohio, USA, 2015

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To the Editor: Senecavirus A (SVA), formerly Seneca Valley virus, is a single-stranded positive-sense, nonenveloped RNA virus (*1*). The RNA genome of SVA is 7.2 kb long and is translated into a polyprotein in a host

cell. The polyprotein is then posttranslationally cleaved into mature proteins, including 4 structural viral capsid proteins (VP 1–4) in the N terminus and 7 nonstructural proteins (2A, 2B, 2C, 3A, 3B, $3C^{pro}$, and $3D^{pol}$) in the C terminus (*1*). SVA was discovered as a contaminant of PER.C6 cells and is closely related to viruses in the genus *Cardiovirus* (*1*). Genomic characterization has led to classification of SVA in a new genus, *Senecavirus*, family *Picornaviridae*. A retrospective study conducted in the United States showed that the samples collected during 1988–2001 were SVA positive, and genetic analysis revealed that the sequences of all 7 SVA isolates are considerably similar to the first US SVA strain (SVV-001), suggesting that SVA may have been circulating in the US pig population for a long time (*2*).

Idiopathic vesicular disease (IVD) is a vesicular disease of pigs, and etiology is unknown (*3*). The clinical signs of IVD are fever, lameness, and vesicular lesions on various body parts including the oral cavity, snout, and coronary bands (*3*). Despite not being a debilitating disease, IVD is noteworthy because it causes lesions clinically indistinguishable from those of other vesicular animal diseases, including foot-and-mouth disease (FMD), vesicular stomatitis, swine vesicular disease, and vesicular exanthema of swine. IVD has been reported in several countries, including the United States (*4*–*7*), and has been recognized in several US states, including Florida, Indiana, and Iowa (*4*,*8*,*9*). Several lines of evidence show that SVA may be associated with IVD outbreaks in Canada, the United States, and Brazil (*3*,*7*,*10*). We describe the detection and genomic characterization of SVA isolated from pigs with vesicular lesions in Ohio.

In October 2015, the Animal Disease Diagnostic Laboratory of the Ohio Department of Agriculture received vesicle tissue, a vesicle swab sample, and whole blood from a sow with vesicular disease for rule-out testing for FMD virus (FMDV). The sow was lame on both front feet and had ruptured vesicular lesions on the snout and coronary bands of both front feet (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/22/7/15-1897-Techapp1.pdf). FMDV-specific real-time reverse transcription PCR was applied to the nucleic acid samples extracted from the 3 samples by using a MagMAX Pathogen RNA/DNA kit (Life Technologies, Carlsbad, CA, USA). All samples were negative for FMDV. We then performed 2 conventional reverse transcription PCRs with primers targeting 2 regions of the SVA genome (VP3/VP1, 3D/3′ untranslated region) on the same set of samples; the vesicle tissue and swab samples were SVA positive. Subsequently, we determined the whole-genome sequence of SVA by using 7 pairs of SVA-specific primers (online Technical Appendix Table 1).

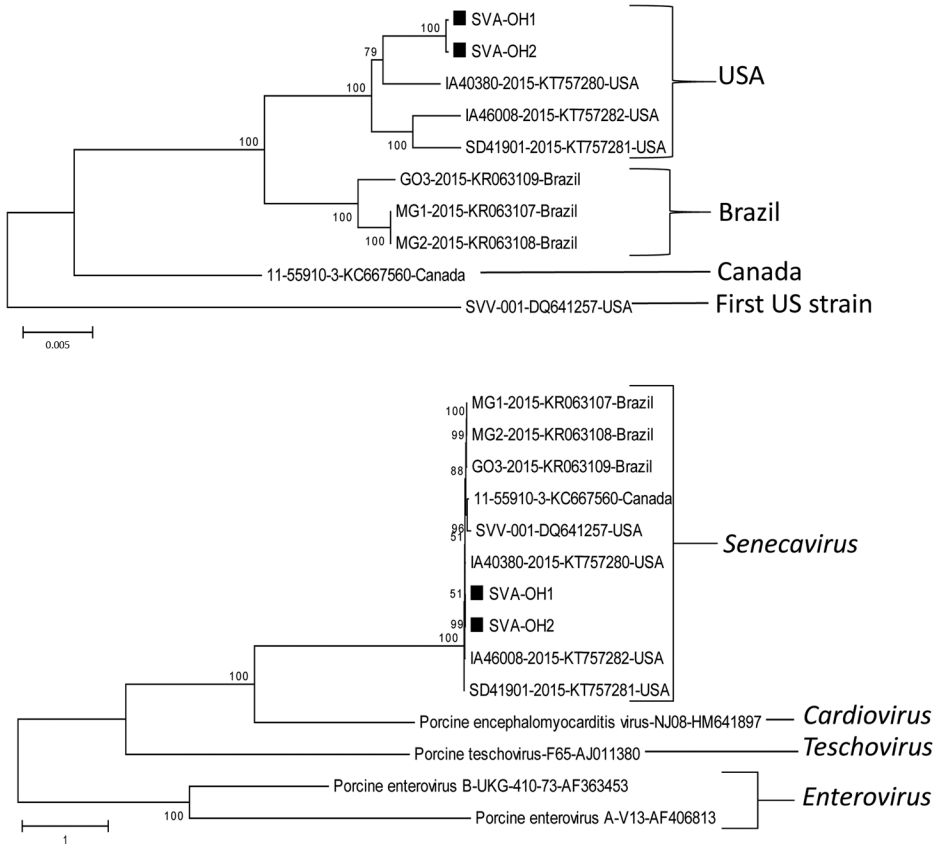

Figure. Phylogenetic trees constructed on the basis of the whole-genome sequences of isolates from the genera *Senecavirus* (SVA), *Cardiovirus*, *Teschovirus*, and *Enterovirus* of the family *Picornaviridae*, including the SVA-OH1 and -OH2 isolates (black squares) from pigs in Ohio, USA. Dendrograms were constructed by using the neighbor-joining method in MEGA version 6.05 (http://www.megasoftware.net). Bootstrap resampling (1,000 replications) was performed, and bootstrap values are indicated for each node. Reference sequences obtained from GenBank are indicated by strain name and accession number. Scale bars indicate nucleotide substitutions per site.

