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February 2014

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On the Cover

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Picking Cotton (1981)

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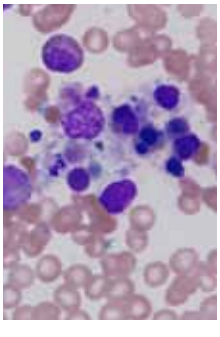

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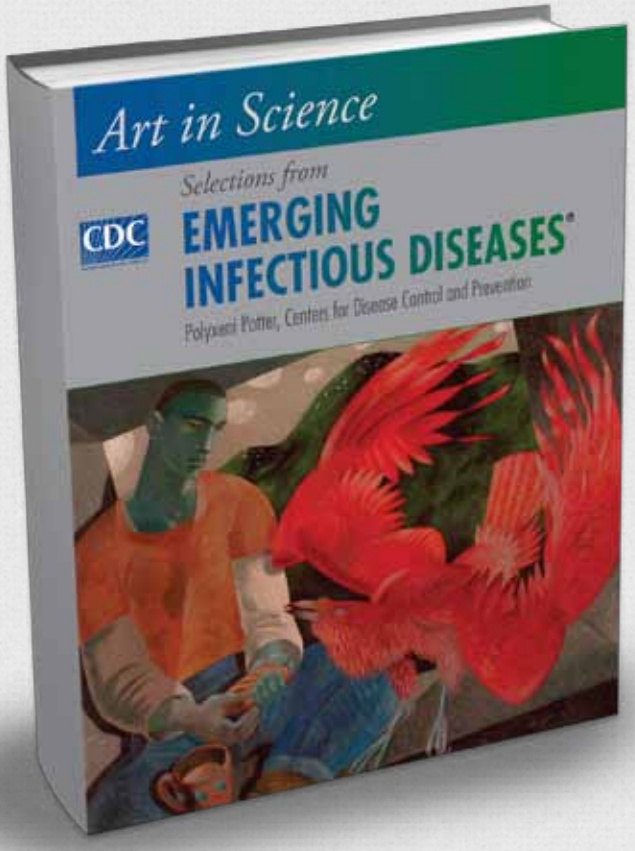

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Poxvirus Viability and Signatures in Historical Relics

Andrea M. McCollum, Yu Li, Kimberly Wilkins, Kevin L. Karem, Whitni B. Davidson, Christopher D. Paddock, Mary G. Reynolds, and Inger K. Damon

Although it has been >30 years since the eradication of smallpox, the unearthing of well-preserved tissue material in which the virus may reside has called into question the viability of variola virus decades or centuries after its original occurrence. Experimental data to address the long-term stability and viability of the virus are limited. There are several instances of well-preserved corpses and tissues that have been examined for poxvirus viability and viral DNA. These historical specimens cause concern for potential exposures, and each situation should be approached cautiously and independently with the available information. Nevertheless, these specimens provide information on the history of a major disease and vaccination against it.

Chinese writings from 1122 BCE contain references to smallpox-like disease, and it has been hypothesized that smallpox caused the death of Ramses V in Egypt in ≈1157 BCE because poxvirus-like lesions were seen on the mummy (*1*,*2*). The most recent epidemics of smallpox occurred through the 1900s, and the last naturally occurring case of smallpox was seen in Somalia in 1977 (*3*). Historical tissue specimens and artifacts yield useful information about the history of and vaccination against smallpox. However, the absolute viability of poxviruses in well-preserved samples has not been determined. Thus, it is not known what risks these artifacts might pose to persons who come into contact with them.

Smallpox is caused by variola virus (genus *Orthopoxvirus*). Illness is characterized by 3 phases: incubation, prodrome, and rash. The incubation phase is ≈10–14 days. During the prodromal period, which lasts 2–4 days, persons with smallpox typically have fever, malaise, vomiting, headache, backache, and myalgia. The rash phase can be moderate or severe and is characterized by a centrifugal distribution of lesions in the same stage of development (Figure 1) in any 1 area of the body. Lesions, including their crusts, contain infectious virus through all stages of the rash. Thus, contact with infectious lesion exudate and tissue (including crusts) can result in virus transmission. However, the most common route of transmission is inhalation of infectious respiratory droplets. Patients who survive an infection often have life-long scarring, and they maintain some level of immunity to orthopoxvirus infection (*1*,*4*,*5*).

Elimination and eradication of smallpox were feasible, in part, because there is no animal reservoir for variola virus. The World Health Organization (WHO) announced worldwide eradication of smallpox in 1980. Successful eradication was accomplished by vaccinating populations and contacts of ill persons with live vaccinia virus, a closely related orthopoxvirus that confers immunity to variola virus. Once smallpox was eradicated, WHO recommended that routine vaccination be discontinued and that the vaccine be used only for select groups at risk for exposure to orthopoxviruses. Thus, persons born after 1980 are likely to not have residual immunity (*1*,*6*). Intentional arm-to-arm transfer of virus by dried scab or lesion exudate from a recent vaccinee was common in nineteenth century Great Britain (*7*). Crusts were collected, stored, and sent to others to aid vaccination before mass production and distribution of vaccine stocks. Scab material from patients with smallpox was often used for variolation, the practice of deliberately infecting a person with smallpox to (hopefully) induce a mild infection and subsequent immunity. Variolation continued into the twentieth century in some regions (*1*).

Present-day stocks of variola virus are maintained at 2 WHO reference laboratories: the Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA) and the State Research Center of Virology and Biotechnology (VECTOR) (Koltsovo, Russia). There is concern that if variola virus is present outside these 2 laboratories, its

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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Figure 1. Patient with smallpox. Photograph by Jean Roy, provided by the Public Health Image Library, Centers for Disease Control and Prevention, Atlanta, GA, USA.

accidental or intentional release could cause illness in a population increasingly composed of unvaccinated persons. Anecdotal reports and formal scientific evidence have not ruled out the possibility that the virus may survive prolonged periods in preserved skin and tissue material, such as those that might be on display in museums, or in unearthed human remains. For example, permafrost is an environment that closely mirrors laboratory freezer storage of live virus, and the maintenance of viable smallpox virus in human remains found in such an environment has been debated (*8*). Environmental contamination with potentially live variola virus recovered from historical relics could threaten our confidence that the disease has been eradicated. In addition to immediate public health concern about such relics, there is much to be gained from investigation of artifacts in terms of scientific and historical interests.

We reviewed experimental data that address virus longevity in a variety of environments. There are several accounts of historical smallpox specimens in the form of unearthed remains and lesion crusts. The Poxvirus Laboratory at CDC recently reviewed this data and reexamined specimens from the inventory to revisit the existence of sections of intact DNA by using more modern methods. We also address the role of public health and scientific interest in such specimens.

We found published articles by searching PubMed for material on virus viability and historical specimens. Search terms included viability smallpox, viability variola, viability orthopoxvirus, and smallpox and corpse. References from articles that cited previous work on virus viability or historical specimens were also reviewed. Studies were also included if they contained experimental data on the viability of an orthopoxvirus on fomites or preserved tissue material (e.g., crusts). Studies or reports on historical specimens were included if there was suspicion of variola virus.

Existing specimens at the CDC Poxvirus Laboratory (tissues from Egypt, Italy, and England) were reexamined by using modern molecular techniques. In addition, we examined newer relic specimens (tissues from Kentucky and New York, New York, and crusts from Virginia, New Mexico, and Arkansas) for molecular signatures of poxviruses. Non-variola orthopoxvirus DNA signatures were amplified by using real-time PCR (*9*).

Experimental Data

The infectiousness of preserved skin and tissue material from patients with smallpox has been a matter of concern, particularly as worldwide smallpox eradication was achieved (*1*,*10*–*12*). Circumstantial evidence had long placed infectious fomites as the cause of many outbreaks; however, there is little evidence that fomites were a frequent cause for disease transmission (*13*–*15*). Nevertheless, smallpox lesion material is infectious, and it is conceivable that such material was present on fomites, such as clothing, linens, and letters, and that those fomites were responsible for transmission of variola virus (*15*,*16*).

During the smallpox era, one source of live virus was lesion crusts or scabs. Crusts were successfully used for variolation in many areas before vaccination with vaccinia virus. Virus content in crusts is not correlated with the vaccination status of the patient, severity of illness, or time during the course of infection (*17*). Thus, crusts from any patient with smallpox could harbor infectious virus. Experimental studies on the infectiousness of lesion crusts, specifically in preserved specimens, are limited. However, a few experimental studies share some common conclusions about the infectious nature of crusts (Table 1).

During smallpox outbreaks in the 1940s, Downie and Dumbell (*18*) tested dried crusts and vesicle fluid that were obtained from patients with smallpox (vesicle fluid was dried on glass slides before examination). Specimens were

Table 1. Viability of infectious variola virus in various materials*

Study, year, (reference)	Type of material	Storage conditions	Maximum storage time viable virus was recovered†
Downie and Dumbell, 1947 (*18*)	Lesion crusts	Room temperature, exposed to daylight	196 d
		Room temperature, kept in dark	417 d
		Refrigerated and then room temperature, exposed to light	>196 d after refrigeration, >341 d total)
		Refrigerated and then room temperature, kept in dark	>196 d after refrigeration, >341 d total)
		In a vacuum over calcium chloride	782 d
	Saline extract of crusts	Refrigerated	432 d
	Vesicle fluid on glass slides	Room temperature, exposed to daylight	35 d
		Room temperature, kept in the dark	84 d
	Vesicle fluid diluted in broth	Refrigerator	270 d
MacCallum and McDonald, 1957 (*19*)	Crusts embedded in raw cotton	Room temperature, indirect light	530 d
		30°C, kept in the dark, 58%, 73%, and 84% relative humidity	70, 70, and 60 d, respectively
Wolff and Croon, 1968 (*20*)	Crusts	Room temperature, kept in an envelope	4,745 d (13 y)
Huq, 1977 (*21*)	Crusts	35°C, 65%–68% relative humidity	21 d
		26°C, <10% and 85%–90% relative humidity	84 and 56 d, respectively
		4°C, 10% and 60%–62% relative humidity	112 d
		−20°C	112 d
Rao, 1972 (*15*)	Vesicle fluid on glass slides	Direct sunlight	<1 h
	Vesicle fluid in capillary tubes	Direct sunlight	<2 h

*Specimens from patients with smallpox were used for all studies.
†For several studies, this is the last sampling time point and either no material was left to continue the experiment or no further samplings were conducted.

stored at room temperature and sampled at regular intervals. Viable virus was detected from vesicle fluid contained on a glass slide stored in daylight for ≤35 days and in the dark for ≤84 days. Moreover, crusts that had been stored for 417 days at room temperature and for 432 days in a refrigerator also contained viable virus. Further testing was not possible because of insufficient crust material. Nevertheless, that study was one of the first to show that viable virus could be isolated from patient material many months after collection and that optimal storage likely included dark and cool conditions (*18*).

In the mid-twentieth century, there was concern for inadvertent importation of variola virus into Great Britain in raw cotton shipped in from tropical areas (*22*). Suspicion was raised for this vehicle of importation after outbreaks occurred in British workers who handled raw cotton. An experiment was conducted to test the viability of variola virus derived from smallpox lesion crusts found in imported raw cotton (*19*). Viable virus was obtained ≤530 days from crusts stored in indirect light at room temperature. Crusts stored at higher humidity (73% and 84%) were viable until 70 and 60 days, respectively. Similar results were obtained from a study in Bangladesh, which found viable virus could be isolated from crusts stored at lower temperatures (*21*). However, crusts stored at higher temperatures and humidity did not retain viable virus after several weeks or months (*21*).

Wolff and Croon (*20*) conducted the longest study of variola viability in crusts from smallpox patients. For the study, crusts were collected from patients, individually placed in envelopes, and stored at room temperature. Viability of virus in these crusts was tested yearly for 13 years. Although the number of viable particles decreased with time, live variola virus was isolated from crusts 13 years after their initial storage. The experiment was discontinued after 13 years because crust specimens were depleted.

Further examination of variola virus viability on clothing and other objects indicated that the virus is not viable after exposure to direct sunlight for 30 min to 3 h; even indirect sunlight had an effect on viability (*15*). Although experimental studies have not yielded a well-defined period at which viable variola virus can survive in a preserved state (either deliberate experimental preservation or part of the natural process of tissue preservation), there is an overriding conjecture reached by these studies. If stored in cool, dry, and dark conditions, variola virus can survive in lesion crusts or tissues for months or years. Because each historical specimen and account is unique and the circumstances of preservation differ, it is essential to test suspicious specimens for viable variola virus.

Historical and Scientific Accounts of Specimens

There have been several published and unpublished reports of suspected smallpox specimens surfacing since eradication (Table 2). Some reports involve scabs or crusts, and others involve entire corpses. These specimens offer an illuminating glimpse into the past, but their presence may also cause some concern for public safety in the event that any of these specimens contain viable variola virus. We present the historical and scientific accounts of each of these specimens with their respective laboratory results, which represent published and more recent data from the CDC Poxvirus Laboratory.

Corpses

An anecdote from eighteenth century England describes an outbreak of smallpox believed, at the time,

Table 2. Historical artifacts tested for variola virus and other viruses

Location, date of origination, description of the artifact (date discovered)	Laboratory testing*				
	Live virus isolated	Evidence by electron microscopy	Viral DNA isolated	Human DNA isolated	Other testing
Egypt, 1157 BCE, mummy of Ramses V with lesions; lesions were present in a centrifugal distribution and had an appearance similar to smallpox (1898, 1979)	No (*2*)	No (*2*)	No†	No†	Viral particles and faint immunologic reactivity with variola antibody; negative radioimmunoassay result for smallpox (*23*)
Egypt, 1200–1100 BCE, piece of skin from male mummy with a typical smallpox rash (1911)					Portion of skin did not show definite pathologic characteristics of smallpox (*24*)
Italy, sixteenth century, corpse exhumed from a crypt; lesions were umbilicated, monomorphic, and in a centrifugal distribution (1986)	No (*25*)	Yes (*25,26*)	No, by molecular hybridization (29); no, by DNA isolation and real-time PCR†	No†	Orthopoxvirus antigens not detected by hemagglutination or enzyme immunoassay (*25*)
Canada, 1640–1650, bones from an adult man located in a burial plot on Native American land; the tribe was known to have had a smallpox epidemic in 1634 (1966)					Bone analysis result was consistent with osteomyelitis variolosa (*27*)
Russia, late seventeenth to early eighteenth centuries, corpses exhumed from permafrost; 1 grave had multiple bodies and evidence suggested quick postmortem burial; samples were analyzed from 1 corpse (2004)			Yes, variola virus–related DNA (*28*)		
England, 1729–1856, piece of skin with lesions attached to a skeleton exhumed from a crypt (1985)	No (*29*)		No†	No†	
Russia, nineteenth century, corpses in permafrost recovered during flooding; corpses were from an area of a smallpox outbreak in the nineteenth century (1991)	No (*30*)				
Kentucky, USA, 1840–1860, mummified remains of a body with lesions discovered at a construction site (2000)	No†		No†*		
New York, New York, USA, City, mid-1800s, mummified remains of a body with lesions contained within an iron coffin discovered at a construction site (2011)	No†	No†	No†	Yes, from a tooth†	
Virginia, USA, 1876, scab from the arm of an infant to be used for community vaccination; found in letter sent from son to father in Virginia; scab was on display at a museum (2011)	No†		Yes, non-variola Orthopoxvirus DNA†	Yes†	
New Mexico, USA, late nineteenth century, scabs from vaccination sites contained in an envelope, which was contained within a book (2003)	No†		Yes, non-variola Orthopoxvirus DNA†	No†	
Arkansas, USA, 1871–1926, suspected smallpox scabs on display at a museum (2004)	No†		No†	No†	

*Published laboratory results are accompanied by the reference (number in parentheses).
†Previously unpublished results.

to be caused by exposure to a long-buried corpse. The grave of a person with smallpox who died 30 years earlier was unearthed in the process of preparing a second grave nearby, and several of the funeral attendees became ill with smallpox (*12*,*31*). Whether these grieving attendees contracted smallpox from the graveside or from another ill person in the community, a likely occurrence during an outbreak, is unknown. However, occupationally derived smallpox infections beset mortuary workers and those who had close contact with bodies of deceased patients with smallpox. In these cases, the disease was likely contracted by contact with virus in or on the corpse or on contaminated clothing or linens (*19*,*32*). These infections may have occurred because of exposure to a recently deceased patient with smallpox, but a question remains with us now: can live virus be maintained in well-preserved ancient corpses and mummies?

Egyptian Mummies—Twelfth Century BCE

An early examination of evidence for variola virus was conducted on a piece of skin from a male mummy housed at the Cairo Museum of Antiquities. The mummy had vesicular cutaneous lesions distributed in a pattern characteristic of smallpox. A portion of skin processed for light microscopy did not show definitive pathologic characteristics of smallpox. However, these ancient tissues were not ideally preserved for histological examination (*24*). The discovery of lesions present in a typical distribution on the mummified body of Ramses V implicated smallpox as the young pharaoh's cause of death and shed new light on ancient Egyptian history, as well as that of variola virus (*2*). Centuries after his death, skin taken from the shroud of the mummy of Ramses V showed some viral particles and had faint immunologic reactivity (*23*); however, the sampling method was noted to have potentially been flawed and no live virus or viral DNA was isolated or amplified from specimens (*2*). Human DNA was also not detected in these specimens. Thus, although there is no laboratory data to firmly support a postmortem diagnosis, the visual appearance was suggestive of a variola infection before his death (*2*).