We completed sequencing the whole genomes for the vesicle tissue (SVA-OH1) and vesicle swab sample (SVA-OH2). On the basis of BLAST (http:blast.ncbi.nlm.nih.gov/Blast.cgi) searches, the SVA-OH1 and -OH2 isolates had 99% nt identity to 3 new US strains (USA/IA40380/2015, USA/SD41901/2015, USA/IA46008/2015) and 98% nt identity to 3 Brazil strains (SVV/BRA/MG1/2015, SVV/BRA/MG2/2015, SVV/BRA/GO3/2015) from GenBank. The Ohio isolates also shared 96% and 94% nt identity with Canada strain (11-55910-3) and the first US SVA strain (SVV-001), respectively. Further analysis showed that, in comparison with these 8 strains with complete genome sequences available in GenBank, the 2 Ohio SVA isolates had 22 unique nucleotide mutations in the genome: 1 in the VP4 gene, 5 in VP2, 2 in VP3, 1 in VP1, 4 in 2B, 3 in 2C, 3 in 3A, 1 in 3B, and 2 in 3D (online Technical Appendix Table 2). Among the 22 unique mutations, there were 2 nonsynonymous mutations at position 2082 in the VP3 gene of both isolates and position 5037 in the 3A gene of SVA-OH1 and 1 unique synonymous mutation only in SVA-OH2.

Phylogenetic analysis of the complete genome further supports that the 2 Ohio SVA isolates are closely related to each other and clustered together with the 3 recently isolated US strains, were less closely related to the isolates of the Brazil cluster, and were more distantly related to the isolate from Canada and the original SVA strain reported from United States (Figure). Consistent with the previous findings (*1*), all SVA isolates from different countries clustered together under the genus *Senecavirus*, which is most closely related to the genus *Cardiovirus* of the family *Picornaviridae* (Figure).

Our findings that a pig with clinical signs of IVD was infected with SVA and our genetic analysis demonstrating that the 2 Ohio SVA isolates are closely related to the other SVA strains from different countries provide further support for SVA involvement in IVD in pigs. More support could be provided by future studies, including continued surveillance of SVA and confirmation of the Koch postulates.

Acknowledgments

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References

1. Hales LM, Knowles NJ, Reddy PS, Xu L, Hay C, Hallenbeck PL. Complete genome sequence analysis of Seneca Valley virus-001, a novel oncolytic picornavirus. J Gen Virol. 2008;89:1265–75. http://dx.doi.org/10.1099/vir.0.83570-0
2. Knowles NJ, Hales LM, Jones BH, Landgraf JG, House JA, Skele KL, et al. Epidemiology of Seneca Valley virus: identification and characterization of isolates from pigs in the United States. XIVth meeting of the European Study Group on the Molecular Biology of Picornaviruses; 2006 Nov 26–Dec 1; Saariselka, Inari, Finland; 2006. p. G2 [cited 2016 Apr 26]. http://www.europic.org.uk/Europic2006/europic_2006_posters.htm
3. Singh K, Corner S, Clark SG, Scherba G, Fredrickson R. Seneca Valley virus and vesicular lesions in a pig with idiopathic vesicular disease. J Vet Sci Technol. 2012;3:123.
4. Amass SF, Schneider JL, Miller CA, Shawky SA, Stevenson GW, Woodruff ME. Idiopathic vesicular disease in a swine herd in Indiana. Journal of Swine Health and Production. 2004;12:192–6.
5. Montgomery JF, Oliver RE, Poole WS. A vesiculo-bullous disease in pigs resembling foot and mouth disease. I. Field cases. N Z Vet J. 1987;35:21–6. http://dx.doi.org/10.1080/00480169.1987.35369
6. Munday BL, Ryan FB. Vesicular lesions in swine—possible association with the feeding of marine products. Aust Vet J. 1982;59:193. http://dx.doi.org/10.1111/j.1751-0813.1982.tb16008.x
7. Pasma T, Davidson S, Shaw SL. Idiopathic vesicular disease in swine in Manitoba. Can Vet J. 2008;49:84–5.
8. Gibbs EPJ, Stoddard HL, Yedloutchnig RJ, House JA, Legge M. A vesicular disease of pigs in Florida of unknown etiology. Florida Veterinary Journal. 1983;12:25–7.
9. Kresse JI, Taylor WD, Stewart WW, Eernisse KA. Parvovirus infection in pigs with necrotic and vesicle-like lesions. Vet Microbiol. 1985;10:525–31. http://dx.doi.org/10.1016/0378-1135(85)90061-6
10. Vannucci FA, Linhares DC, Barcellos DE, Lam HC, Collins J, Marthaler D. Identification and complete genome of Seneca Valley virus in vesicular fluid and sera of pigs affected with idiopathic vesicular disease, Brazil. Transbound Emerg Dis. 2015;62:589–93. http://dx.doi.org/10.1111/tbed.12410

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Senecavirus A in Pigs, United States, 2015

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To the Editor: Senecavirus A (SVA) has been sporadically identified in pigs with idiopathic vesicular disease in the United States and Canada (*1*–*3*). Clinical symptoms observed include ruptured vesicles and erosions on the snout and lameness associated with broken vesicles along the coronary band. A recent report characterized SVA in pigs in Brazil with similar clinical symptoms in addition to a higher proportion of deaths than would be expected in pigs 1–4 days of age (*4*,*5*). Several outbreaks of this infection in pigs were reported in the summer of 2015 in the United States; the more severe clinical features resembled those seen in outbreaks in Brazil (*6*). Subsequent testing by PCR of 2,033 oral

fluid samples from material submitted during 441 routine diagnostic testing procedures (from 25 states) identified 5 SVA-positive cases (1%) (*7*). Besides affecting animal health, SVA infection is notable because its clinical symptoms resemble those caused by foot-and-mouth disease and vesicular stomatitis viruses. When vesicular disease is observed in US swine, mandatory reporting and testing of animals for foreign animal diseases are required.

In June 2015, we collected 25 nasal and 25 rectal swab specimens from healthy pigs at 5 pig markets in North Carolina (250 total samples), representing pigs from 5 producers per market; the pigs were commingled for <12 hours. Primary markets 1 and 2 were slaughterhouses that purchased top quality pigs. Secondary market 3 was a slaughterhouse that purchased lower quality pigs (primarily underweight or herniated pigs). Market 4 was a broker that purchased pigs for culling and resold them for slaughter. Market 5 was a culled pig slaughterhouse. At markets 1–4, animals were ≈20 weeks of age; at market 5, animals were >10 weeks of age.

We sampled the same sites a second time in August 2015. Again, we performed metagenomic sequencing on swab specimens pooled by producer (5 specimens per pool, 50 total pools per sampling) (*8*). Reads most similar to SVA were identified in numerous pools from samplings and at 4 different markets. Quantitative reverse transcription PCR (qRT-PCR) was performed at the Kansas State Veterinary Diagnostic Laboratory (Manhattan, KS, USA) on the original pooled samples and was positive for SVA (cycle threshold [C_t] <37) for 26 (52%) pools from June and 18 (36%) pools from August. Sites 2 (n = 1 pool positive), 3 (n = 10), 4 (n = 5), and 5 (n = 10) had positive results in June, and sites 3 (n = 10), 4 (n = 1) and 5 (n = 7) had positive results in August. Both specimen types had an approximately equal number of positive results. We carried out virus isolation on swine testicle cells (positive samples from the second sampling), and 100% cytopathic effects were observed for 5 samples that tested positive for SVA by qRT-PCR with C_t values 16–21.

Templated assembly of the metagenomic sequencing reads with the SVA prototype strain SVV-001 genome (GenBank accession no. DQ641257) yielded near complete genomes from 5 pools (GenBank nos. KT827249–KT827253). The polyprotein-encoding region of the genomes showed >99% pairwise identity to each other and were most similar to sequences determined from recent outbreaks in Brazil (97%–98% nucleotide and >99% amino acid identity). Analysis of the P1 region of the genome found >99% nucleotide identity between 2015 US SVA sequences and 97% identity to SVA from Brazil. The contemporary US SVA sequences were more distantly related to SVA from an outbreak in Canada in 2011 (95% identity) and to historical US sequences (87%–92% identity). To investigate SVA phylogeny, we performed ClustalW (http://www.genome.jp/tools/clustalw/) alignment of P1 nucleotide sequences, followed by maximum-likelihood analysis using the best-fitting Kimura 2-parameter plus gamma distribution model of evolution. The 2015 US SVA sequences were most closely related to SVA sequences from Brazil; these sequences shared a common ancestor in Canada/11-55910-2011 (Figure).

Our results suggest that SVA commonly circulates in secondary and culled swine markets in North Carolina and

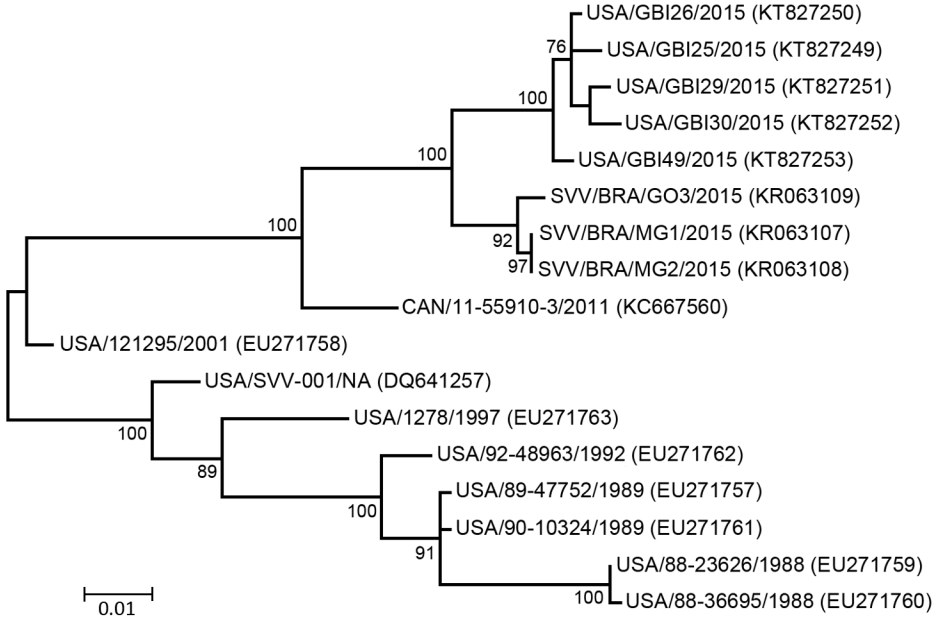

Figure. Phylogenetic tree of Senecavirus A P1 sequences. Maximum-likelihood analysis in combination with 1,000 bootstrap replicates as implemented in MEGA 6.06 (http://www.megasoftware.net) was used to derive the tree on the basis of nucleotide sequences. GenBank accession numbers are shown in parentheses. SVV in some isolate names indicates Seneca Valley virus, the original name for Senecavirus A. Scale bar indicates number of nucleotide changes per site.

that these strains are most similar to strains characterized in 2014–2015 in Brazil, which were associated with idiopathic vesicular disease and neonatal death. Little diagnostic testing is performed on culled animals, which may in part explain the discrepancy between 1% of oral fluids submitted for diagnostic testing being positive for SVA (*7*), compared with 72% of culled swine swab specimen pools in this study . The sole sample from primary markets that was positive for SVA by qRT-PCR had a C_t of 36.9, just below the negative cutoff of 37.