Archeologic Excavations

There have been 2 examples of corpses exhumed from crypts during archeologic excavations in the twentieth century. In both examples, the corpses had what were described as typical variola lesions, and the bodies had been contained in cool, dark environments. No live virus, viral DNA, or human DNA remained within these corpses. However, a corpse from sixteenth century Italy showed immunologic electron microscopy results that were consistent with those expected for orthopoxvirus infection (*25,26,29*). An archeologic excavation of a known Native American grave site (1640–1650) in Ontario, Canada, recovered bones from an adult male. The bones had visual scarring and an appearance consistent with osteomyelitis variolosa, a disease manifestation of smallpox in the bones and joints. On the basis of extensive document review and bone analysis, the investigators determined that the person likely had smallpox before 1639 and survived the infection with long-term osteomyelitis variolosa (*27*).

Permafrost in Russia

Two corpses with questionable lesions and that had been contained within permafrost in Siberia have been unearthed: one was unearthed naturally during flooding, and the other during an archeologic excavation. Dating of the corpses to the late seventeenth or early eighteenth century matched with written accounts of smallpox epidemics in the local communities for one of the sites, but no live virus was obtained from these remains (*28,30*). The more recent archeologically excavated corpse was sampled as soon as graves and mummified remains were exposed to the surface. The corpse yielded DNA closely related to more recent variola virus specimens. This finding provided further insight into the strain of variola that was circulating in northeastern Siberia during the late seventeenth or early eighteenth centuries (*28*).

Construction Sites in Kentucky and New York, New York, USA—Nineteenth Century

There are 2 accounts of remains with suspicious lesions that were accidently unearthed during construction at a burial site. In 2000, mummified remains were discovered at a construction site in Kentucky. No live virus or viral DNA was isolated from these remains. More recently in 2011, the remains of a woman buried in an iron coffin were uncovered during construction at a known African-American cemetery in New York, New York. Preservation of the body was remarkable because of the airtight environment provided by the iron coffin (*33*). The presence on the body of lesions with the characteristic deep-seated, umbilicated appearance and in a centrifugal distribution of smallpox lesions immediately prompted concern for unearthed smallpox (Figure 2). No live virus or viral DNA was isolated from or visualized in any of multiple specimens taken from the body and evaluated by cell culture, molecular methods, or immunohistochemical stains. Human DNA was isolated from a tooth pulp specimen. Thus, the results do not conclusively verify the hypothesis of smallpox as the cause of death. However, visual inspection cast little doubt on this hypothesis.

Crusts from Patients

Some accounts from the eighteenth century report that material used in variolation (often scab material) was stored

Figure 2. Mummified remains of a woman buried in an iron coffin, New York, New York, USA, mid-1800s. Photograph provided by Don Weiss.

for ≤8 years before successful use (*34*). Thus, long-term storage and subsequent use of variola virus from preserved specimens have long been recognized. However, during the era of eradication, 45 scab specimens were collected from variolators and tested 9 months after collection; live virus was not isolated from any of the specimens (*35*). Nevertheless, stored crusts have caused immediate concern for potential exposures and their discovery has caused immediate exposure mitigation and testing.

In the past 10 years, suspected variola crusts have been discovered in the United States on 3 occasions. In Virginia, a crust labeled as a smallpox scab was on display at a museum and was accompanied by a letter describing its origin (Figure 3, panel A). The letter and crust were sent from 1 family member to another in Virginia in 1876, and the correspondence stated that the crusts came from the arm of an infant and were to be used to vaccinate others. No live virus was isolated from this crust. However, non-variola orthopoxvirus DNA and human DNA were successfully extracted. This rare letter and scab are evidence to support arm-to-arm vaccination in the United States around the same time that it was also performed in Great Britain (*7*).

A second incident of suspected smallpox scabs on display at a local museum occurred in Arkansas (Figure 3, panel B). These relics were donated by the family of a physician who practiced in Arkansas during 1871–1926. In 1905, there was a large smallpox outbreak in Arkansas (*36*). No live variola virus, viral DNA, or human DNA were isolated from the specimens. The crusts were affixed to blocks of wood with a dense resin, and the resin may have been inhibitory to the PCR or DNA stability. The origins and species of these specimens will continue to remain a mystery.

In 2003, a librarian in New Mexico opened a book and an envelope containing lesion crusts fell out of the book (Figure 3, panel C). The envelope was labeled "scabs from vaccination of W.B. Yarrington's children," and the book was dated 1888. Similar to the relic from Virginia, no live virus was isolated from this material, but non-variola orthopoxvirus DNA was isolated. In this instance, human DNA was not amplified. The question of precisely what virus was used in vaccination in the United States in the nineteenth century is intriguing from the perspective of historical significance and the evolution of orthopoxviruses.

Public Health

Historical specimens come to the attention of public health authorities when there is a perception that they may constitute a potential risk to those who are handling or may have handled the artifacts. This concern extends to specific groups of persons who might work routinely with historical specimens, including archeologists and museum archivists, as well as those who may stumble upon these specimens on an irregular basis, such as construction workers or the general public. Although live variola virus has never been isolated from historical tissues, this finding does not eliminate the possibility of live variola virus resurfacing from well-preserved tissue material (*10*,*12*). Moreover, variola virus has been absent for >30 years, and there is an increasingly large population of susceptible persons who have never been vaccinated against smallpox.

The discovery of a series of corpses and mummies with suspected smallpox lesions in the late 1970s and 1980s sparked a series of commentaries over the risks to archeologists and anthropologists and the potential need for vaccination of workers (*19,23,33,34*). This proposition has been hotly debated, and opponents have argued that live variola virus has never been isolated from archeologic specimens and that live virus vaccination carries its own risks. This debate underscores the lack of firm scientific evidence to enable an informed assessment of risk to those who come into contact with artifacts and relics potentially contaminated with variola virus. The inability to exclude the possibility of risk led to the vaccination of 3 archeologists who handled a corpse with suspect lesions in London in 1985 (*29*). Current recommendations from the Advisory Committee on Immunization Practices do not specifically address vaccination for those who work with antiquities, including corpses and tissue material (*6*). Although routine vaccination is not recommended, prudent preparation and recognition of potential smallpox relics is advised for those who work with potentially contaminated tissues and corpses (*29*).

If a suspected smallpox relic or body of a person who died of smallpox has been discovered, local and state public health departments are an excellent resource. Public health officials can work closely with those who have handled any suspect artifacts, determine risks, help mitigate concern, and arrange for appropriate testing. Testing

can be performed on a suspected specimen to definitively determine if live virus is present. The WHO smallpox reference laboratories can perform these tests and have successfully participated in inquiries involving historical specimens (Table 1).

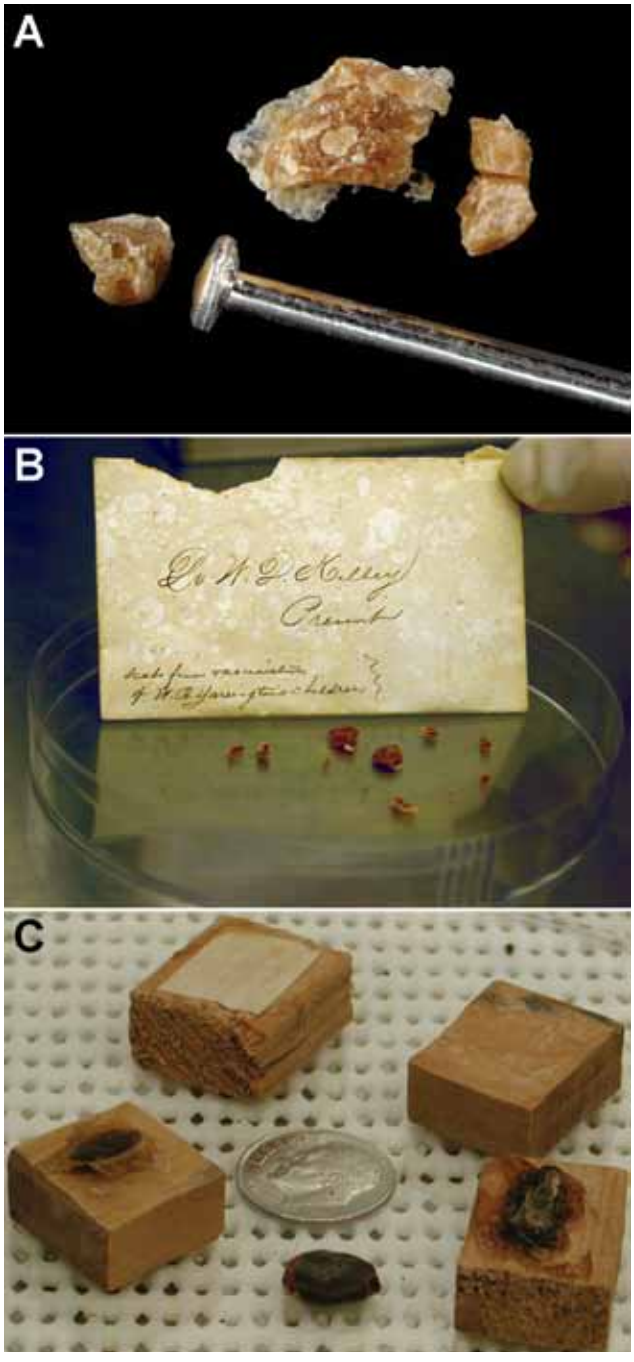

Figure 3. Recovered crusts. A) Lesion crust material from Virginia, USA, photographed after gamma irradiation. Photograph by James Gathany. B) Lesion crust material from an envelope contained within a book, New Mexico, USA, nineteenth century. Photograph by Russell L. Regnery. C) Lesion crust material from a jar on display in a museum, Arkansas, USA. Photograph provided by Erin Goldman.

Conclusions

Aside from immediate public health concerns surrounding a suspected smallpox specimen, historical cases help highlight disease history in terms of the society and patient in question. Historical specimens might also help explain the history of smallpox epidemics and vaccine development. Recent exhumation of a corpse from permafrost in Siberia led to sequence characterization of an older strain of variola virus, which shed light on the evolutionary history of the virus (*28*).

Today, the smallpox vaccine consists of an intradermal inoculation with vaccinia virus, and the premise and method of this vaccination has not changed since the time of Jenner (*7*). However, the species of virus that Jenner used to vaccinate persons is still debated (*37*). Irrespective of the debate, most scientists agree that Jenner and generations of persons since him have used an orthopoxvirus species in vaccinations to confer immunity to smallpox. Accounts of vaccination exist in historical records, but descriptions of which virus was used, how it was used, and who was performing procedures (e.g., physicians, communities) are sparse. Thus, our understanding of the history of smallpox vaccination is incomplete. Information obtained from historic relics helps build an understanding and picture of vaccination before the twentieth century (*1*).

Modern molecular approaches can be used with historical specimens to confirm the presence of variola or another orthopoxvirus and elucidate the evolutionary history of the virus. Full genome, gene, or partial gene sequencing of isolates enables investigating the history of 1 virus compared with others. Long-term stability of smallpox virus DNA is not well characterized. However, constant low temperatures, such as those in crypts and permafrost, are believed to be key to the stability of DNA molecules. Theoretically, DNA can survive up to ≈1 million years in cold environments (*38*). Specific characteristics that make orthopoxviruses stable and viable over long periods are unknown. However, for viruses embedded in tissue (such as those in crusts or skin specimens), it is reasonable to postulate that being surrounded by a protein or organic matrix may provide some protection to the virus.

Archival specimens offer opportunities to delve into the past and capture a glimpse of the history of an eradicated disease. There are no published reports of residual live microbes found in archeologic relics. Furthermore, on the basis of experiences in the past several decades, risks for transmission of live organisms from such relics would seem to be nonexistent; nevertheless, archeologic specimens should be handled with caution. Each situation should be approached independently and with vigilance and attention. Special attention to the scientific value of a specimen will yield useful data about smallpox and vaccination history that might provide useful information about the virus and affected populations.

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Dr McCollum is an epidemiologist in the Poxvirus and Rabies Branch, Centers for Disease Control and Prevention, Atlanta, Georgia. Her research interests include the epidemiology, ecology, and population genetics of infectious diseases.

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Address for correspondence: Andrea M. McCollum, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop A30, Atlanta, GA 30333, USA; email: amccollum@cdc.gov

Anncaliia algerae Microsporidial Myositis

Matthew R. Watts, Renee C.F. Chan, Elaine Y.L. Cheong, Susan Brammah, Kate R. Clezy, Chiwai Tong, Deborah Marriott, Cameron E. Webb, Bobby Chacko, Vivienne Tobias, Alexander C. Outhred, Andrew S. Field, Michael V. Prowse, James V. Bertouch, Damien Stark, and Stephen W. Reddel

The insect microsporidian *Anncaliia algerae* was first described in 2004 as a cause of fatal myositis in an immunosuppressed person from Pennsylvania, USA. Two cases were subsequently reported, and we detail 2 additional cases, including the only nonfatal case. We reviewed all 5 case histories with respect to clinical characteristics, diagnosis, and management and summarized organism life cycle and epidemiology. Before infection, all case-patients were using immunosuppressive medications for rheumatoid arthritis or solid-organ transplantation. Four of the 5 case-patients were from Australia. All diagnoses were confirmed by skeletal muscle biopsy; however, peripheral nerves and other tissues may be infected. The surviving patient received albendazole and had a reduction of immunosuppressive medications and measures to prevent complications. Although insects are the natural hosts for *A. algerae*, human contact with water contaminated by spores may be a mode of transmission. *A. algerae* has emerged as a cause of myositis, particularly in coastal Australia.

Author affiliations: Centre for Infectious Diseases and Microbiology Public Health, Westmead, New South Wales, Australia (M.R. Watts, C.E. Webb, A.C. Outhred); Pathology West–Institute for Clinical Pathology and Medical Research Westmead, Westmead (M.R. Watts, C.E. Webb); University of Sydney, Sydney, New South Wales, Australia (M.R. Watts, C.E. Webb, E.Y.L. Cheong, A.C. Outhred, S.W. Reddel); Concord Repatriation General Hospital, Concord West, New South Wales, Australia (R.C.F. Chan, E.Y.L. Cheong, S. Brammah, S.W. Reddel); Prince of Wales Hospital, Randwick, New South Wales, Australia (K.R. Clezy, C. Tong, J.V. Bertouch); St Vincent's Hospital, Darlinghurst, New South Wales, Australia (D. Marriott, A.S. Field, D. Stark); John Hunter Hospital, Newcastle, New South Wales, Australia (B. Chacko); Liverpool Hospital, Liverpool, New South Wales, Australia (V. Tobias); and Port Macquarie Base Hospital, Port Macquarie, New South Wales, Australia (M.V. Prowse)

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Microsporidia is a phylum of eukaryotes that contains almost 160 genera (*1*). Related to fungi, they are obligate intracellular organisms that spread among hosts through a spore stage (*1*). The microsporidian *Anncaliia algerae*, former genera *Nosema* and *Brachiola*, is an emerging human pathogen that primarily infects insects (*2*–*7*). *A. algerae* has caused severe myositis in patients taking immunosuppressive medication for rheumatoid arthritis or solid-organ transplantation (*3*,*5*,*8*). It also has led to skin abscesses and an infection of the false vocal cord in patients receiving chemotherapy for hematologic malignancies and caused keratitis in a man with no significant medical history (*2*,*4*,*6*).

Two other species of *Anncaliia* are reported to cause myositis in humans (*9*). *A. vesicularum* caused infection localized to the skeletal muscle in a patient with HIV (*9*). Another *Anncaliia* species, probably *A. connori*, led to disseminated infection in an infant who had thymic dysplasia and malabsorption syndrome (*9*,*10*). Autopsy demonstrated microsporidia in myocytes from the heart and diaphragm and the muscularis of the gut and arteries (*10*). Organisms also were seen in the alveolar septae, renal tubular cells, and parenchyma of the adrenal glands and liver (*10*). Microsporidia of the genera, *Trachipleistophora*, *Pleistophora*, and *Tubulinosema* also can cause myositis in immunocompromised hosts (*11*–*17*); the infection can be localized or part of a disseminated infection. A recent case report from Thailand described an infection caused by a novel microsporidia, related to *Endoreticulatus* spp, in the skeletal muscle, urinary tract, and bone marrow of a previously healthy man (*18*).

A. algerae myositis was first described in 2004 in a patient from Pennsylvania, USA, who had rheumatoid arthritis (*3*). Two patients in subsequent reports had histories of lung transplantation, 1 with a recent renal transplantation (*5*,*8*). We describe 2 additional cases of *A. algerae* myositis in patients with histories of rheumatoid arthritis, including 1 who survived. Four of these 5 case-patients were from

the east coast of Australia. We have reviewed all 5 case histories with respect to clinical characteristics, diagnosis, and management (Tables 1, 2) and summarized organism life cycle and epidemiology.

Case Reports

We have designated persons with the cases of *A. algerae* myositis described here as case-patients A and B and those with cases reported in 2004, 2012, and 2013 as case-patients 1, 2, and 3, respectively (*3*,*5*,*8*). We reviewed the medical records of case-patients 2 and 3 to obtain additional information.

Case-patient A

In 2011, a 67-year-old man from coastal New South Wales, Australia, sought care at hospital for an 8-week history of watery diarrhea; weight loss; and increasing arthralgias, fatigue, lethargy, and generalized myalgias. He had rheumatoid factor–positive rheumatoid arthritis that was diagnosed when he was in his early twenties, with fluctuating joint disease, but no extra-articular involvement. Therapy preceding the illness included long-term methotrexate (20 mg weekly); leflunomide (20 mg/d), which was stopped 1 week before he sought care at a hospital; prednisone (5 mg/d); etanercept (antitumor necrosis factor α therapy) for 5 months; and nonsteroidal anti-inflammatory drugs (NSAIDs). Serum creatinine and liver function tests were moderately elevated. The methotrexate, etanercept, and NSAIDs were stopped; he responded to intravenous rehydration; his serum creatinine normalized; and he was discharged after 3 days.

The man's symptoms progressed, dysphagia developed, and he was readmitted to the hospital 2 weeks later. He had generalized weakness in the upper and lower limbs of Medical Research Council grade 3+ to 4, distal greater than proximal, absent or reduced reflexes, and no clinical sensory abnormalities. Mild peripheral edema was present. He had mild glossitis but no mouth ulcers. He was noted to be at significant risk for aspiration because of bulbar muscle weakness and was not permitted to eat or drink by mouth; nasogastric feeding was begun. Prednisone was increased to 25 mg/d. His condition deteriorated despite 5 days of intravenous immunoglobulin for possible Guillain-Barré syndrome, and he was transferred to a Sydney tertiary referral hospital.

No cause for the diarrhea was found on fecal bacterial culture; examination for ova, cysts, and parasites; and modified trichrome staining for microsporidia (Table 2). A pulmonary infiltrate was present on computed tomographic (CT) scan. Results of magnetic resonance imaging of the brain and spinal cord were normal. Neurophysiologic studies showed reduced motor and sensory amplitudes and F wave persistence; electromyography demonstrated marked fibrillations and positive sharp waves, with only a mildly reduced pattern, suggesting mixed myositis and neuropathy.

An immediate vastus lateralis muscle biopsy was performed. Light microscopy demonstrated a necrotizing myositis with numerous ovoid spores (Figure 1). Electron microscopy confirmed the diagnosis of microsporidial myositis with features characteristic of *A. algerae* (Figure 2). DNA was extracted from the muscle biopsy specimen, and part of the small subunit ribosomal RNA gene was amplified by PCR in accordance with a published method (*5*). Sequence analysis was consistent with *A. algerae*.

Prednisone was reduced to 5 mg, and leflunomide was washed out with cholestyramine. Albendazole (400 mg 2×/d), sulfadiazine (1 g 4×/d) and pyrimethamine (50 mg/d)

Table 1. Epidemiologic and clinical characteristics of *Anncaliia algerae* myositis case-patients*

Characteristic	Case-patient A	Case-patient B	Case-patient 1 (*3*)	Case-patient 2 (*5*)	Case-patient 3 (*8*)
Age, y/sex	67/M	66/M	57/F	49/M	56/M
Residence	Port Macquarie, NSW, Australia	Sydney, NSW, Australia	PA, USA	Woolongong, NSW, Australia	Rutherford, NSW, Australia
Distance from residence to open land, m	<100 to golf course	<100 to golf course	ND	<200 to coastal woodland	<100 to golf course
Background illness	RA	RA	RA, T2DM	Lung Tx, T1DM, CD	Lung Tx, kidney Tx
Immunosuppression	MTX, CS, LEF, ETN	MTX	MTX, CS, LEF, IFX	AZ, TAC, MMF, CS	TAC, MMF, CS
Fever	Yes	Yes	Yes	Yes	Yes
Fatigue	Yes	Yes	Yes	Yes	Yes
Weight loss	Yes	Yes	ND	Yes	Yes
Weakness	Yes	Yes	Yes	Yes	Yes
Generalized pain	Yes	Yes	Yes	Yes	Yes
Dysphagia	Yes	Yes	ND	Yes	Yes
Glossitis	Yes	Yes	ND	Yes	Yes
Peripheral edema	Yes	Yes	ND	Yes	Yes
Diarrhea	Yes	No	ND	Yes	Yes
CNS abnormalities	No	Delirium	Cerebrovascular infarction	Delirium, seizures†	Delirium

*NSW, New South Wales; PA, Pennsylvania; ND, not described in publication; RA, rheumatoid arthritis; T2DM, type 2 diabetes mellitus. Tx, transplantation; T1DM, type 1 diabetes mellitus; CD, Crohn disease; MTX, methotrexate; CS, corticosteroids; LEF, leflunomide; ETN, ertanercept; IFX: infliximab; AZ, azathioprine; TAC, tacrolimus; MMF, mycophenolate mofetil; CNS, central nervous system.
†Magnetic resonance imaging consistent with cerebral vasculitis; might have been caused by coexistent *Aspergillus* infection.

Table 2. Diagnostic test results, management, and outcome for persons with *Anncaliia algerae* myositis*

Variable†	Case-patient A	Case-patient B	Case-patient 1 (*3*)	Case-patient 2 (*5*)	Case-patient 3 (*8*)
Hemoglobin, g/L (130–180)‡	95	122	ND	100	96
Lymphocytes $\times 10^9$/L (1.5–4.0)	0.3	0.4	ND	0.2	0.1
CK U/L, peak (<200)	2,028	6,630	6,337	685	441
ALT, U/L (<45)	154	93	ND	66	50
AST, U/L (<45)	320	210	ND	129	70
ESR, mm/hr (0–14)	85	26	ND	38	30
CRP, mg/L (<3)	152	134	ND	16	41
Serum albumin, lowest, g/L (33–48)	21	19	ND	19	14
Serum creatinine, μmol/L (60–100)§	44	202	ND	81	216
Urinary protein, g/24 h (<0.1)¶	0.56	1.8	ND	NT	1.53
Fecal stain#	No microsporidia	NT	ND	No microsporidia	No microsporidia
Neurophysiology/EMG	Myopathy; axonal neuropathy	Myopathy; axonal neuropathy	ND	Myopathy; axonal neuropathy	Myopathy; axonal neuropathy
Negative biopsy/fluid sites	CSF	Esophagus, stomach, duodenum	Tracheal aspirate	Bone marrow, lung, rectum, BAL, CSF	NT
Positive biopsy sites	Vastus lateralis	Vastus lateralis	Quadriceps femoris**	Deltoid, tongue	Deltoid
A. algerae sequence	Yes	Yes	Yes	Yes	Yes
Immunosuppression reduced	Yes	Yes	Yes	Yes	Yes
Albendazole	Yes	No	Yes	No	Yes
Outcome	Survived >18 mo	Died, aspiration pneumonia	Died, stroke	Died, palliated	Died, aspiration pneumonia

*ND, not described in publication; CK, creatinine kinase; ALT, alanine transaminase; AST, aspartate transaminase; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; EMG, electromyography; NT, not tested; CSF, cerebrospinal fluid; BAL, bronchoalveolar lavage.
†Biochemical and hematologic values are from the most recent visit to hospital, unless otherwise indicated. Reference values are in parentheses.
‡Anemia was normocytic.
§Case-patient A: serum creatinine was higher (150 μmol/L) at the first admission; case-patient B: baseline creatinine was ≈160μμmol/L; case-patient 3: preexisting renal impairment, with transplant.
¶Case-patient A: urinary myoglobin was negative; case-patient B urinary albumin:creatinine ratio 8 mo previously was normal; case-patient 3: urinary protein 2 mo previously was 0.68 g/24 h.
#Modified trichrome stain on concentrated feces.
**Component of quadriceps femoris not specified in publication.

were begun. Fevers developed, and the patient's condition continued to deteriorate for a week, reached a nadir, and progressively improved, despite cessation of albendazole after 3 weeks because of vomiting and abnormal liver function test results. Creatine kinase (CK) normalized at 12 months after hospital admission. At 18 months follow-up, there was no indication of infection, and the patient had regained full strength. His rheumatoid arthritis was controlled with NSAIDs, prednisone (5 mg/d), and injectable gold. Follow-up neurophysiology showed full recovery of nerve conduction velocities and amplitudes.

Case-patient B

In 2006, a 66-year-old man from suburban Sydney, New South Wales, sought care at hospital for a 5-week history of progressive generalized muscle pain, lethargy, weight loss, poor appetite, low mood, difficulty sleeping, fevers, pain in the mouth, difficulty swallowing, and discomfort on opening the mouth. He had rheumatoid factor–positive rheumatoid arthritis diagnosed 2 years previously, with no extra-articular manifestations. He was treated with methotrexate for 22 months, with the dose gradually increased to 25 mg per week. This drug was stopped 2 weeks before hospital admission because of concerns about toxicity. Prednisone was begun 4 weeks before hospital admission (maximum dose 50 mg/d for 1 week).

The patient had fevers (maximum temperature 39.5°C); tachycardia; oropharyngeal mucositis; glossitis; macroglossia; a tongue ulcer; decreased tongue movements; trismus; peripheral edema; tenderness on palpation of the limbs; mild bilateral facial weakness; and upper and lower limb weakness, marked in the hands. Pain limited grading of the weakness; however, mobilization was possible. Sensation was normal, and reflexes were present.

Investigation results included methotrexate level <0.1 μmol/L, IgG (subtype 1) 1.65 g/L (reference 4.9–11.4) and (subtype 2) 0.78 g/L (reference 1.50–6.4), and troponin I 0.7 μg/L (reference <0.1) (Table 2). The urine contained no cellular casts. Transthoracic echocardiography showed normal left ventricular function. Noncontrast CT scan of the brain showed no abnormalities. Neurophysiologic studies revealed a mild axonal type neuropathy, and electromyography results were consistent with an active myopathic process in multiple muscles.

Broad-spectrum antimicrobial drug therapy, acyclovir, and intravenous immunoglobulin were given. The corticosteroid dose was reduced with a weaning regimen. Vastus lateralis muscle biopsies were performed ≈2 weeks into

hospitalization. Histopathologic examination demonstrated large numbers of round, predominantly extracellular, 3- to 4-μm organisms. Specimens from esophageal, gastric, and duodenal biopsies did not contain organisms. Liposomal amphotericin B was begun to treat a presumed fungal infection.

The patient remained febrile, and his condition worsened. Nasogastric feeding was begun because of bulbar muscle weakness. Several other complications developed, including atrial fibrillation, delirium, and acute renal failure requiring dialysis. He died 4 weeks after admission after a nasopharyngeal bleed that led to aspiration pneumonia. Subsequent electron microscopy of the muscle biopsy demonstrated myositis caused by *A. algerae* (Figure 2). As with case-patient A, the partial sequence of the small subunit ribosomal RNA gene from the muscle biopsy was homologous with *A. algerae*.

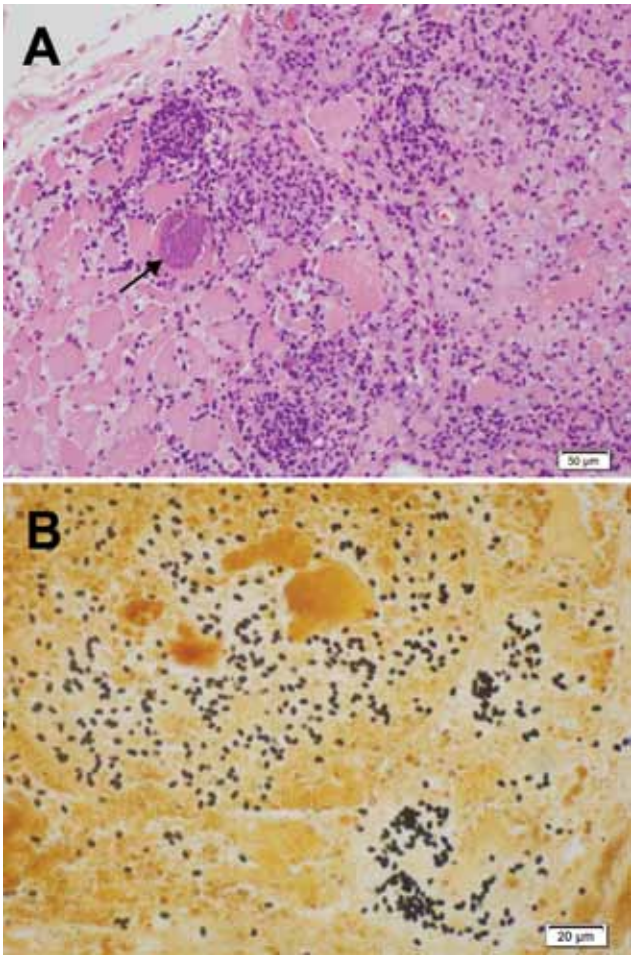

Figure 1. Light micrographs of muscle biopsy tissue from a 67-year-old man (case-patient A), New South Wales, Australia, showing microsporidial myositis caused by *Anncaliia algerae*. A) Necrotizing myositis with prominent inflammation and spores within the necrotic cytoplasm of a myocyte (arrow). Hematoxylin and eosin stain. B) Numerous dark brown to black, 3- to 4-μm ovoid spores in necrotic myocytes. Warthin-Starry stain.

Discussion

In the cases reported here, *A. algerae* myositis caused fever, weight loss, fatigue, generalized muscle weakness and pain, dysphagia, glossitis, peripheral edema, and diarrhea (Table 1). The limb edema might have been multifactorial and associated with local inflammation of the muscle; hypoalbuminemia; and impaired renal function, including proteinuria (*19*). The urinary myoglobin was negative in case-patient A, and urinary sediment staining for microsporidia was either not performed or not documented in any of the case-patients. Three case-patients had diarrhea, but no microsporidia were observed in gut biopsy specimens or in stained fecal samples (*5*,*8*). Although oropharyngeal ulceration and mucositis in case-patients B and 3 might have been caused by medication, it also might represent *A. algerae* infection (*4*,*8*).

The cardiac and central nervous system disorders may be associated with the *A. algerae* infection, related to comorbidities or nonspecific features of severe illness. Case-patient B had delirium and atrial fibrillation with increased troponin I. Case-patient 2 had delirium, seizures, and an increased troponin I, 1.32 μg/L (reference <0.01). Seizures were associated with *Trachipleistophora anthropophthera* dissemination to the brain (*16,17*). However, the signs of cortical vasculitis and ischaemia on magnetic resonance imaging of the brain in case-patient 2 may have been due to angio-invasive fungal infection, considering the concurrent *Aspergillus* lung abscess. Case-patient 1, who had type 2 diabetes mellitus, died of a cerebrovascular infarction (*3*).

Laboratory indicators of muscle damage and inflammation included elevated CK, aspartate aminotransferase, alanine aminotransferase, erythrocyte sedimentation rate, and C-reactive protein and decreased serum albumin (*20*) (Table 2). Peak CK levels were lower in the transplant patients. Lymphopenia and decreased immunoglobulin levels might have been related to preadmission medications, including corticosteroid therapy (*21*). Although the underlying causes are yet to be determined, anemia and proteinuria were common features. The resolution of the neuropathy on nerve conduction studies in case-patient A might indicate recovery from peripheral nerve infection.

After fixation in formalin and staining, biopsy specimens can be examined under light microscopy (*3*,*5*). Muscle biopsies show necrotic myocytes containing spores, which stain poorly with hematoxylin and eosin (Figure 1, panel A) (*5*). Silver stains, particularly the Warthin-Starry, clearly demonstrate 3- to 4-μm ovoid spores (Figure 1, panel B) (*5*,*22*). In case-patient 1, muscle was immunofluorescently stained by using antibodies to *A. algerae* (*3*). The differential diagnoses include the yeast forms of fungi and leishmania amastigotes.

Transmission electron microscopy of biopsies can confirm a diagnosis of microsporidial myositis and

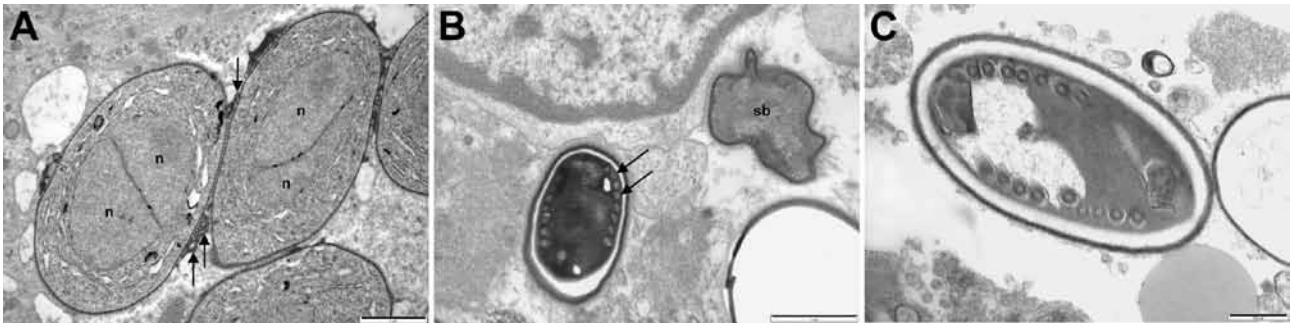

Figure 2. Electron micrographs of muscle biopsy tissue from a 66-year-old man (case-patient B) (A) and a 67-year-old man (case-patient A) (B,C) showing *Anncaliia algerae*. A) Early proliferative stage meronts with diplokaryotic nuclei (n) and vesiculotubular appendages (arrows) attached to the plasmalemma. Scale bar indicates 1 µm. B) Degenerate crenated sporoblast (sb) and a mature spore with visible coils of the polar tubule (arrows). Scale bar indicates 1 µm. C) Mature spore with 9 polar tubule coils in a single row, pale endospore, and a dense exospore. Scale bar indicates 500 nm.

differentiate *Anncaliia* spp. from *Trachipleistophora hominis* (*3*,*5*,*23*). Although electron microscopy is highly specific for microsporidiosis, the number of organisms within a biopsy specimen limits sensitivity. Features that define *Anncaliia* spp. include binary fission; diplokaryotic nuclei; lack of a parasitophorous vacuole, with the thickened plasmalemma of meronts and sporonts in direct contact with host cytoplasm; and vesiculotubular appendages projecting from the plasmalemma (Figure 2, panel A) (*5*,*23*). Degenerate sporoblasts and spores appear crenated (Figure 2, panel B). Mature spores have a dense exospore coat, a pale endospore, and single rows of 8–11 polar tubule coils (Figure 2, panels B, C). This number of polar tubule coils and the absence of deep protoplasmic extensions are features of *A. algerae*; however, species identification also relies on nucleic acid sequence homology (*5*,*23*). DNA extracted from clinical specimens can be amplified by PCR (*3*,*5*). The primers target areas of the large and small subunit ribosomal RNA genes and sequence analysis enables identification of the organism.