Further research is needed to address possible correlation between SVA and health status of animals sold at lower value to cull markets. A notable distinction between contemporary SVA in the United States and Brazil, however, is that all the US samples originated from healthy animals that showed no clinical symptoms. Given the high genetic similarity between contemporary US SVA sequences and those from Brazil, additional cofactors likely affect clinical disease.

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References

1. Singh K, Corner S, Clark SG, Scherba G, Fredrickson R. Seneca valley virus and vesicular lesions in a pig with idiopathic vesicular disease. J Vet Sci Technol. 2012;3:123 [2015 Sep 2015]. http://www.omicsonline.org/seneca-valley-virus-and-vesicular-lesions-in-a-pig-with-idiopathic-vesicular-disease-2157-7579.1000123.php?aid=9061
2. Pasma T, Davidson S, Shaw SL. Idiopathic vesicular disease in swine in Manitoba. Can Vet J. 2008;49:84–5.
3. Hales LM, Knowles NJ, Reddy PS, Xu L, Hay C, Hallenbeck PL. Complete genome sequence analysis of Seneca valley virus-001, a novel oncolytic picornavirus. J Gen Virol. 2008;89:1265–75. http://dx.doi.org/10.1099/vir.0.83570-0
4. Vannucci FA, Linhares DCL, Barcellos DESN, Lam HC, Collins J, Marthaler D. Identification and complete genome of Seneca valley virus in vesicular fluid and sera of pigs affected with idiopathic vesicular disease, Brazil. Transbound Emerg Dis. 2015;62:589–93. http://dx.doi.org/10.1111/tbed.12410
5. Leme RA, Zotti E, Alcântara BK, Oliveira MV, Freitas LA, Alfieri AF, et al. Senecavirus A: an emerging vesicular infection in Brazilian pig herds. Transbound Emerg Dis. 2015;62:603–11. http://dx.doi.org/10.1111/tbed.12430
6. American Association of Swine Veterinarians. Senecavirus A (Seneca Valley virus) in swine-breeding herd cases [cited 2015 Sep 25]. https://www.aasv.org/SVV/SVA_Sow_ISU_9215.pdf
7. American Association of Swine Veterinarians. SVV oral fluids testing sheds light on distribution [cited 2015 Sep 25]. https://www.aasv.org/news/story.php?id=8361
8. Hause BM, Collin EA, Anderson J, Hesse RA, Anderson G. Bovine rhinitis viruses are common in U.S. cattle with bovine respiratory disease. PLoS ONE. 2015;10:e0121998. http://dx.doi.org/10.1371/journal.pone.0121998

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Novel Senecavirus A in Swine with Vesicular Disease, United States, July 2015

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To the Editor: Senecavirus A (SVA; formerly known as Seneca Valley virus [SVV] belongs to the genus *Senecavirus*, family *Picornaviridae* (*1*,*2*). SVA was first isolated in 2001 as a contaminant of the PER.C6 cell line and designated as SVV-001 (*1*,*3*). Since its discovery, SVA has been infrequently detected in swine with idiopathic vesicular disease (IVD) (*4*–*6*), which clinically resembles foot-and-mouth disease, swine vesicular disease, vesicular exanthema of swine, and vesicular stomatitis. The virus has also been retrospectively detected in previous cases with various clinical conditions in the United States during 1988–2001 (*7*). However, the clinical significance of SVA in swine could not be determined (*7*,*8*).

In late July 2015, the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) received reports of 4 apparently unrelated cases of IVD affecting exhibition and commercial swine. The first 3 cases originated from unrelated farms located in southwest and central Iowa and were observed at 2 county fair exhibitions. The fourth case was observed in a commercial finisher farm in South Dakota. Affected animals exhibited acute lameness, anorexia, lethargy, and transient fever without associated mortality; they also exhibited coronary band hyperemia and vesicles, which occasionally progressed to cutaneous ulcers, as previously reported (*5*,*6*). Small vesicles were also evident on the snout, within the oral cavity, or both; these vesicles variably progressed to ulceration. No specific microscopic lesions beyond the ulcerative changes were present in specimens submitted to ISUVDL.

We collected vesicular lesion swab specimens and blood samples from all affected animals, and all tested negative for the viruses causing vesicular diseases mentioned previously (foot-and-mouth disease, swine vesicular disease, vesicular exanthema of swine, and vesicular stomatitis). No other common swine pathogens except

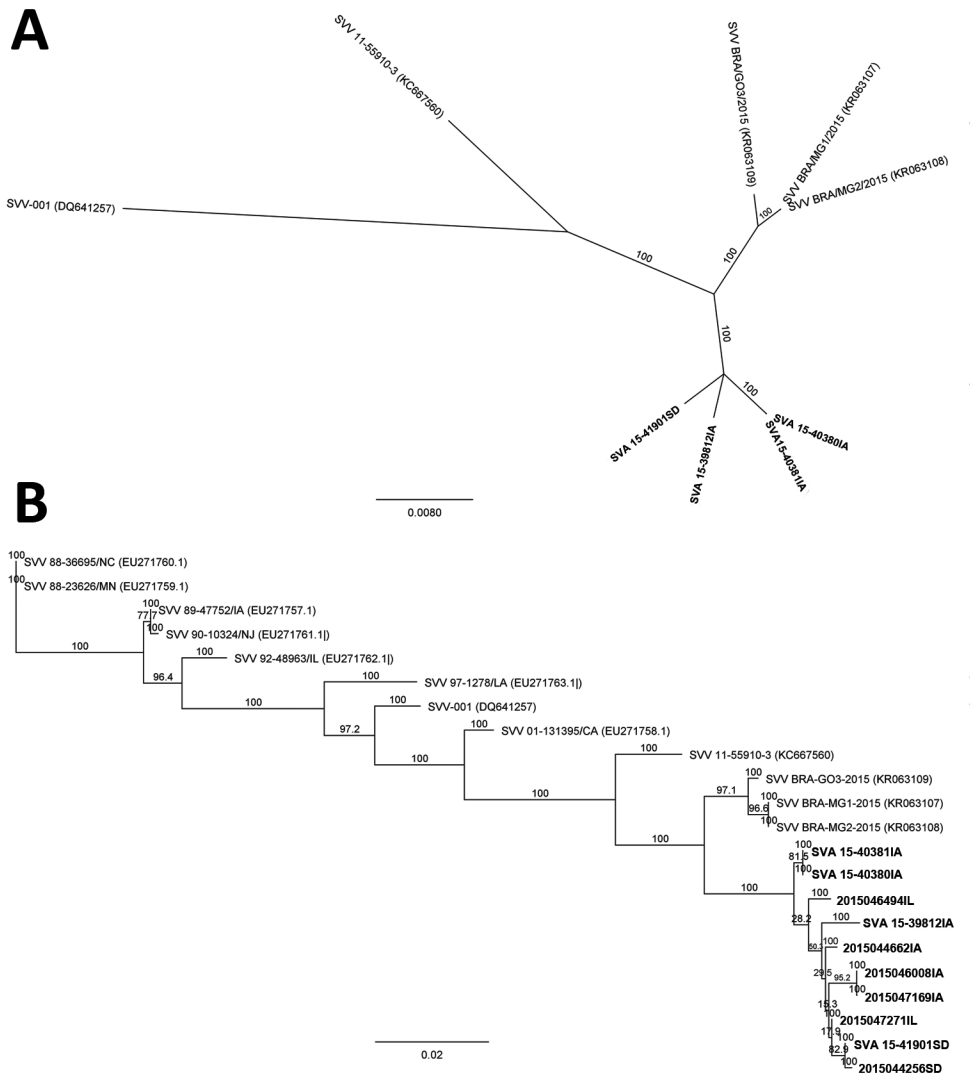

Figure. Phylogenetic relationships of 2015 US Senecavirus A (SVA) isolates (SVA15-39812IA, SVA15-40380IA, SVA15-40381IA, and SVA15-41901SD) with the prototype SVA isolate (SVV-001), a 2011 Canada swine SVA isolate (11-55910-3), and 2015 Brazil swine SVA isolates (SVV-BRA-G03-2015, SVV-BRA-MG1-2015, and SVV-BRA-MG2-2015). A) Full-length genomic sequences of 4 isolates from Iowa and South Dakota (bold) compared with reference isolates. B) Viral protein 1 sequences of 4 isolates from Iowa and South Dakota and 6 additional sequences from Iowa, Illinois, and South Dakota (2015044256SD, 2015044662IA, 2015046008IA, 2015046494IL, 2015047169IA, and 2015047271IL) (bold) compared with reference isolates. Trees were determined by using the neighbor-joining method with 1,000 bootstrap replicates. GenBank accession numbers for reference isolates are provided in parentheses. Scale bars indicate nucleotide substitutions per site.

SVA were detected at ISUVDL. By using a quantitative real-time reverse transcription PCR assay, we targeted a conserved region between the 5′ untranslated region and protein L (602–710 bp) and detected SVA RNA in vesicular fluids, epithelial scrapings of the snout, coronary band lesions, and/or hoof lesions with quantities ranging from 2×10^7 to 1.2×10^{11} genomic copies/mL. We also identified the virus in serum and fecal samples, indicating SVA viremia and shedding. In a follow-up submission from the South Dakota premise, we detected SVA in nearly all of the tissues tested; inguinal lymph nodes and tonsils contained the highest SVA loads. Seroconversion to SVA in all affected swine was evident by indirect fluorescent antibody test titers ranging from 1:160 to 1:1,280 at 2–3 weeks after the clinical outbreak.

Our attempts to isolate the virus by using ST cells (ATCC CRL-1746; ATCC, Manassas, VA, USA) and NCI-H1299 (ATCC CRL-5803) (*8*) yielded cytopathic SVA isolates with titers up to 1×10^9 PFU/mL from multiple vesicular lesion swabs or scrapings. We designated a representative isolate from each Iowa case as SVA15-39812IA, SVA15-40380IA, and SVA15-40381IA and the South Dakota case as SVA15-41901SD. Sequencing of viral protein (VP) 1 as previously described (*7*) demonstrated that each SVA isolate had a VP1 sequence identical to that of the virus in clinical specimens.