For case-patient A, who survived, a muscle biopsy was performed as soon as it became evident that empiric corticosteroid and intravenous immunoglobulin therapy was not effective. The corticosteroid dose was minimized; etanercept, methotrexate, and leflunomide had been stopped; and leflunomide was washed out with cholestyramine (*24*). Measures were taken to avoid aspiration pneumonia. On histopathology, microsporidiosis was considered, and a regimen with albendazole was begun. For case-patients B and 2, in whom microsporidiosis was diagnosed postmortem, antifungal agents were used. In vitro studies indicated that amphotericin B had no activity against the tested microsporidia (*25*).

Albendazole targets microtubules and prevents formation of intranuclear spindles in microsporidia (*25*). Microsporidial myositis caused by species other than *A. algerae* has been responsive to albendazole alone or in combination with other agents (*9*,*12*,*18*). Although albendazole has not been tested for activity against *A. algerae*, the survival of case-patient A suggests clinical effectiveness. Side effects include headache, gastrointestinal upset, and elevated levels of transaminases (*25*).

In case-patient A and a case-patient with *Trachipleistophora* myositis, the combination of albendazole, sulfadiazine, and pyrimethamine was successful (*12*). However, the contribution of sulfadiazine and pyrimethamine to the regimen is unclear because in vitro testing has indicated these agents are ineffective against the microsporidian *Encephalitozoon cuniculi* (*26*). Metronidazole, itraconazole, atovaquone, and clindamycin were among the medications used for case-patient 1 (*3*). The activity of these agents against microsporidia varies (*25*,*27*). Fumagillin and its analogs have been used topically to treat corneal infections and orally for gastrointestinal microsporidiosis (*27*), but because of possible side effects, they have not been used to treat microsporidial myositis (*27*).

Like other microsporidia, *A. algerae* depends on host cells to obtain metabolites (*28*). Genes that code for a diversity of kinases and transport proteins are regarded as an adaptation to the broad range of insect hosts (*7*,*28*). *A. algerae* was previously investigated as a biologic control agent for mosquitoes because heavily infected larvae rarely survive to adulthood (*7*). Other than in humans, no naturally occurring infections have been identified in mammals.

Mosquitoes become infected during their aquatic immature stages, either through exposure to spores in the water or by feeding on dead insect hosts (*7*,*29*). Infection occurs when the polar tubule is extruded from the spore and sporoplasm is introduced into the host cell through the tubule (*7*). In the initial proliferative phase, asexual reproduction occurs (*7*,*29*). Sporoloblasts then mature into spores (*7*,*29*). Although spores survive outside the host, they become nonviable when dried at room temperature for 5 days (*30*,*31*). Information is limited about the life cycle of

A. algerae in Australia. *Culex sitiens* Wiedemann, an abundant coastal mosquito that usually bites birds, is currently the only documented insect host (*7*,*32*).

The exact mode of transmission of *A. algerae* to humans is yet to be established. Contact with water that contains spores generated in insect hosts is thought to be the most likely mechanism (*6*,*33*). Mechanical inoculation of spores by crushing a feeding mosquito into a bite wound has also been proposed as means a transmission (*3*,*7*). However, spores from mosquito tissue have substantially lower germination rates in human plasma or serum than those first exposed to water (*34*). Direct inoculation from a mosquito bite also is considered less likely because the saliva of laboratory-infected mosquitoes did not contain *A. algerae* (*30*). The Australian case-patients lived on the eastern seaboard, within 500 km of each other, in a warm temperate environment. They were exposed to mosquito habitat through proximity to bodies of water on golf courses or coastal woodland.

All the 5 case-patients reviewed were taking immunosuppressant therapy, and 2 had coexistent diabetes mellitus. Before infection, methotrexate, leflunomide, corticosteroids, and antitumor necrosis factor a therapy were used as treatments in the case-patients with rheumatoid arthritis. Epidemiologic studies have suggested that methotrexate and leflunomide are not associated with a substantial increase in serious infection, although this lack of association does not exclude a contribution with combination immunosuppression (*35*–*37*). Antitumor necrosis factor a therapy and corticosteroids are associated with increased risk (*35*,*38*,*39*). The severity of rheumatoid arthritis is also a risk factor for serious infection (*35*). However, before the addition of corticosteroids 4 weeks before hospital admission, methotrexate was the only immunosuppressive agent used by case-patient B, and he had no erosive or extraarticular disease.

Case-patients 2 and 3 were solid-organ transplant recipients (*5*). The immunosuppressive agents used in the months preceding the development of *A. algerae* myositis were mycophenolate mofetil, tacrolimus, azathioprine, and corticosteroids. Systemic infections caused by *Encephalitozoon* spp. in solid-organ transplants have been associated with these therapies (*40*). Studies with *E. cuniculi* have indicated the importance of cytotoxic CD8+ T-cell immunity for protection against severe and persistent infection (*1*).

A. algerae myositis is uncommon infection and has a high case-fatality rate. Whether it has emerged because previous cases have gone unrecognized or because epidemiologic factors have changed is unclear. *A. algerae* transmission occurs possibly through contact with water that contains spores amplified by insect hosts. The signs and symptoms of *A. algerae* infection primarily indicate myositis. Whether other organs and tissues may be infected has yet to be confirmed. In case-patient A, who survived, microsporidiosis was diagnosed on biopsy, and clinical management was based on the reduction of immunosuppression, measures to prevent complications, and a treatment regimen based on albendazole. Genomic analysis for drug targets and susceptibility testing of *A. algerae* may lead to increased therapeutic options. *A. algerae* myositis is an additional differential diagnosis to be considered in immunocompromised persons.

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Dr Watts is an infectious diseases physician, clinical microbiologist, and National Health and Medical Research Council postgraduate scholar affiliated with the Centre for Infectious Diseases and Microbiology Public Health; Pathology West–Institute for Clinical Pathology and Medical Research Westmead, Westmead Hospital, New South Wales, and the Marie Bashir Institute for Infectious Diseases and Biosecurity, University of Sydney. His research interests include zoonotic and parasitic infections.

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Address for correspondence: Matthew R. Watts, Centre for Infectious Diseases and Microbiology, Westmead Hospital, University of Sydney, Darcy Rd, Westmead, NSW 2145, Australia; email: watts.idmicro@gmail.com

Human Antibody Responses to Avian Influenza A(H7N9) Virus, 2013

Li Guo,[1] Xi Zhang,[1] Lili Ren,[1] Xuelian Yu,[1] Lijuan Chen,[1] Hongli Zhou, Xin Gao, Zheng Teng, Jianguo Li, Jiayu Hu, Chao Wu, Xia Xiao, Yiyi Zhu, Quanyi Wang, Xinghuo Pang, Qi Jin,[2] Fan Wu,[2] and Jianwei Wang[2]

Understanding host antibody response is crucial for predicting disease severity and for vaccine development. We investigated antibody responses against influenza A(H7N9) virus in 48 serum samples from 21 patients, including paired samples from 15 patients. IgG against subtype H7 and neutralizing antibodies (NAbs) were not detected in acute-phase samples, but ELISA geometric mean titers increased in convalescent-phase samples; NAb titers were 20–80 (geometric mean titer 40). Avidity to IgG against subtype H7 was significantly lower than that against H1 and H3. IgG against H3 was boosted after infection with influenza A(H7N9) virus, and its level in acute-phase samples correlated with that against H7 in convalescent-phase samples. A correlation was also found between hemagglutinin inhibition and NAb titers and between hemagglutinin inhibition and IgG titers against H7. Because of the relatively weak protective antibody response to influenza A(H7N9), multiple vaccinations might be needed to achieve protective immunity.

In March 2013, an emerging virus, influenza A(H7N9), of novel avian origin was identified in humans in China (*1,2*). As of August 1, 2013, a total of 134 infections and 45 deaths had been reported (www.moh.gov.cn/zhuzhan/yqxx/201309/1f465a32fa8b476c93a4075e07742685.shtml). Other avian influenza virus subtypes, including H5N1, H6N1, H7N1, H7N2, H7N3, H7N7, H9N2, and H10N7 (*3–10*), have been transmitted directly from poultry to humans and, with the exception of 1 subtype H7N7 infection that caused death, cause mild symptoms (*5–8*). In contrast, most subtype H7N9 infections cause severe lower respiratory infections; estimated mortality rate is 30% (*2*). Clinical observations indicate that subtype H7N9 tends to cause severe symptoms in elderly (>60 years of age) patients, and these symptoms last longer (median 41.7 days) than those caused by subtype H5N1 infections (median 18.7 days) (*11*). The potential for subtype H7N9 virus to cause a pandemic among humans raises great public health concern (*12–14*).

Host immunity affects disease severity, disease duration, and vaccine response with regard to influenza virus infections and viral pathogenesis. Neutralizing antibodies (NAbs) are one of the most critical factors in virus clearance and disease outcome (*15,16*). NAbs, whether given as postexposure treatment or preexposure prophylaxis, protect animals from infection (*17*). In contrast, low-avidity antibodies against influenza virus might have adverse effects during infection (*15*). To determine human antibody responses to influenza A(H7N9) virus, we examined serum samples from infected patients.

Methods

Patients and Samples

From March 30 through August 12, 2013, a total of 48 serum samples were collected from 21 patients with influenza A(H7N9) virus infection in Shanghai and Beijing, China: 21 acute-phase (collected ≤7 days after symptom

Author affiliations: MOH Key Laboratory of Systems Biology of Pathogens, Beijing, People's Republic of China (L. Guo, L. Ren, J. Li, Q. Jin, J. Wang); Institute of Pathogen Biology, Beijing (L. Guo, L. Ren, H. Zhou, X. Gao, J. Li, C. Wu, X. Xiao, Q. Jin, J. Wang); Shanghai Municipal Center for Disease Control and Prevention, Shanghai, People's Republic of China (X. Zhang, X. Yu, Z. Teng, J. Hu, Y. Zhu, F. Wu); and Beijing Municipal Center for Disease Control and Prevention, Beijing (L. Chen, Q. Wang, X. Pang)

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[1]These authors contributed equally to this article and are co–first authors.

[2]These authors contributed equally to this article and are co–senior authors.

onset), 18 convalescent-phase (collected >14 days after symptom onset), and another 9 samples (collected 102–125 days after symptom onset from patizents from whom paired samples were collected). Because of some patient deaths, ethical considerations, and the difficulty of following up with patients in isolation units, most convalescent-phase samples were collected from patients immediately before hospital discharge. Hence, paired convalescent-phase serum samples were obtained from 15 patients at various times (17–37 days after symptom onset). Only acute-phase serum samples were obtained from the other 6 patients. For the 15 patients for whom paired serum samples were available, 2 convalescent-phase samples were collected from 3 patients at different times (Table 1) and 1 convalescent-phase sample was collected from each of the other 12 patients. To trace serum conversion, an additional 9 serum samples were collected 102–125 days after symptom onset from the patients for whom paired serum samples were available.

For confirmation of influenza A(H7N9) virus infection, respiratory samples (throat swab or sputum samples) were obtained from each patient at the time of hospital admission. From these samples, total RNA was extracted by using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany); RNA for subtype H7N9 was detected by a real-time reverse transcription PCR (RT-PCR) kit provided by the Chinese National Influenza Center, Chinese Center for Disease Control and Prevention. In accordance with protocol recommendations, specimens were considered positive if the cycle threshold was ≤38.0. Clinical data were retrieved through a retrospective review of the medical records for each patient. The severity of subtype H7N9 infections was determined according to the guidelines for the management of pneumonia in children and adults (*18,19*).

For use as controls, 100 serum samples were collected from healthy blood donors in Beijing, China, from October through December 2008, and another 77 serum samples were collected from open-market poultry workers in Shanghai, China, in May and June 2013. All procedures involving subtype H7N9 virus were performed in a Biosafety Level 3 laboratory.

Table 1. Clinical characteristics of patients infected with influenza A(H7N9) virus, China, 2013*

Patient no.	Age, y/sex	Concurrent conditions	Days of admission after symptom onset	ICU	Complications	Mechanical ventilation	Days to commencing antiviral treatment after admission	Oseltamivir	Outcome
1	66/M	Arthritis, prostatitis	6	No	No	No	6	Yes	R
2	73/M	Chronic bronchitis	7	No	No	No	8	Yes	R
3	67/M	Hypertension	5	No	No	No	5	Yes	R
4	62/M	Hypertension	4	NA	No	No	7	Yes	R
5	76/F	Diabetes mellitus, hypertension, coronary disease	7	NA	No	Yes	No	No	R
6	81/F	Coronary disease, rheumatoid disease	4	NA	No	No	6	Yes	R
7	83/F	Diabetes mellitus, hypertension	6	Yes	Respiratory failure, MODS	Yes	8	Yes	D
8	68/M	Diabetes mellitus, hypertension	6	No	No	No	7	Yes	R
9	53/M	Diabetes mellitus	7	Yes	Respiratory failure	Yes	8	Yes	R
10	54/M	Hypertension	5	No	No	No	5	Yes	R
11	79/M	Hypertension	5	NA	No	Yes	9	Yes	R
12	47/M	None	6	No	No	Yes	6	Yes	R
13	75/F	Diabetes mellitus, hypertension	6	NA	No	Yes	No	No	R
14	61/F	None	3	Yes	Respiratory failure, MODS	Yes	NA	Yes	D
15	7/F	None	1	Yes	No	Yes	1	Yes	R
16†	4/M	None	6	No	No	No	6	Yes	R
17	52/M	Thyroid tumor	7	No	Respiratory failure	Yes	7	Yes	D
18	77/M	Hypertension, atrial fibrillation	5	NA	NA	Yes	5	Yes	D
19	56/M	NA	3	Yes	ARDS, DIC, hemorrhagic shock	Yes	3	Yes	D
20	89/M	Hypertension, diabetes mellitus	4	NA	MODS	No	No	No	D
21	80/M	Coronary disease, cirrhosis	7	NA	Respiratory failure, acute heart failure	Yes	7	Yes	D

*ICU, admission to intensive care unit; R, recovered; NA, not available; D, died; MODS, multiple organ dysfunction syndrome: ARDS, acute respiratory distress syndrome; DIC, disseminated (or diffuse) intravascular coagulation.

Written, informed consent was obtained from all participants. The study was approved by the required ethics review boards.

ELISA

Bound IgG titers for serum samples were evaluated by using an influenza virus hemagglutinin IgG ELISA. The hemagglutinin proteins of subtype H7N9 (A/Anhui/1/2013[H7N9]) and of seasonal subtypes H1N1 (A/California/04/2009[H1N1]) and H3N2 (A/Brisbane/10/2007[H3N2]) viruses were expressed in human embryonic kidney 293 cells (H3 hemagglutinin) or a baculovirus-insect cell system (H1 and H7 hemagglutinin) and purified by using 6× His tag (Sino Biologic, Beijing, China). The hemagglutinin activities of the recombinant H1, H3, and H7 hemagglutinin proteins were confirmed by using an Octet system (FortéBIO, Menlo Park, CA, USA) (data not shown). Recombinant H1, H3, and H7 hemagglutinins were used as coating antigens in all ELISA tests.

For this process, 96-well plates (Costar, Bethesda, MD, USA) were coated overnight with the purified hemagglutinins (25 ng/well) in 100 μL of coating buffer (0.05 mol/L carbonate/bicarbonate, pH 9.6) at 4°C and blocked with 300 μL of 1% bovine serum albumin for 2 h at 37°C. The amount of coating protein was optimized by a chessboard titration protocol (*20*). Serial-diluted serum samples (100 μL each) were added in duplicate for 1 h at 37°C. After 6 washes with 300 μL of phosphate-buffered saline containing 0.5% Tween-20, 100 μL of a 1:40,000 dilution of horseradish peroxidase–conjugated goat anti-human IgG (Sigma-Aldrich, St. Louis, MO, USA) was added to each well for 1 h at 37°C. The plates were then washed 6 times with 300 μL of phosphate-buffered saline containing 0.5% Tween-20 and incubated with 100 μL of substrate 3, 3′, 5, 5′-tetramethylbenzidine (Sigma-Aldrich) at 37°C for 15 min. The reactions were terminated by adding 50 μL of 2 mol/L hydrogen sulfate. The absorbance at 450 nm (A450) was determined for each serum sample. To evaluate the background signal, we used bovine serum albumin instead of human serum as the background signal control. The cutoff value was defined as twice the background signal (*21*). The antibody end-point titer was defined as the reciprocal of the highest dilution of serum that had a reading above the cutoff value.