We obtained almost full-length genomic sequences (7,116–7,221 nt) of the 4 SVA isolates by using next-generation sequencing technology (*9*) and through de novo assembly (GenBank accession nos. KU051391–4). Sequence alignments showed that the isolates shared 98.9%–100% nucleotide identity with each other but diverged by 2.1%–2.2% from SVA isolate SVV-BRA-MG1-2015 (GenBank accession no. KR063107.1), by 3.9%–4.0% from SVA isolate 11-55910-3 (accession no. KC667560.1), and by 6.1%–6.4% from SVA isolate SVV-001

(accession no. DQ641257.1). Phylogenetically, the new US SVA isolates formed their own clade separated from all other SVA isolates (Figure, panel A). Such a branching out remained even when VP1 sequences, which are typically used for picornavirus phylogenetic analyses, were compared (Figure, panel B). All 4 isolates, along with VP1 sequences of SVA from 6 additional submissions from commercial farms in Iowa, Illinois, and South Dakota (2015044256SD, 2015046008IA, 2015046494IL, 2015047169IA, and 2015047271IL) and the Iowa State Fair (2015044662IA), were clustered (98.7%–100% identity) and separated from recent SVA isolates from Brazil (*6*) with 97.8%–98.0% identity. The viruses were further distant from other historical SVA isolates, showing 86.2%–95.7% identity.

Laboratory findings suggest that SVA infection was the etiology of these cases because no other common pathogen was detected across the cases examined; index swine were viremic, shed SVA in feces and nasal secretions, and seroconverted to the virus; a high level of SVA was present in areas with vesicular lesion; and evidence of disease spread among pen mates. SVA detected in these cases were genetically distinct from previously reported SVA, suggesting that the virus has evolved, possibly leading to higher adaption to swine and change in pathogenicity. Although SVA is not a new virus, numerous unrelated cases of vesicular disease at exhibitions and commercial farms within such a short period is unusual. The fact that swine producers in Brazil have experienced an epidemic of vesicular diseases, in which SVA similar to the recent US SVA was implicated, warrants further studies to characterize the pathogenesis and associated risk factors of this novel SVA in swine.

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The authors thank staff at Iowa Department of Agriculture and Land Stewardship and practicing veterinarians for case submissions. ISUVDL technical staff are acknowledged for their assistance in sample processing and testing. Testing for foreign animal disease agents causing vesicular disease in swine (i.e., foot-and-mouth disease virus, swine vesicular disease virus, and vesicular exanthema of swine virus) and vesicular stomatitis virus was conducted at the National Veterinary Services Laboratories of the US Department of Agriculture's Animal and Plant Health Inspection Service in Plum Island, New York. The next-generation sequencing was performed at the Iowa State University DNA Facility.

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References

1. Hales LM, Knowles NJ, Reddy PS, Xu L, Hay C, Hallenbeck PL. Complete genome sequence analysis of Seneca Valley virus-001, a novel oncolytic picornavirus. J Gen Virol. 2008;89:1265–75. http://dx.doi.org/10.1099/vir.0.83570-0
2. Racaniello VR. *Picornaviridae*: the viruses and their replication. In: Knipe DM, Howley PM, editors. Fields virology. 6th ed. Vol. 1. Philadelphia: Lippincott, Williams and Wilkins; 2013. p. 610–64.
3. Hales LM, Jones BH, Vasko AJ, Knowles NJ, Police SR, Hallenbeck PL. Epidemiology of Seneca Valley virus (SVV-001), a novel oncolytic picornavirus for the systemic treatment of patients with solid cancers with neuroendocrine features. Mol Ther. 2006;13:S187. http://dx.doi.org/10.1016/j.ymthe.2006.08.550
4. Pasma T, Davidson S, Shaw SL. Idiopathic vesicular disease in swine in Manitoba. Can Vet J. 2008;49:84–5.
5. Singh KCS, Clark SG, Scherba G, Fredrickson R. Seneca Valley virus and vesicular lesions in a pig with idiopathic vesicular disease. J Vet Sci Technol. 2012;3:123. http://dx.doi.org/10.4172/2157-7579.1000123
6. Vannucci FA, Linhares DC, Barcellos DE, Lam HC, Collins J, Marthaler D. Identification and complete genome of Seneca Valley virus in vesicular fluid and sera of pigs affected with idiopathic vesicular disease, Brazil. Transbound Emerg Dis. 2015;62:589–93. http://dx.doi.org/10.1111/tbed.12410
7. Knowles NJ, Hales LM, Jones BH, Landgraf JG, House JA, Skele KL, et al. Epidemiology of Seneca Valley virus: identification and characterization of isolates from pigs in the United States. Presented at: Northern Lights EUROPIC 2006: 14th Meeting of the European Study Group on the Molecular Biology of Picornaviruses; 2006 Nov 26–Dec 1; Saariselka, Inari, Finland. Abstract G2.
8. Yang M, van Bruggen R, Xu W. Generation and diagnostic application of monoclonal antibodies against Seneca Valley virus. J Vet Diagn Invest. 2012;24:42–50. http://dx.doi.org/10.1177/1040638711426323
9. Chen Q, Li G, Stasko J, Thomas JT, Stensland WR, Pillatzki AE, et al. Isolation and characterization of porcine epidemic diarrhea viruses associated with the 2013 disease outbreak among swine in the United States. J Clin Microbiol. 2014;52:234–43. http://dx.doi.org/10.1128/JCM.02820-13

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Artist Unknown. ***Hercules and the Erymanthian Boar*****,** mid-17th century. Bronze, with red-brown lacquer patina, Height: 17½ in / 44.5 cm. Metropolitan Museum of Art, New York, New York, USA; The Jack and Belle Linsky Collection, 1982.

An Immortal Hero, an Enduring Challenge

Byron Breedlove and Paul M. Arguin

Hercules[1] has endured as perhaps the most popular figure from Greek mythology for nearly 3 millennia. Central to his myth is the story cycle about the 12 presumably impossible labors he carried out for the loathed King Eurystheus. Sometimes overlooked is that Hercules performed those tasks as penance for having murdered his own wife and children during a fit of madness.

For the fourth labor, Eurystheus ordered Hercules to capture the vicious Erymanthian Boar, a menacing beast that would descend from its lair on the mountain of Erymanthus each day, trampling the farmlands and attacking man and beast. Surprising the boar in its lair, Hercules

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[1]In this essay, we use the Roman name Hercules to indicate the Greek hero Herakles.

drove his quarry into the deep snow, where he subdued the exhausted boar and then carried it to the king's court. According to the myth, when the cowardly King Eurystheus heard the snorting, grunting beast and realized that Hercules had succeeded, the king hastily hid in a buried pithos jar, imploring Hercules to remove the boar.

Hercules' capture of the Erymanthian Boar, a tempting subject for artists, has been immortalized on coins and amphora; on a Roman sarcophagus; in paintings, films, and sketches; and through myriad sculptures. The identity of the sculptor who created the elegant bronze statue reproduced for his month's cover is not known. This work is thought to be modeled on earlier works by an imitator or student of the Flemish sculptor Giambologna (1529–1608), the court sculptor to the Medici grand dukes in Florence. He had produced a set of bronze statuettes of the 12 labors and supervised the work and training of numerous assistants.

This bronze portrays the aftermath of the chase. The figure of Hercules resolutely strides toward the court, clasping the huge boar over his shoulder with his left arm, balancing it with the club he holds aloft in his right hand. The textures of the bronze offer visual and tactile contrasts. Hercules' body is cast in smooth bronze, which heightens his physical prowess and musculature. The struggling boar's bristly hide, Hercules' hair and beard, and the patterns in his wooden club reveal the artist's nimble touch in working with bronze. A Metropolitan Museum of Art commentary notes that "The present statuette is extremely light in weight, with a dark but warm brown patina and richly variegated tool marks, such as the punch marks that articulate the club."

Although his strength and vigor are realized through this statue, Hercules would not have redeemed himself through his struggles and suffering alone, and ultimately become immortal, without also using his cunning and skill. He frequently sought the counsel of others in the course of his undertakings. During this labor, it was the centaur Chiron (though some versions say it was a different centaur named Pholus) who advised Hercules to drive the boar into the snow.

While completing his fourth labor, Hercules was potentially exposed to dangers that also threatened mortal men of his time and ours: possible exposure to zoonotic diseases. Zoonotic diseases are caused by any of more than 200 pathogenic agents, including bacteria, viruses, parasites, and fungi, transmitted directly or indirectly from animals to humans. For instance, the Erymanthian Boar could have felled Hercules via microbiology instead of muscle and tusk.

Hercules' risks did not stop with the Boar. In slaying and then skinning the formidable Nemean Lion and stalking and capturing the sacred Hind of Ceryneia, Hercules was at risk for various zoonotic diseases. Cleaning the Augean stables exposed him to enteric pathogens from the vast quantities of manure generated by teeming herds of cows, sheep, horses, and goats. Additional exposures occurred when he wrestled the Cretan Bull and stole the Cattle of Geryon. Hercules' efforts to dispel the Stymphalian Birds and capture the Mares of Diomedes may have exposed him to zoonoses acquired from birds and equids. And finally, why was the great 3-headed dog Cerberus so furious and out of control—rabies perhaps?

The human web of daily activities crosses many ecosystems. Animals provide food, transportation, companionship, and if you are Hercules, occasionally a wrestling adversary. Managing and limiting infectious risks associated with the intricate connections among humans, animals, and our environments prove to be challenging, complex endeavors. It's not hyperbole to label this undertaking a Herculean task.

Bibliography

1. Gibbons MW. Giambologna: narrator of the Catholic reformation [cited 2016 May 9]. http://ark.cdlib.org/ark:/13030/ft9n39p3vz/
2. Metropolitan Museum of Art. Hercules and the Erymanthian Boar [cited 2016 Apr 18]. http://www.metmuseum.org/art/collection/search/207005
3. Tufts Digital Library. The Erymanthian Boar [cited 2016 Apr 23]. http://www.perseus.tufts.edu/Herakles/boar.html
4. The Walters Art Museum. Hercules carrying the Erymanthian Boar [cited 2016 May 9]. http://art.thewalters.org/detail/8509/hercules-carrying-the-erymanthian-boar/
5. World Health Organization. Zoonoses and the human-animal-ecosystems interface [cited 2016 May 19]. http://www.who.int/zoonoses/en/

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Upcoming Issue

- Probable Rabies Virus Transmission through Organ Transplantation, China, 2015
- Multistate US Outbreak of Rapidly Growing Mycobacterial Infections Associated with Cosmetic Surgical Procedures in Dominican Republic
- Hemolysis after Oral Artemisinin Combination Therapy for Uncomplicated *Plasmodium falciparum* Malaria
- Cutaneous Melioidosis Cluster Caused by Contamination of Wound Irrigation Fluid
- Exposure-Specific and Age-Specific Attack Rates for Ebola Virus Disease in Ebola-Affected Households, Sierra Leone
- Time Lags between Exanthematous Illness Attributed to Zika Virus, Guillain-Barré Syndrome, and Microcephaly, Salvador, Brazil
- Virulence and Evolution of West Nile Virus, Australia
- Enterovirus D68 Infection in Children with Acute Flaccid Myelitis, Colorado, USA, 2014
- Assessment of Community Event–Based Surveillance for Ebola Virus Disease, Sierra Leone, 2015
- Middle East Respiratory Syndrome Coronavirus Transmission in Extended Family, Saudi Arabia, 2014
- Possible Role of Fish and Frogs as Paratenic Hosts of *Dracunculus medinensis*, Chad
- *Onchocerca lupi* Nematodes in Dogs Exported from the United States into Canada
- Lyssavirus in Indian Flying Foxes, Sri Lanka
- Q Fever and Spotted Fever Group Rickettsiosis Seroconversions among US Marines Deployed to Afghanistan, 2001–2010
- *Borrelia miyamotoi* Infection in Patients from Upper Midwestern United States, 2014–2015
- *Baylisascaris procyonis*–Associated Meningoencephalitis in a Previously Healthy Adult, California, USA
- Use of Unamplified RNA/cDNA–Hybrid Nanopore Sequencing for Rapid Detection and Characterization of RNA Viruses
- Survival and Growth of *Orientia tsutsugamushi* in Conventional Hemocultures