Antibody Avidity Analysis

For avidity analysis, an ELISA assay was performed as described (*22*). Serum was added at a dilution with an expected A450 of ≈1.0. Avidity was determined by incubating samples with urea at 4, 5, 6, and 7 mol/L for 30 min at room temperature before washing, followed by incubation with the horseradish peroxidase–conjugated goat anti-human IgG. The avidity index was defined as the ratio of the absorbance with urea to that without urea (*15,22*).

Microneutralization Assay

Microneutralization assays were performed as described (*21*). In brief, serial dilutions of serum (starting at 1:10) were preincubated with 100 doses (50% tissue culture infective doses) of the influenza A/Anhui/1/2013 (H7N9) strain. After 2 h of incubation, the mixture was incubated with MDCK cells in 96-well plates (Costar). The virus/serum mixtures were removed after 2 h, and serum-free Dulbecco modified Eagle medium with 1 μg/μL of TPCK-trypsin (L-1-tosylamide-2-phenylethyl chloromethyl ketone-treated trypsin) (Sigma-Aldrich) was added to each well. The cytopathic effects were evaluated 96 h after incubation at 35°C in 5% CO_2. For each antibody dilution, 4 duplicate wells were used. NAb titers were defined as the reciprocal of the highest serum dilution at which 50% of wells were protected.

Hemagglutination Inhibition Assay

Hemagglutination inhibition (HI) assays were performed by using a b-propriolactone–inactivated A/Anhui/1/2013 (H7N9) strain according to World Health Organization protocol (www.who.int/influenza/gisrs_laboratory/cnic_serological_diagnosis_hai_a_h7n9.pdf). All HI assays were performed in V-bottom 96-well plates with 1% horse erythrocytes (*23*).

Statistical Analyses

The Mann-Whitney U test and Student *t*-test were used for continuous variables. Serum IgG titers between groups were tested by using the Wilcoxon rank-sum test. HI titers were tested by using the Student *t*-test. All statistical calculations involving the geometric mean titers (GMTs) of antibodies were performed with log-transformed titers. Correlations between acute-phase serum antibodies against H1 and H3 and convalescent-phase serum antibodies against H7 were assessed by using the Spearman rank correlation coefficient test. $p<0.05$ was considered statistically significant.

Results

A total of 21 patients with influenza A(H7N9) virus infection were enrolled in this study; 19 were from Shanghai and 2 were from Beijing, 15 were male and 6 were female, 19 were adults (47–89 years of age, median 68 years, mean 68.4 years), 1 was a 7-year-old girl, and 1 was a 4-year-old boy (Table 1). Only the 4-year-old boy experienced mild infection; 7 adults died. Patients were hospitalized 1–7 days after symptom onset. Major clinical manifestations included fever, cough, sputum, sore throat, myalgia, chills, dyspnea, and diarrhea. Most patients who died had complications of respiratory failure, multiple organ failure, or acute respiratory distress syndrome. Laboratory tests showed that some patients

Table 2. Laboratory data for patients infected with influenza A(H7N9) virus, China, 2013*

Patient no.	H7N9 C_t for FluA, H7, N9 tests	Concomitant infection	ALT	AST	Creat	BUN	WBC	Temp	Serum collection, d†
1	28.47, 32.87, 32.55	No	39	77	74.3	NA	3.5	39.5	20
2	23.46, 24.34, 22.91	No	15	41	75.4	4.3	3.2	39.3	24
3	21.44, 34.76, 29.70	No	62	45	84.2	5.78	5.95	39.7	26
4	24.41, 25.91, 28.59	No	NA	NA	NA	NA	10.99	39.7	35
5	34.64, 31, Negative	No	36	40	65.0	6.8	4.7	39.0	35
6	31.99, 26.1, Negative	No	NA	NA	NA	NA	4.9	40.1	25, 31
7	29.89, 30.91, 27.63	*Candida albicans*	66	79	84.0	7.5	6.62	38.2	17, 27
8	30.26, 30.96, 30.6	No	31	35	84.0	41.0	4.1	38.9	25
9	37.49, 36.6, 36.95	No	33	78	102.0	43.0	5.64	39.5	24, 30
10	29.7, 26.43, 21.68	No	129	89	85.0	4.76	8.62	38.6	29
11	34.88, 36.18, 18.43	No	14	39	196.0	6.3	8.31	38.6	33
12	33.91, 33.66, Negative	No	86	144	83.0	3.6	7.11	39.0	37
13	30.48, 37.94, Negative	No	37	55	46.0	6.8	8.0	38.5	18
14	16.3, 16.1, 16.4	No	NA	NA	NA	NA	3.79	40.0	31
15	30.8, 33.0, 32.7	No	NA	NA	NA	NA	6.96	38.6	33
16	37.26, 38.48, 27.65	No	NA	NA	NA	NA	7.68	39.0	NA
17	30.73, 32.1, 28.85	No	100	30	NA	NA	11.2	39.5	NA
18	36.05, 35.75, 37.52	No	132	239	123.2	12.8	8.9	39.4	NA
19	27.21, 30.4, 30.62	*Acinetobacter baumannii*	25	19	88.7	2.85	7.93	39.8	NA
20	28.72, 27.76, 30.08	Fungus	NA	NA	NA	NA	9.5	38.5	NA
21	27.84, 35.23, 29.6	No	36	207	162.7	17.94	4.06	36.5	NA

*C_t, cycle threshold; ALT, alanine transaminase, U/L; AST, aspartate transaminase, U/L; Creat, creatinine, μmol/L; BUN, blood urea nitrogen, μmol/L; WBC, white blood cell (leuckocyte) count, $\times 10^9$/L; temp, body temperature, °C; NA, not available.
†Convalescent-phase; days after symptom onset.

had liver or renal damage (Table 2). Major radiographic findings included pneumonia, increased markings, fuzzy patch lesions, and patch effusion shadows in lungs (Table 3). Of the 21 patients, 18 received antiviral treatment. The cycle threshold of real-time RT-PCR detection of subtype H7N9 virus ranged from 21.44 to 37.49 (median 30.48, mean 30.69) for the FluA (M gene), 24.34 to 38.00 (median 31.55, mean 31.92) for the H7, and 18.43 to 37.52 (median 29.65, mean 29.27) for the N9 tests, indicating a relatively low to medium viral load in most patients. Concomitant fungal infections occurred in 2 patients, and a concomitant *Acinetobacter baumannii* infection occurred in 1 patient (Table 2).

A hemagglutinin ELISA was used to determine IgG titers (Figure 1). The end points for antibodies against hemagglutinin were obtained by determining A450 at a 2-fold serial dilution of each serum sample from patients and controls, starting at a dilution of 1:50 (Figure 1, panel A). High levels of IgG against H1 and H3 hemagglutinins were detected in the 2 control groups, as were GMT values of 2,070.40 and 1,118.10 in open-market poultry workers, and 1,476.80 and 1,448.50 in healthy blood

Table 3. Radiographic findings for patients infected with influenza A(H7N9) virus, China, 2013

Patient no.	Radiographic findings
1	Increased markings in both lungs, cloud floccule shadow in left lower zone
2	Increased markings in both lungs, visible small fuzzy patch shadow at right lower diaphragm
3	Pneumonia in left upper lung
4	Patch consolidation with dim edges in middle and lower zones of right lung
5	Pneumonia with partial consolidation in right lower lobe
6	Pneumonia in right lower lung
7	Increased markings in both lungs
8	Increased markings in both lungs. Visible patch lesions and strip lesions in 2 lower lobes
9	Patch lesions beside the right lung hilum, together with nodules and fuzzy strip shadows. Fuzzy patch lesions in left middle zone
10	Diffused effusion in both lungs
11	Increased marking in both lungs, fuzzy patch shadows in middle and upper lobes of right lung
12	No active lesion in either lung
13	Patch effusion shadows in right lower lung
14	Pneumonia in both lungs
15	Pneumonia in both lungs
16	Not applicable
17	Inflammation in the right lower lung
18	Pneumonia, increased markings in both lungs
19	Pneumonia, increased markings in both lungs
20	Fuzzy shadow in the left lower lung
21	Inflammation and consolidation in both lungs

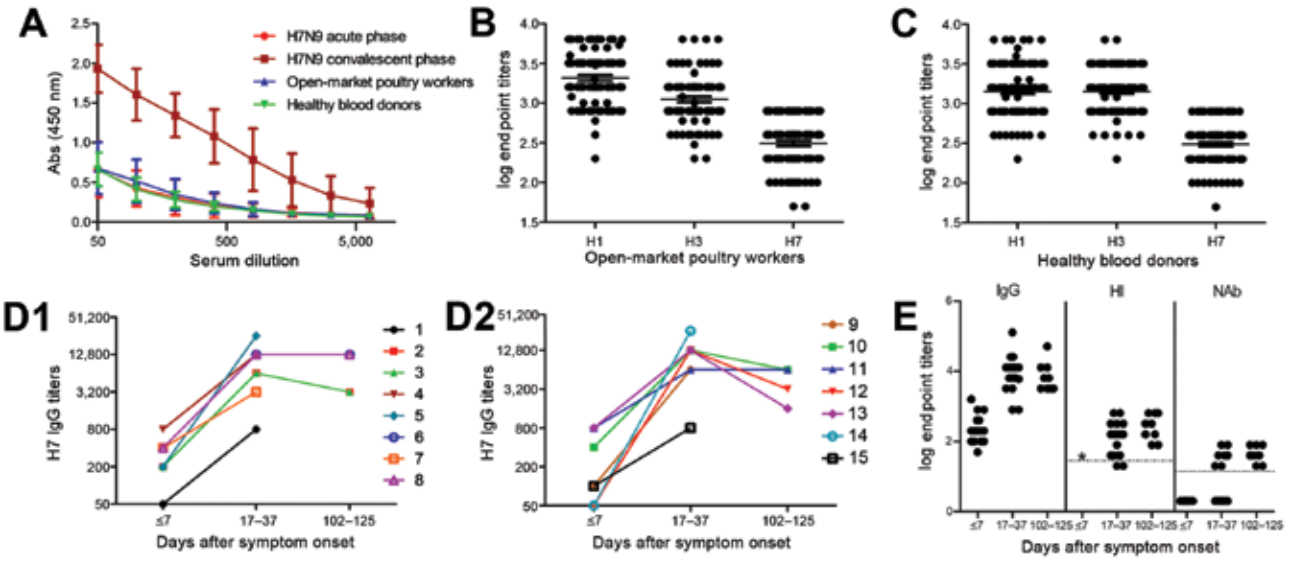

Figure 1. Serum antibodies (Abs) in patients infected with influenza A(H7N9) virus and in control populations (poultry-market workers and healthy blood donors), China, 2013. A) Dilution curves of IgG against subtype H7 in serum samples. Bars indicate SE. B and C) Titers of IgG against H7, H1, and H3 in poultry-market workers (B) and healthy blood donors (C). D) Increasing titers of IgG against subtype H7 after symptom onset in patients from whom paired serum samples were collected. E) Levels of IgG against H7, neutralizing antibodies (NAbs), and hemagglutination inhibition (HI) in serum samples after symptom onset. IgG against hemagglutinins of H7 and seasonal influenza A viruses (subtypes H1 and H3) in patients with subtype H7N9 virus infection and control populations were titrated by ELISA by using recombinant hemagglutinin antigens. NAbs were assessed by microneutralization assay the influenza A/Anhui/1/2013 (H7N9) strain. HI antibodies (Abs) were assessed by HI assay that used a b-propriolactone–inactivated influenza A/Anhui/1/2013 (H7N9) strain. B–E) Serum IgG, NAb, and HI titers were transformed to $\log_{10}$. For NAb-negative samples, titers of 2 were used for $\log_{10}$ transformation. Serum with titers >40 were considered HI positive for H7-specific antibody. The HI dotted line denotes a titer of $\log_{10}40 = 1.60$. Serum with titers ≥20 were considered NAb positive for H7-specific antibody. The NAb dotted line denotes a titer of $\log_{10}20 = 1.30$. *, not available.

donors, respectively. These findings suggest preexposure to seasonal influenza subtypes (H1 and H3) by both control groups (Figure 1, panels B, C). The low levels of IgG signal against H7 detected in the control groups (GMT 280.80 for poultry workers and 313.70 for healthy blood donors; median 400.00 for the 2 groups) (Figure 1, panels A–C) most likely resulted from antibody cross-reactivity, thus serving as the baseline of the assay for IgG against H7. The titers of IgG against H7 of the acute-phase serum samples (GMT 282.80, median 400.00) (Figure 1, panel A) did not differ significantly from those of the 2 control groups ($p>0.05$, Mann-Whitney U test).

The tested samples probably contained no preexisting antibodies against the H7 subtype. In contrast, IgG titers in the convalescent-phase samples increased greatly on days 17–37 (GMT 7,412.40, median 9,600.00) (Figure 1, panels A, D, E); GMT was 26.2-fold higher for convalescent-phase than for acute-phase samples. To monitor the antibody dynamics in patients, we further analyzed IgG titers against H7 after patients recovered from subtype H7N9 virus infection. No obvious changes were detected in serum samples collected 102–125 days after symptom onset (GMT 7,465.80, median 6,400.00) ($p>0.05$, Wilcoxon rank-sum test) (Figure 1, panel D).

NAbs against subtype H7N9 virus were not detectable in serum from poultry-market workers or healthy blood donors (data not shown). No NAbs were detected in samples collected before day 28, although they were detected in most samples (7 of 9 patients) collected 29–37 days after symptom onset (NAb titers ranged from 20 to 80, GMT 40, median 40) (Figure 1, panel E). This finding differs from that observed for influenza A(H1N1)pdm09 and subtype H5N1 virus infections, in which NAbs were detected 14–21 days after symptom onset (*16,24*). NAbs were positive in all serum samples collected from the 9 recovered patients 102–125 days after symptom onset (NAb titers 20–80, GMT 40, median 40). However, the NAb titers did not significantly increase compared with those of the convalescent-phase samples collected at 17–37 days (Figure 1, panel E) ($p = 0.906$, Student *t*-test).

Because we lacked sufficient blood samples, we did not analyze HI in acute-phase serum. HI titers in convalescent-phase serum were measured in parallel to those in serum obtained at 102–125 days. HI titers ranged from 20 to 640 (GMT 117.60, median 160.00) for the convalescent-phase serum collected at 17–37 days and from 80 to 640 (GMT 260.00, median = 320.00) for serum collected at 102–125 days. The differences were not significant ($p = 0.886$, Student *t*-test) (Figure 1, panel E).

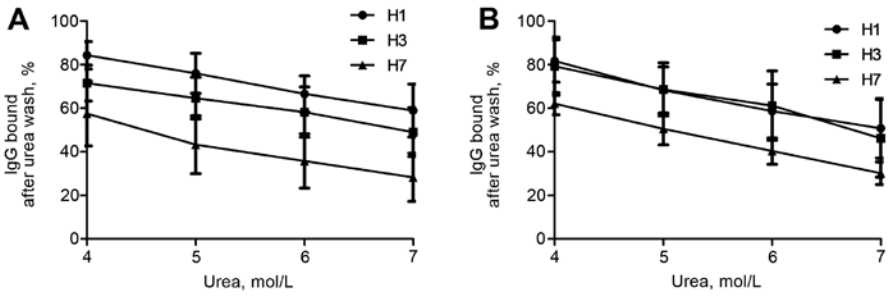

Figure 2. Avidity analysis of antibodies in patients infected with influenza A(H7N9) virus, China, 2013. The avidities of IgG against influenza viruses were determined by ELISA assay with 4–7 mol/L urea. Shown are avidities of IgG against H1, H3, and H7 hemagglutinin of convalescent-phase serum samples collected 17–37 days (A) and 102–125 days (B) after symptom onset. Bars indicate SE.

Further characterization of the antibody response by avidity analysis showed that the percentage of IgG bound to H7 was much lower than that bound to H1 or H3 in convalescent-phase serum collected 17–37 days after symptom onset when treated with different urea concentrations in the ELISA assay ($p<0.05$, Student *t*-test) (Figure 2, panel A). Similar results were observed for serum collected 102–125 days after symptom onset (Figure 2, panel B; $p>0.05$, Student *t*-test). These data suggest that IgG avidity to H7 is significantly lower than that to seasonal influenza A viruses.

In parallel to IgG detection for control groups, we also evaluated IgG levels against seasonal influenza virus hemagglutinins H1 and H3 by ELISA of the 15 paired serum samples (Figure 3, panel A). IgG against H1 and H3 was detected in the acute-phase serum (GMT 1,114.00 and 933.30, median 1,600.00 and 800.00, respectively), indicating preexposure of these patients to seasonal influenza virus. IgG titers did not differ significantly between acute-phase serum from patients with subtype H7N9 infection (most were older persons) and that from control groups ($p>0.05$, Mann-Whitney U test; Figure 1, panels B, C). This finding differs from that observed for patients with A(H1N1)pdm09virus infections, among whom high levels of HI antibody against A(H1N1)pdm09virus in older populations has been reported (*25,26*). This disparity might result from different detection methods (IgG vs. HI) and different times and doses of exposure to influenza virus.

Of note, a boost of IgG titers against H1 and H3 in convalescent-phase serum collected at 17–37 days (GMT 13,128.50 and 16,345.40, median 12,800.00 and 16,400.00, respectively) was observed, compared with that of the acute-phase serum, in which a boost was not detected ($p<0.01$, Wilcoxon rank-sum test). IgG titers against H1 were lower in serum collected at 102–125 days (GMT 5,486.40, median 6,400.00) than in serum collected at 17–37 days ($p<0.01$, Wilcoxon rank-sum test) (Figure 3, panel A) but were still higher than those in the acute-phase serum ($p<0.01$, Wilcoxon rank-sum test). Titers of IgG against H3 did not obviously change in serum collected at 102–125 days (GMT 10,972.70, median 12,800.00) compared with that collected at 17–37 days ($p>0.05$, Wilcoxon rank-sum test) (Figure 3, panel A). Moreover, a significant correlation ($R^2 = 3036$, $p = 0.0411$) was observed between the levels of IgG against H3 in acute-phase serum and that against H7 in convalescent-phase serum (Figure 3, panel B) but not between the levels of IgG against H1 in acute-phase serum and IgG against H7 in convalescent-phase serum ($R^2 = 0.009446$, $p = 0.2851$) (Figure 3, panel C), indicating that there is a heterologous boost of IgG against H3 by H7 hemagglutinin.

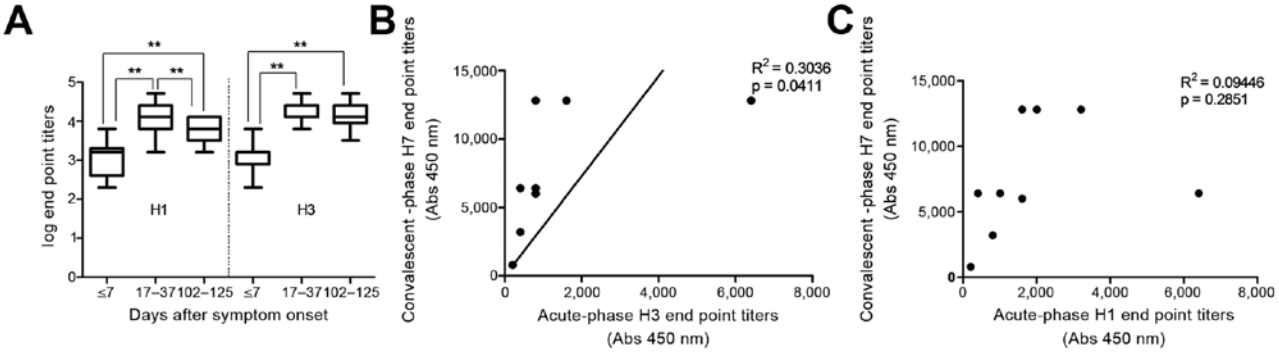

Figure 3. Association between antibody responses against H7 and seasonal subtypes in patients infected with influenza A(H7N9) virus, China. A) Levels of IgG against H1 and H3 in serum samples after symptom onset. IgG in samples taken at acute-phase (≤7 days), convalescent-phase (17–37days,) and 102–125 days after symptom onset were titrated by ELISA with recombinant H1 and H3 hemagglutinin antigens, respectively. IgG titers were transformed to $\log_{10}$. Bars indicate SE. B and C) Correlation between IgG against H3 (B) and H1 (C) in acute-phase serum and against H7 in convalescent-phase serum. **p<0.01.

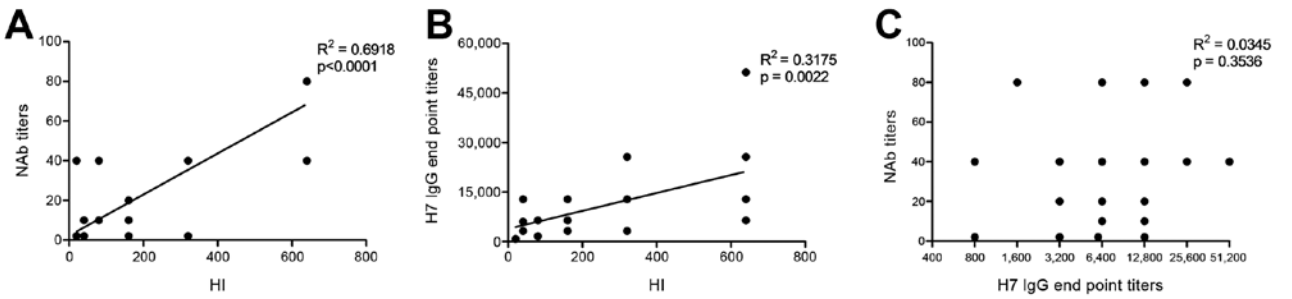

Figure 4. Correlation analysis among titers of hemagglutination inhibition (HI), neutralizing antibodies (NAbs), and IgG against H7 in patients infected with influenza A(H7N9) virus, China, 2013. A) NAb vs. HI. B) IgG against H7 vs. HI. C) NAb vs. IgG against H7.

Further evaluation that used data obtained from the serum collected 17–37 days and 102–125 days after symptom onset revealed a significant correlation between titers of HI and NAb ($R^2 = 0.6918$, $p<0.0001$) and between titers of HI and IgG against H7 ($R^2 = 0.3175$, $p = 0.0022$) (Figure 4, panels A, B). However, no correlation was found between titers of IgG against H7 and NAb ($R^2 = 0.0345$, $p = 0.3536$) (Figure 4, panel C), indicating that titers of IgG against H7 might may not be an ideal indicator for protective immunity against infection with influenza A(H7N9) virus.

Discussion

The protective antibody response against influenza A(H7N9) virus was relatively weak; the NAb response was lower than that for influenza A(H1N1)pdm09 and H5N1 viruses. Studies have shown that the GMT of convalescent-phase NAbs against A(H1N1)pdm09virus is 1:101.1 at 21–42 days after symptom onset (*24*), but the NAb titers against subtype H5N1 virus range from 80 to 2,560 at 14 days after symptom onset in convalescent-phase serum samples (*16*). Moreover, the serum IgG avidity for H7 was lower than that for H1 and H3. Of note, a significant boost of titers of IgG against H3 in convalescent-phase serum and a correlation between the level of IgG against H3 in the acute-phase serum and the IgG level against H7 in the convalescent-phase serum were observed.

Our findings raise several questions about the role of humoral responses to subtype H7N9 virus infection and disease outcome. Does the relatively weak NAb response of the host against subtype H7N9 play a role in the severity and duration of infections? And does the low avidity of IgG against H7 hemagglutinin correlate with subtype H7N9 pathogenesis?

With regard to the first question, it has been reported that for other influenza A viruses, the presence of NAbs correlates with recovery time and the outcomes of the disease (*24*). We speculate that the relatively weak NAb response against subtype H7N9 might directly contribute to the severity of the symptoms. In this regard, we note that 1 patient, for whom no Nabs were detectable in convalescent-phase serum at days 17 and 27, died and that another patient, for whom NAb titer was low (1:20) on day 31, also died, although titers of IgG against H7, H1, and H3 in both patients were very high.

There are several possible explanations for the relatively low NAb response. Immunogenicity of subtype H7N9 virus is probably weaker than that of influenza subtype H5N1 and A(H1N1)pdm09 viruses. An immune-informatic analysis predicted that the T-cell epitope contents are low in subtype H7N9 proteins, probably leading to a lower immune response against subtype H7N9 virus (*27*). Furthermore, it has been reported that the humoral response to subtype H7N7 vaccine is lower than that to subtypes H5N1 and H9N2 after vaccination with a dose that should have stimulated effective immune response (*28*). Another reason for the lower NAb response could be inefficient T-cell helper response. The isotype switch of antibody production, such as that from IgM to IgG, as well as the process of antibody affinity maturation, requires T-cell help (*29*). This point should be investigated in future studies, such as analysis of peripheral blood mononuclear cells, which were not available in our study. Patients' immune status might also provide an explanation. Most patients in this study had underlying diseases, which might attenuate the immune responses (*30*). In addition, age can also play a role in attenuated immunity; the severity of infection with subtype H7N9 virus increases with age (*2*). It is possible that the relatively low NAb response against subtype H7N9 infection is caused by several of the aforementioned factors.

With regard to the second question, whether the low avidity of IgG against H7 hemagglutinin correlates with subtype H7N9 pathogenesis, previous studies demonstrated that low-affinity antibodies against A(H1N1)pdm09 virus could form pathogenic immune complexes to impair multiple organ functions and were associated with disease outcome (*21*). In this study, we found that although high titers of IgG against H7 developed in patients infected with subtype H7N9 virus, binding avidity to this subtype is much lower than that to seasonal influenza A viruses. In particular, we found that among tested patients, although IgG against H7

was highest (1:25,600) among the 2 deceased patients from whom paired serum samples were obtained, IgG avidity for subtype H7N9 was also very low. Whether the nonprotective, low-avidity antibody response plays a major role in the pathogenesis of subtype H7N9 virus in addition to the relatively weak NAb response needs to be elucidated.

In our study, we observed a significant boost of antibody against H3 and H1 in patient serum samples. Titers of IgG against H3 in acute-phase serum were correlated with those against H7 in convalescent-phase serum. Perhaps subtype H7N9 virus infection triggered a cross-reactive response between H7 and H3 or H1 hemagglutinins. Primary infection with influenza virus can lead to a heterosubtypic hemagglutinin antibody response (*31*). Moreover, hemagglutinin stalks are structurally conserved within each hemagglutinin subgroup. The induction of anti-stalk antibodies during influenza virus infection provides the cross-reactivity and protection against infections by different influenza A virus subtypes (*17, 32–39*). However, in our study, the cross-reactivity of H7 versus H1 was lower than that of H7 versus H3 (Figure 3). Because H3 and H7 subtypes belong to subgroup 2 of hemagglutinin, and H1 hemagglutinin belongs to subgroup 1, it is reasonable that such cross-reactivity is stronger between H7 and H3 than between H7 and H1 and H5 (*40*). Because a substantial proportion of persons all over the world have experienced H3N2 virus infection, the effects of such boost responses in infection with subtype H7N9 should be further investigated (*17*).

Our main study limitation was the small number of patients for whom paired serum samples were available. These few paired samples were insufficient for certain analyses, such as comparing differences across age groups and sex. Another limitation was the timing of convalescent-phase serum sample collection, which occurred immediately before hospital discharge. The varied sampling times might have influenced the accurate identification of NAb occurrence and NAb titer comparisons among patients. In addition, clinical information was incomplete for some patients; and given the small sample size, we were unable to correlate antibody responses with disease severity.

In summary, our findings indicate a relatively weak protective antibody response against influenza A(H7N9) virus in tested patients. This low response might provide insights useful for potential vaccine development against subtype H7N9; multiple vaccinations might be needed to achieve protective immunity.

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Dr Guo is a scientist working at MOH Key Laboratory of Systems Biology and Christophe Mérieux Laboratory of Institute of Pathogen Biology, Chinese Academy of Medical Sciences. Her research activities focus on the etiology and immunology of respiratory viruses.

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Address for correspondence: Jianwei Wang, MOH Key Laboratory for Systems Biology of Pathogens and Christophe Merieux Laboratory, IPB, CAMS-Fondation Mérieux, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College, #9 Dong Dan San Tiao, Dongcheng District, Beijing 100730, P. R. China; email: wangjw28@163.com

Seven-Valent Pneumococcal Conjugate Vaccine and Nasopharyngeal Microbiota in Healthy Children

Giske Biesbroek, Xinhui Wang,[1] Bart J.F. Keijser,[1] Rene M.J. Eijkemans, Krzysztof Trzciński, Nynke Y. Rots, Reinier H. Veenhoven,[2] Elisabeth A.M. Sanders, and Debby Bogaert

Seven-valent pneumococcal conjugate vaccine (PCV-7) is effective against vaccine serotype disease and carriage. Nevertheless, shifts in colonization and disease toward nonvaccine serotypes and other potential pathogens have been described. To understand the extent of these shifts, we analyzed nasopharyngeal microbial profiles of 97 PCV-7–vaccinated infants and 103 control infants participating in a randomized controlled trial in the Netherlands. PCV-7 immunization resulted in a temporary shift in microbial community composition and increased bacterial diversity. Immunization also resulted in decreased presence of the pneumococcal vaccine serotype and an increase in the relative abundance and presence of nonpneumococcal streptococci and anaerobic bacteria. Furthermore, the abundance of *Haemophilus* and *Staphylococcus* bacteria in vaccinees was increased over that in controls. This study illustrates the much broader effect of vaccination with PCV-7 on the microbial community than currently assumed, and highlights the need for careful monitoring when implementing vaccines directed against common colonizers.

Vaccination is one of the most effective methods to prevent infectious diseases by direct protection of persons against a specific pathogen and by eradication of these specific pathogens from the population, leading to so called herd effects or indirect protection (*1*). Over the past decade, a 7-valent pneumococcal conjugate vaccine (PCV-7) was introduced in national immunization programs for newborns in most high-income countries, and the newer-generation 10-valent and 13-valent vaccines are being progressively introduced in developing countries (*2*).

The specific serotypes of the first licensed 7-valent pneumococcal vaccine are common colonizers of the upper respiratory tract of children during the first years of life, in which these serotypes generally reside as part of the nasopharyngeal microbiota (bacterial community) (*3*). However, these bacteria might occasionally spread beyond this niche and cause otitis media, pneumonia, sepsis, or meningitis (*4*). Vaccines show effectiveness against vaccine-serotype disease, nasopharyngeal acquisition of pneumococci, and pneumococcal transmission. However, nonvaccine pneumoccal serotypes fill the vacant nasopharyngeal niche, leaving overall pneumococcal carriage similar or only temporarily decreased (*5*,*6*) and lead to a gradual increase in nonvaccine serotype disease (*7*). In addition, several studies have raised awareness of the replacement of vaccine serotypes in the bacterial community with other potential pathogens, such as *Haemophilus influenzae* and *Staphylococcus aureus* in carriage or disease (*8*–*11*). This replacement is likely explained by the highly interactive nature of the microbiota in the natural habitat of the specific bacterium (*12*).

The recent availability of high-throughput, deep-sequencing techniques has made it possible to obtain more insight in the microbiota in humans, including the not yet cultivated fraction of bacteria. These techniques have elucidated that bacteria of the human microbiota outnumber human host cells by 10-fold, and microbiota composition varies greatly between body sites and persons. Colonization is a dynamic process of interactions among microbes

Author affiliations: University Medical Center Utrecht, Utrecht, the Netherlands (G. Biesbroek, X. Wang, K. Trzciński, E.A.M. Sanders. D. Bogaert); TNO, Zeist, the Netherlands (G. Biesbroek, B.J.F. Keijser); Julius Center for Health Sciences and Primary Care, Utrecht (R.M.J. Eijkemans); Netherlands Vaccine Institute, Bilthoven, the Netherlands (N.Y. Rots); and Spaarne Hospital, Hoofddorp, the Netherlands (R.H. Veenhoven)

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[1]These authors contributed equally to this article.

[2]Deceased.

and between microbes and the host and result in balanced bacterial ecosystems that benefit health. Perturbations of these interactive microbial structures (e.g., by environmental change or vaccinations) alter the bacterial network structures and may thereby influence the presence and containment of other microbiota members, and these alterations have effects on health and susceptibility to disease (*13*,*14*).

Given the changes in pneumococcal serotypes, as well as well as *S. aureus* and *H. influenzae* carriage after vaccination with PCV-7 (*7*,*8*), we questioned whether the effects of PCV-7 could be even more extensive than initially believed. We therefore studied the effects of PCV-7 on the complete nasopharyngeal microbiota of healthy children in a randomized controlled trial by using deep-sequencing techniques. The study was initiated shortly before nationwide implementation of PCV-7 in the Netherlands, therefore before herd effects appeared, which enabled us to measure the direct effects of the vaccine (*15*).

Methods

Study Design and Population

Nasopharyngeal samples were obtained from a randomized controlled trial that studied efficacy of reduced-dose schedules of PCV-7 on pneumococcal carriage in 1,005 healthy children in the Netherlands. The methods of this trial have been described (*15*). In brief, participants were randomly assigned to receive 1) PCV-7 at 2 and 4 months of age (2-dose group) of age; 2) PCV-7 at 2, 4, and 11 months of age (2 + 1-dose group); or 3) no PCV-7 (unvaccinated control group).

For the present study, we selected nasopharyngeal samples of the group of children that received 3 vaccinations with PCV-7 (n = 336) and of the group of children that received no vaccinations with PCV-7 (controls) (n = 331). To avoid seasonal influences on microbiota composition (*3*), we selected samples from children whose first birthday was during October 2006–January 2007. To avoid interference from background DNA, only samples from those children with sufficient bacterial density at 12 and 24 months of age (i.e., samples with DNA levels ≥1 pg/μL) were selected for 454 pyrosequencing, as described (*16*).