Complete list of articles in the August issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

July 18–22, 2016
21st International AIDS Conference
Durban, South Africa
http://www.aids2016.org/

August 24–28, 2016
Options IX for the Control of Influenza
Chicago, IL, USA
http://2016.isirv.org/

October 9–13, 2016
6th International Calicivirus Conference
Savannah, GA, USA
https://www.faingroup.com/Calicivirus/

October 29–November 2, 2016
American Public Health Association
Denver, CO, USA
https://www.apha.org/events-and-meetings/annual/past-and-future-annual-meetings

November 13–17, 2016
ASTMH
American Society of Tropical Medicine and Hygiene
Atlanta, GA, USA
https://www.astmh.org/annual-meeting?utm_source=ASTMH%2DInformz&utm_medium=email&utm_campaign=default

December 3–8, 2016
ASLM
African Society for Laboratory Medicine
Cape Town, South Africa
http://aslm2016.org/

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Article Title

Pregnancy, Labor, and Delivery after Ebola Virus Disease and Implications for Infection Control in Obstetric Services, United States

1. You are evaluating a 26-year-old woman at 8 weeks' estimated gestational age of her first pregnancy. She has no complaints, and the only significant risk factor she has for her pregnancy is a history of Ebola virus disease (EVD) 6 months ago. She has been asymptomatic and free of the virus for the past 4 months. What should you consider regarding special considerations for the maternity care of this patient?

A. Ebola virus is rarely transmitted vertically among women with active infection
B. Ebola virus is not shed into breast milk
C. Ebola virus may persist in amniotic fluid after the period of active infection
D. Good evidence exists that the Ebola virus persists in the female genital tract for months after acute symptoms have resolved

2. According to the current report on Ebola virus, which of the following statements about the labor and delivery of the patient with a history of EVD is most accurate?

A. The infant was delivered at a normal weight for gestational age and had normal Apgar scores
B. Her first detailed anatomy ultrasound in the United States demonstrated microcephaly
C. She underwent induction of labor because of early signs of fetal distress
D. Epidural anesthesia was contraindicated because of the potential for Ebola virus retained in the cerebrospinal fluid

3. Which of the following statements regarding precautions taken during the patient's labor and delivery is most accurate?

A. The care team neglected to call public health officials
B. No additional precautions were recommended beyond routine standards for delivery
C. The number of care providers attending the delivery was limited to 3
D. One member of the environmental staff team had contact with the patient's room once daily

4. Which of the following laboratory assessments yielded a positive result in the current case of this patient?

A. Cord blood for immunoglobulin G (IgG) against Ebola virus
B. Amniotic fluid for IgG against Ebola virus
C. Amniotic fluid for immunoglobulin M (IgM) against Ebola virus
D. Neonatal ear swabs for Ebola virus by real-time reverse transcription polymerase chain reaction

CME Questions

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Current Guidelines, Common Clinical Pitfalls, and Future Directions for Laboratory Diagnosis of Lyme Disease, United States

1. You are advising a large health maintenance organization regarding Lyme disease diagnostics. According to the review by Moore and colleagues, which of the following statements about current US testing guidelines for Lyme disease is correct?

A. Patients with an erythema migrans lesion and epidemiologic risk who live in or have traveled to Lyme-endemic areas require laboratory testing to confirm the diagnosis
B. The diagnostic test of choice for all patients presenting with signs of extracutaneous Lyme disease is a 3-tiered serologic test
C. Recommended diagnostic testing is an enzyme-linked immunoassay (EIA or ELISA) or immunofluorescence assay (IFA) followed by a reflex Western immunoblot
D. The US Food and Drug Administration (FDA) has approved IFA, but not EIA, for first-tier serologic testing for Lyme disease

2. According to the review by Moore and colleagues, which of the following statements about appropriate use and interpretation of tests for Lyme disease is correct?

A. To accurately order and interpret tests for Lyme disease, clinicians must consider the patient's history, timeline of symptoms, and pretest probability
B. Sensitivity of 2-tiered testing is high during early infection
C. Sensitivity of 2-tiered testing is 30% to 40% for disseminated Lyme disease
D. Specificity of 2-tiered testing is low during early infection, so clinicians should use alternative laboratory tests

3. According to the review by Moore and colleagues, which of the following statements about recent developments in Lyme disease diagnostics would most likely be correct?

A. Used as a standalone test, C6 EIA is more prone to false-negative results than the current 2-tiered test in patients with early Lyme disease
B. Novel 2-tiered approaches still require Western immunoblotting for the second-tier
C. Proteomics and metabolomics are useful only for diagnosis
D. The Centers for Disease Control and Prevention/ National Institutes of Health repository of sera from patients with Lyme disease, potentially cross-reactive conditions, and healthy control participants allows validation of novel diagnostic tests

CME Questions

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words in the main body of the text or include more than 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section comprises case series papers and concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

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Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

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