Nasopharyngeal swab specimens from the controlled trial had been obtained during home visits by using a deep transnasal approach with a flexible, sterile, dry, cotton-wool swab (TranswabPernasal Plain; Medical Wire and Equipment Co., Ltd., Corsham, UK). Specimens were immediately inoculated into transswab modified Amies Medium, 483CE (Copan Diagnostics Inc., Murrieta, CA, USA), transported to the laboratory, and stored in saline within 24 h at –80°C until further analyses. All nasopharyngeal swab specimens were cultured for *H. influenzae, Moraxella catarrhalis, S. aureus*, and *Streptococcus pneumoniae* and subjected to pneumococcal serotyping (*15*,*17*,*18*). With each nasopharyngeal swab specimen, a questionnaire on risk factors for pneumococcal carriage in children and prior antimicrobial drug use was completed.

The randomized controlled trial (NCT00189020) was approved by an acknowledged Dutch National Ethics Committee (Stichting Therapeutische Evaluatie Geneesmiddelen) and conducted in accordance with European Statements for Good Clinical Practice, which included the provisions of the Declaration of Helsinki of 1989. Before enrollment, written informed consent was obtained from both parents of each participant.

Construction of Phylogenetic Library

The selected subset was processed for sequencing of the 16S rDNA gene; the 454 GS-FLX-Titanium Sequencer (Life Sciences, Branford, CT, USA) was used for sequencing. The 16SrDNA gene is a conserved gene with variable regions among bacteria. Therefore, sequencing of this gene enables detection of all bacteria in the microbiota, which enables discrimination between bacterial taxa. DNA was extracted and quantified by quantitative PCR specific for conserved regions of the 16S rDNA gene. A barcoded amplicon library was generated by amplification of the V5–V7 hypervariable region of this gene and sequenced unidirectional, which generated ≈1.5 million sequences. Details of the methods have been described (*3*,*16*) and are shown in the online Technical Appendix (wwwnc.cdc.gov/EID/article/20/2/13-1220-Techapp1.pdf). The obtained sequences were processed and classified by using modules implemented in the Mothur V.1.20.0 software platform (*19*–*22*). This platform enables sequence classification on several taxonomic levels on the basis of evolutionary relatedness. The smallest accurate taxonomic level obtained by using 16S rDNA gene sequencing is the operational taxonomic unit (OTU), which is based on 97% similarity in nucleotide composition and enables differentiation just beyond genus level: OTUs do not always discriminate between species, and multiple OTUs might represent a specific genus, each capturing distinct lineages within it.

For each of the samples, rarefaction curves were plotted and sequence coverage was calculated by using the formula 1 – (number of OTUs with a single sequence per sample/number of samples in the study) to ensure that sufficient sequence numbers were analyzed. Sequence data were subjected to weighted UniFrac analysis by using the UniFrac module implemented in Mothur (*23*). The UniFrac metric is a proxy for the distance between microbial communities based on evolutionary relatedness of lineages in each sample. For all samples, we calculated the presence and relative and absolute abundance of all OTUs. The relative abundance was calculated as the proportion of sequences

assigned to a specific OTU divided by the overall number of obtained sequences per sample. In addition, for the absolute abundance, we multiplied the relative abundance of an OTU by the obtained bacterial load per sample measured by quantitative PCR.

Statistical Analyses

Data analyses were performed by using R version 2.7 (http://cran.r-project.org/bin/windows/base/old/2.7.1/), Excel 2011 (Microsoft, Redmond, WA, USA), and SPSS version 15.0 (SPSS Inc., Armonk, NY, USA). We used the Pearson χ^2 test to compare baseline characteristics between PCV-7–vaccinated children and control children. To visualize the weighted UniFrac dendrogram in relation to metadata, we used iTOL version 2 software (*24*). We used univariate and multivariate linear regression models (function lm and analysis of variance in software package R) to study the effect of vaccination with PCV-7 on microbiota profiles. We adjusted for antimicrobial drug use 1 month before sampling, the presence of siblings, and daycare attendance in all multivariate linear regression models. Associations were considered statistically significant after correction for multiple testing by determining the false-discovery rate (q value 0.2). Relative effect sizes and their 95% CIs were calculated for all significant OTUs from the standardized regression coefficients of the fitted linear model, whereby 1 indicates no effect, >1 indicates higher abundance, and <1 indicates less abundance in vaccinated children than in controls.

Interindividual variability between vaccinated and control children at 12 months and 24 months of age was calculated by using Pearson correlations and tested for significance by using the Mann-Whitney U test. We used nonmetric multidimensional scaling (nMDS) to compare microbiota profiles for dissimilarities and Euclidean distances to locate each sample in a low-dimensional space. OTUs were clustered hierarchically by using average linkage and Pearson correlation. The optimal number of clusters was identified by using the Silhouette index. OTU clusters and Pearson correlations between OTUs were displayed by using Cytoscape V2.8.2 (*25*).

Results

Characteristics of Study Population and Culture Results

We sequenced the nasopharyngeal microbiota of 97 children at 12 and 24 months of age who had received PCV-7 at 2, 4, and 11 months of age, and 103 controls. Similar to the main trial (*15*), in this subset of children, baseline characteristics were not different between PCV-7 vaccinees and controls (Table). Also, use of antimicrobial drugs was low, especially in the month before sampling, and no correlation was observed between antimicrobial drug use and vaccination with PCV-7 (partial correlation, r<–0.01).

Consistent with the main trial, vaccine serotype pneumococcal carriage decreased in PCV-7–vaccinated children. However, because of a lower number of children than in main trial and loss of statistical power, we observed only a trend toward increased carriage of nonvaccine-type pneumococci (p = 0.08) at 24 months of age. Furthermore, the increase in *S. aureus* carriage at 12 months of age in vaccinees observed in the main trial was not significant in this subset because of a loss of power (Table) (*18*,*26*).

Table. Baseline characteristics of PCV-7 vaccinated and unvaccinated children (controls) at 12 and 24 months of age*

	12 mo			24 mo		
Characteristic	PCV-7, n = 97	Control, n = 103	p value	PCV-7, n = 97	Control, n = 103	p value
Male sex	54 (56)	53 (51)	NS	54 (56)	53 (51)	NS
Crowding						
Presence of siblings in household	52 (54)	57 (55)	NS	64 (66)	65 (63)	NS
Daycare attendance†	62 (64)	68 (66)	NS	75 (77)	74 (72)	NS
Symptoms of URTI						
URTI (<6 mo)	56 (58)	55 (54)	NS	41 (42)	43 (42)	NS
Current cold‡	40 (41)	38 (37)	NS	36 (37)	39 (38)	NS
Current otitis media	3 (3)	6 (6)	NS	0	0	NS
History of wheezing	15 (16)	13 (13)	NS	10 (10)	9 (9)	NS
Smoke exposure at home	6 (6)	7 (7)	NS	6 (6)	6 (6)	NS
Antimicrobial drug use (<1 mo)§	4 (4)	5 (5)	NS	2 (2)	2 (2)	NS
Carriage detected by culture						
Streptococcus pneumoniae	63 (64.9)	80 (77.7)	0.06	59 (60.8)	75 (72.8)	0.10
Vaccine serotypes	30 (30.9)	51 (49.5)	0.01	16 (16.5)	42 (40.8)	0.001
Nonvaccine serotypes	33 (34.0)	29 (28.2)	NS	43 (44.3)	33 (32.0)	0.08
Moraxella catarrhalis	75 (77.3)	85 (82.5)	NS	67 (96.1)	81 (78.6)	NS
Haemophilus influenzae	56 (57.7)	50 (48.5)	NS	55 (53.4)	59 (57.3)	NS
Staphylococcus aureus	9 (9.3)	5 (4.9)	NS	7 (6.8)	4 (3.9)	NS

*Values are no. (%) unless otherwise indicated. p values were calculated by using the χ^2 test and are shown when there was a trend (p = 0.05–0.1) or a significant difference (p<0.05). PCV-7, 7-valent pneumococcal conjugate vaccine; NS, not significant (p>0.1); URTI, upper respiratory tract infection.
†Defined as at least 4 h/wk of daycare with >1 child from a different family.
‡Presence of mild symptoms of a respiratory tract infection at time of sampling as reported by parents.
§Defined as use of antimicrobial drug within 1 mo before sampling.

Sequence and Microbiota Characteristics

We obtained 1,016,934 high-quality sequences (mean ± SD 2,561 ± 767 sequences/sample). Sequence depth was sufficient to obtain a high degree of sequence coverage for all samples (mean 0.995, median 0.996, range 0.975–1). Sequencing of nasopharyngeal microbiota identified a diverse ecosystem dominated by the well-known bacterial genera *Moraxella*, *Streptococcus*, and *Haemophilus*, but also *Dolosigranulum* and *Corynebacterium* (online Technical Appendix Table 1). In addition, we detected a range of lower abundant bacterial genera (424 OTUs excluding singletons), present in either many (*Escherichia*/*Shigella*, *Neisseria*, and *Gemella* spp.) or few (*Sneathia* and *Porphyromonas* spp.) children. We found that for most children, the microbiota profile was determined mostly by abundance of the 5 predominant OTUs, and did not differ in children at 12 and 24 months of age (Figure 1).

To discriminate potential pathogens in the OTU set, we correlated the culture results of the samples with the corresponding OTUs of *Moraxella*, *Streptococcus*, *Haemophilus*, and *Staphylococcus*. We observed a strong correlation between culture results and the highest ranking OTUs for the respective genera ($p<0.005$) (online Technical Appendix Table 2), which indicated a strong representation of these potential pathogens within these OTUs.

Nasopharyngeal Microbiota Composition in Vaccinees and Controls

To evaluate the effect of vaccination with PCV-7 on the overall microbial community composition, we first calculated the degree of dissimilarity in microbiota composition between vaccinees and controls by using nMDS (*27*). We observed a significant shift in microbiota profiles between vaccinated and nonvaccinated children at 12 months of age (geometric means; $p = 0.01$, by F-test) but not at 24 months of age (Figure 2).

Because nMDS suggested higher variability of community profiles in vaccinees, we calculated interindividual variability in microbiota composition among vaccinees and controls by using Pearson correlations. We confirmed higher interindividual variability (i.e., less similarities between profiles) among vaccinees than in control children at 12 months of age (median correlation coefficient $r = 0.39$ vs. 0.41, $p<0.0001$) than at 24 months of age (median correlation coefficient $r = 0.42$ vs. 0.44, $p = 0.006$). At 12 months of age, this variability was accompanied by a significantly higher number of OTUs per community profile (i.e., higher diversity of bacteria) in vaccinated children (median 20, range 6–82) than in unvaccinated controls (median 17, range 4–46; $p = 0.002$) (online Technical Appendix Table 3).

In univariate and multivariate linear regression models, these changes in overall community composition, variability, and bacterial diversity in vaccinated children were accompanied by significant (false-discovery rate q value <0.2, $p<0.0003$) increases in relative and absolute abundance of anaerobic bacteria (e.g., *Veillonella* spp., relative effect size [RES] 3.90, 95% CI 2.13–7.17; *Prevotella* spp., RES 7.24, 95% CI 4.06–12.94; unclassified *Bacteroidetes* spp., RES 2.41, 95% CI 1.28–4.54; and *Leptotrichia* spp., RES 3.31, 95% CI 1.78–6.16) as well as increases in relative and absolute abundance of several streptococcal OTUs (RES 4.53, 95% CI 2.48–8.30). A trend ($0.005<p< 0.05$) toward higher abundance of gram-positive *Actinomyces* spp. (RES 3.00, 95% CI 1.60–5.62) and *Rothia* spp. (RES 2.43, 95% CI 1.29–4.58), the gram-negative *Neisseria* spp. (RES 2.12, 95% CI 1.13–3.99), and the anaerobes *Fusobacterium* spp. (RES 1.93, 95% CI 1.02–3.64) and *Megasphaera* spp. (RES 1.96, 95% CI 1.03–3.71) was also observed after vaccination with PCV-7. In addition, we found apparent higher absolute abundance of *Haemophilus* (RES 1.33, 95% CI 0.73–2.44) and *Staphylococcus* (RES 1.56, 95% CI 0.83–2.93) species in vaccinated children at age 12 months (Figure 3). At 24 months of age, differences between vaccinees and controls had largely disappeared.

Although antimicrobial drug use was low, we observed a trend ($0.01<p< 0.05$) toward decreased relative abundance of *Dolosigranulum* (RES 0.28, 95% CI 0.061–1.33) and *Corynebacterium* (RES 0.28, 95% CI 0.061–1.30) and increased abundance of *Staphylococcus* (RES 6.29, 95% CI 1.38–28.77) in children who received antimicrobial drugs in the month before sampling.

Microbial Inference Network in Controls and Vaccinees

Because microbial ecosystems form interacting networks of microorganisms, the presence (or abundance) of 1 type of bacteria will most likely affect the presence of others. To obtain better insight into the effect of vaccination on the bacterial community structure, we evaluated the effect of vaccination with PCV-7 on the microbial interaction network by using network inference analysis (Figure 4). OTUs were hierarchically clustered and displayed with their Pearson correlation by using Cytoscape V2.8.2 for control (Figure 4, panel A) and vaccinated (Figure 4, panel B) children. At the age of 12 months, children showed clear shifts in cluster distribution, composition, and interrelatedness after vaccination with PCV-7. In general, as a consequence of vaccination with PCV-7, several independent clusters observed in controls merged into 1 large cluster in vaccinees: this cluster included gram-negative anaerobes (*Prevotella*, *Veillonella*, and *Fusobacterium* spp.) as well as *Actinomyces* and *Neisseria* spp. and several streptococcal species. Bacteria that had expanded as a consequence of vaccination all belonged to the merged cluster or a single distinct cluster

containing mostly *Prevotella*, unclassified *Bacteroidetes*, *Fusobacterium*, *Streptococcus*, and *Neisseria* spp. (cluster 8). The cluster containing the predominating potential pathogens *Staphylococcus* and *Haemophilus* spp. in controls (cluster 1) was divided in vaccinees because of changed behavior, in particular that of the *Staphylococcus* spp. OTU.

Discussion

The novelty of the present study was use of deep-sequencing analyses. By using these analyses, we gained a far broader insight into the effect of PCV-7 on bacterial carriage at the ecologic niche of pneumococci without restricting selection to cultivable or well-known potential pathogens. We showed that vaccination with PCV-7 has a marked effect on the complete microbiota composition of the upper respiratory tract in children. This effect goes far beyond the shifts in pneumococcal serotypes distribution (*7*,*15*) and well-known potential pathogens reported (*8*). Vaccination with PCV-7 resulted in a shift in bacterial community composition and structure, with an increase in presence or abundance of several anaerobes, such as *Veillonella*, *Prevotella*, *Fusobacterium*, and *Leptotrichia* species; gram-positive bacteria, such as *Actinomyces* and *Rothia* species, and nonpneumococcal streptococci; and gram-negative *Neisseria* species.

Shifts in newly acquired or expanded OTUs concern mainly commensal organisms that are in general more predominantly present in the oropharynx than in the nasopharynx (*28*,*29*). Because the reduction in carriage of the 7 specific pneumococcal serotypes after PCV-7 administration correlated highly with emergence and expansion of these oropharyngeal types of species, this finding might suggest that after eradication of a common colonizer, such as vaccine serotype pneumococci, momentum is created for species from surrounding regions to colonize or expand in the vacant nasopharyngeal niche. In support of this hypothesis, Tano et al. (*30*) reported negative associations between *S. pneumoniae*, particularly PCV-7 serotypes, and other streptococcal species in healthy young children. Moreover, Laufer et al. (*31*) reported negative associations between *S. pneumoniae* and the presence of

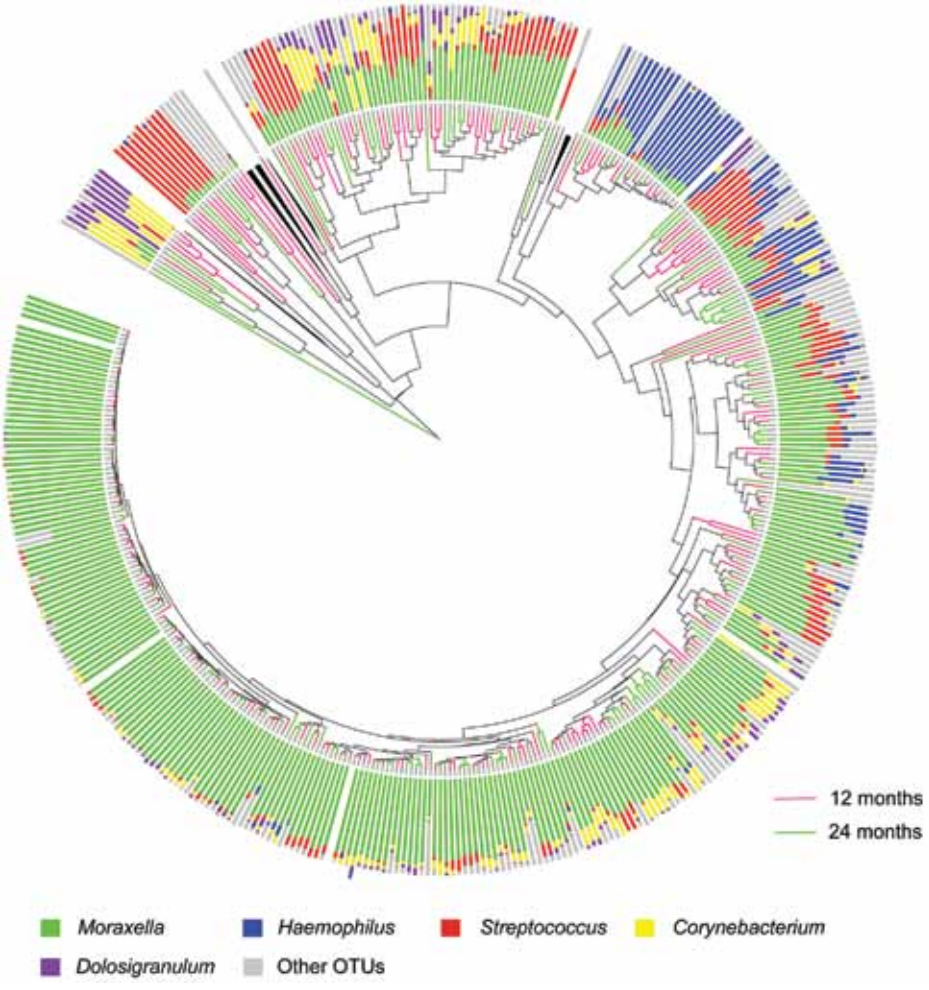

Figure 1. Weighted UniFrac analyses (*23*) of nasopharyngeal samples of children at 12 and 24 months of age vaccinated with 7-valent pneumococcal conjugate vaccine. Clustering of samples was based on evolutionary (phylogenetic) relatedness by using Weighted UniFrac analyses. Clustering is shown in a circle dendrogram. Each branch represents a sample and each adjacent histogram represents the relative abundance of the top 5 operational taxonomic units (OTUs) found in that sample. Differences in length of branches among samples reflect their distance (i.e., dissimilarity) to each other. Branches of reference samples were collapsed and are represented by black triangles. Samples are mostly dominated by *Moraxella, Streptococcus*, and *Haemophilus* spp., or the combination of *Dolosigranulum* and *Corynebacterium* spp., which highly affects sample clustering by Weighted UniFrac. Branches are colored according to age of sampled children (purple = 12 months, green = 24 months). No clear clustering of samples by age was observed.

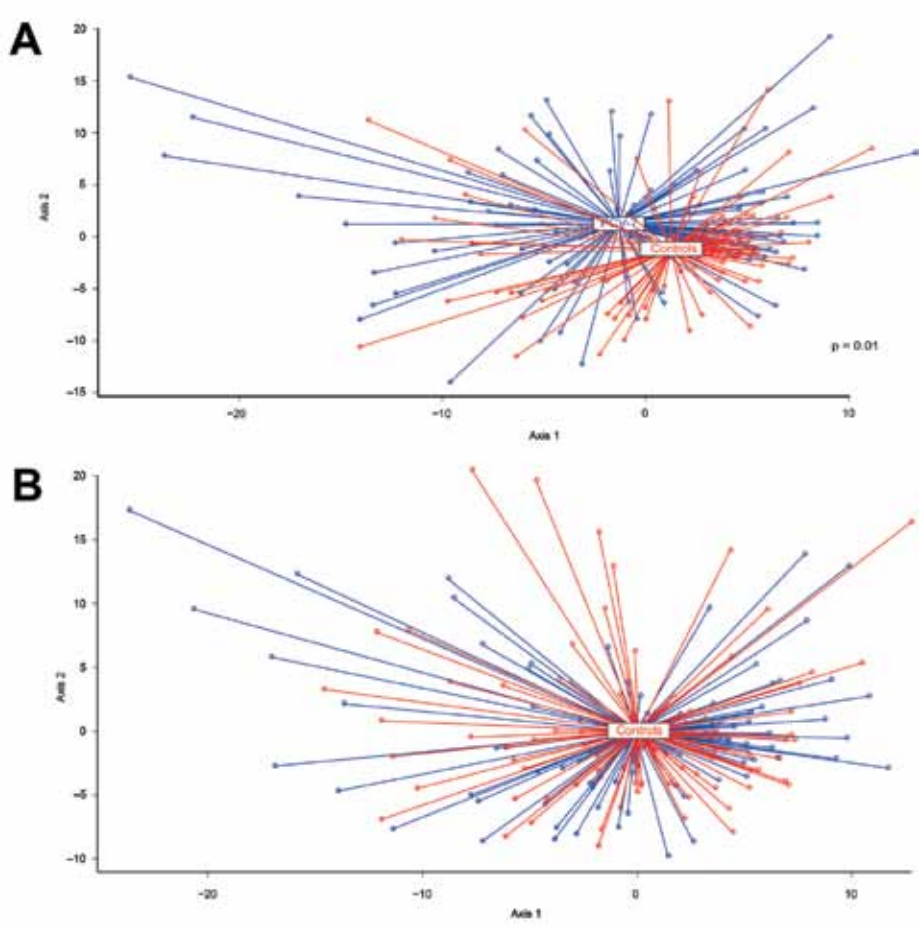

Figure 2. Nonmetric multidimensional scaling (nMDS) of microbiota profiles of children vaccinated with 7-valent pneumococcal conjugate vaccine and control children at 12 and 24 months of age. Microbiota profiles were compared between groups by using nMDS to find dissimilarities between samples and locate samples in a 2-dimensional space. Each circle represents the microbiota profile of a sample. Boxes indicate geometric means of both groups in which the length of the line between the sample (circle) and the geometric mean (box) indicates the distance of that sample from the geometric mean. Longer lines indicate higher distances of samples (i.e., higher variability between sample compositions). A) nMDS plots of vaccinated children (blue lines) and controls (red lines) at 12 months of age. The geometric mean of microbiota profiles differed significantly (p = 0.01, by F-test) between vaccinated children and controls. B) nMDS plots of vaccinated children (blue lines) and controls (red lines) at 24 months of age, showing no differences in geometric means of microbiota profiles between the 2 groups.

Veillonella, *Neisseria*, *Rothia*, and *Actinomyces* spp. in nasal swab specimens from children with upper respiratory tract symptoms, a finding that is consistent with the influx pattern of bacteria we observed after vaccination with PCV-7.

In addition to this shift in microbiota profiles, we also observed increased bacterial diversity and interindividual variability after vaccination with PCV-7. This influx or outgrowth of anaerobes and other bacteria might lead to a disequilibrium with the host. These species might be at a disadvantage again when nonvaccine serotypes fill in the gap, which would lead to a restored host-microbiome equilibrium.This hypothesis could explain why we observed the strongest PCV-7 effect on microbiota in children at 12 months of age (1 month after administration of the last PCV-7 dose) and not at 24 months, because serotype replacement has already become apparent at this later time.

The mechanisms and consequences of this change in community composition and structure after vaccination with PCV-7 remain mostly speculative. In general, temporary disequilibria of bacterial composition (dysbiosis) are associated with an increased risk for disease, as has been shown for the gut (*14*) and oral niches (*32*). Moreover, the combination of some of the emerging bacteria (*Veillonella*, *Actinomyces*, *Rothia*, and *Neisseria* spp.) are associated with increased risk for otitis media (*31*). Nevertheless, in our study, we did not obtain samples during respiratory tract infections and were therefore unable to link observed changes in microbiota structure with susceptibility to respiratory tract infections. Therefore, short-term and long-term surveillance during health and disease seems warranted to understand the full implications of vaccine-induced changes in microbiota structure.

Although increased presence or abundance of *S. aureus* and *H. influenzae* at 12 months of age was not significant in this subset of children, we observed an increase in culture-proven *S. aureus* carriage in the original randomized controlled trial (*18*), as well as further increases in culture-proven *S. aureus* and *H. influenzae* carriage observed in surveillance studies 3–5 years after PCV-7 implementation in the Netherlands (*8*). These findings are consistent with negative associations between *S. pneumoniae* (particularly PCV-7 serotypes) and *S. aureus* (*33*,*34*) and *H. influenzae* (*35*–*37*) observed in healthy nonimmunized children. Nontypeable *H. influenzae* and

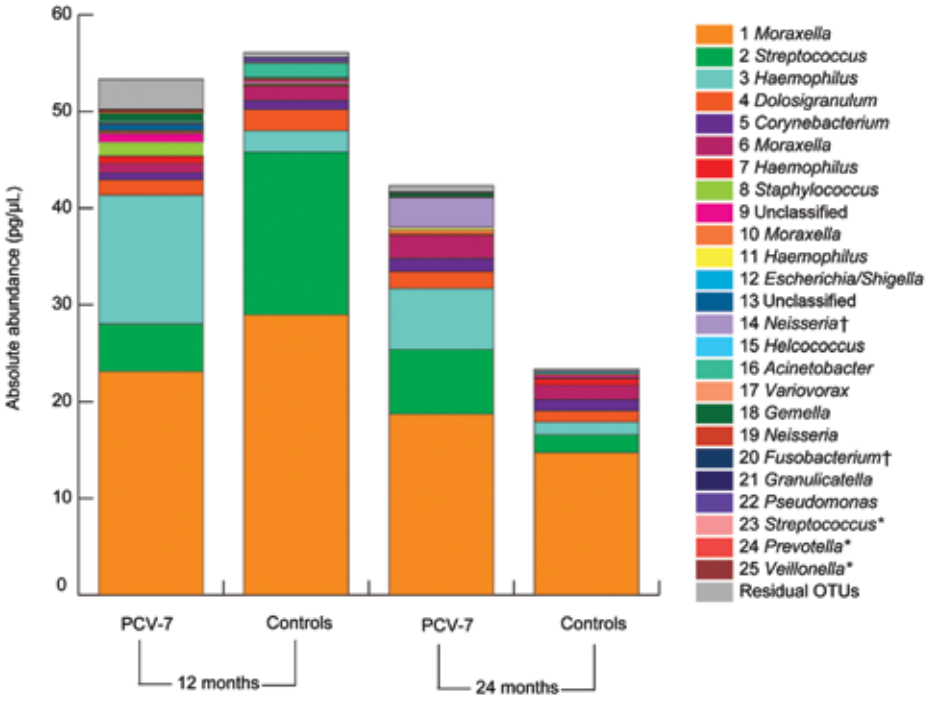

Figure 3. Mean absolute abundances of operational taxonomic units (OTUs) in children vaccinated with 7-valent pneumococcal conjugate vaccine and control children at 12 and 24 months of age. The 25 most abundant OTUs are represented by different colors. *OTUs that showed significantly higher abundance in vaccinated children than in controls ($p<0.0003$). Although not significant, an apparent higher average absolute abundance was observed for *Haemophilus* and *Staphylococcus* spp. in vaccinated children than in control children at 12 months of age. †OTUs that showed a trend toward higher abundance in vaccinated children than in controls ($0.0003<p<0.05$).

S. aureus were also more frequently isolated from persons with acute otitis media after introduction of PCV-7 in national immunization programs (*38–40*), which indicates that carriage may reflect disease dynamics. Together with *S. pneumoniae* nonvaccine serotype replacement, these effects may further jeopardize the net health benefit of vaccinations with PCV.

Some limitations of our study should be recognized. First, this study was limited to a representative subset of the original study of 1,003 infants. Second, to avoid seasonal bias (*3*) in microbiota composition, we analyzed samples from only the winter season. Third, children who received antimicrobial drugs before sampling were not excluded from the analyses because only a small number of children received these drugs and we observed no correlation between antimicrobial drug use and vaccination with PCV. Furthermore, the observed associations between antimicrobial drug use and microbiota composition were also different from the vaccination effect of PCV-7.

One strength of this study was the randomized controlled study design, which enabled us to attribute changes in microbiota profiles directly to the conjugate vaccine independent of secular trends or other external confounders. Furthermore, recruitment in this study was completed well before implementation of PCV-7 in the Dutch vaccination program for newborns, and vaccine-induced changes in this randomized controlled trial setting might therefore become more apparent in the open population several years after introduction due to herd effects (*5*,*7*)

Our study indicates that vaccination against a common colonizer affects microbiota composition and structure. This finding underlines the need for more detailed understanding of microbiota dynamics and interactions between its inhabitants. Overall, because infants might be vulnerable to community disruptions and dysbiosis, we recommend that new trials, such as studies on efficacy of broader pneumococcal coverage vaccines, consider the effect of vaccination on the commensal flora in its totality instead of only on a single species.

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Ms Biesbroek is an MD and PhD candidate at the Wilhelmina Children's Hospital, University Medical Center, Utrecht, the Netherlands. Her research interests include molecular characterization and epidemiology of the microbiota in the respiratory tract in children.

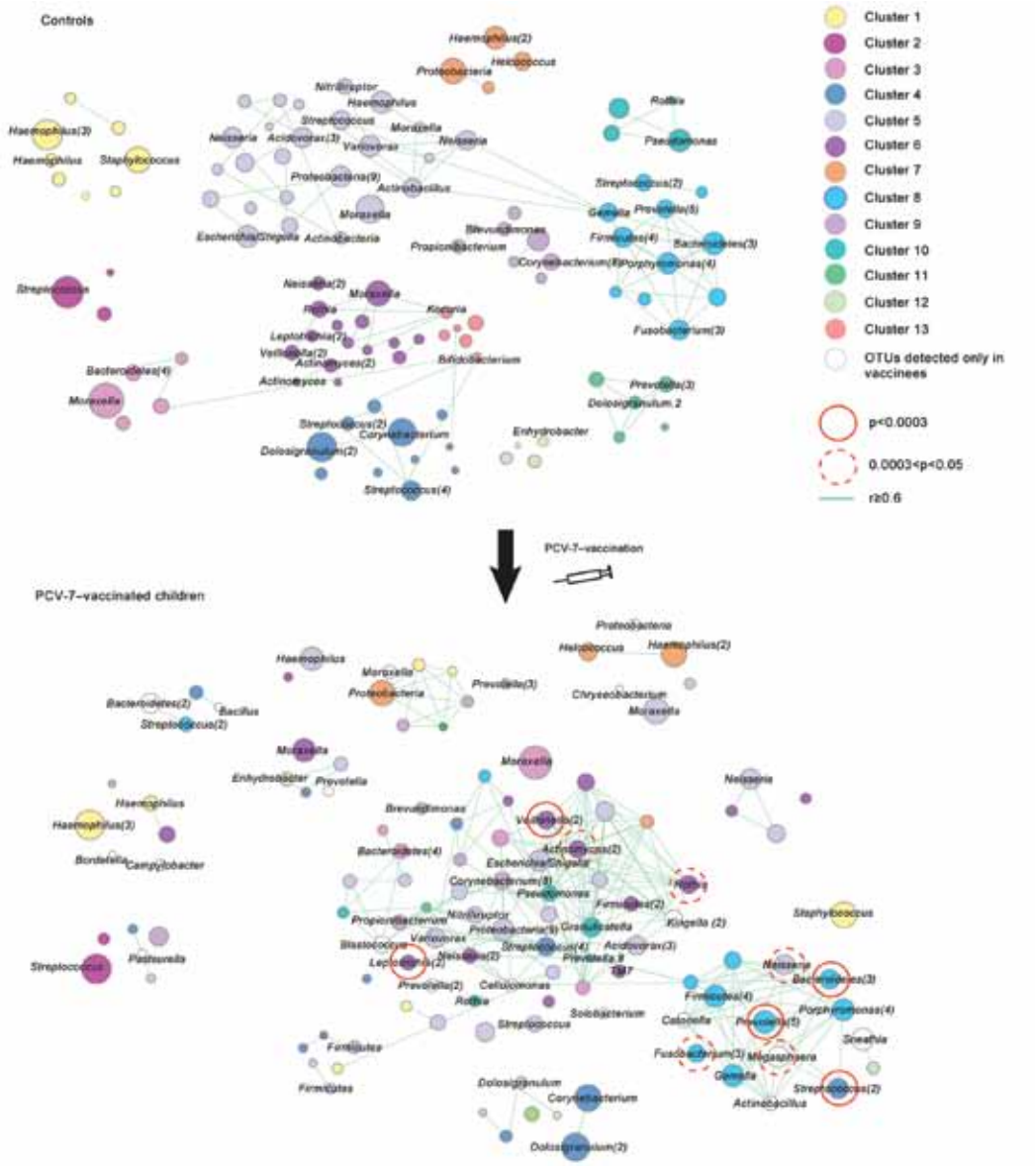

Figure 4. Microbial association network between operational taxonomic units (OTUs) in nonvaccinated children (controls) and children at 12 months of age who were vaccinated with 7-valent pneumococcal conjugate vaccine (PCV-7). Hierarchical clustering with average linkage and Pearson correlation distance is used to identify patterns of co-occurrence or similar abundance patterns between OTUs in the complete sample set of controls and PCV-7–vaccinated children. Results are depicted in a microbial association network. Lines connecting particular OTUs depict positive correlations (correlation coefficient $r \geq 0.6$) between individual OTUs. Clusters of OTUs are discriminated by different colors. To enable visualization of shifts in cluster composition, OTUs in PCV-7–vaccinated children are colored according to the cluster they originated from in control children. Node sizes reflect average relative abundance of the OTU in the selected population (i.e., PCV-7–vaccinated or controls by using a $\log_2$ scaling. OTUs that were significantly higher in vaccinated children are indicated by red circles around nodes. For visualization purposes, we did not depict all OTU names at the nodes. Also, if multiple OTUs of the same genus clustered together, we depicted only 1 node of that genus and indicated the number of representing OTUs for that genus in parentheses. In 12 month-old children (controls), we identified 13 OTU clusters. *Haemophilus influennzae* and *Staphylococcus aureus* clustered together in a small cluster distant from the other OTUs (cluster 1). *Streptococcus pneumoniae* formed, together with 2 other OTUs, a separate cluster that was also distant from the other OTUs (cluster 2). Cluster 3 contained, among others, the largest *Moraxella catarrhalis* OTU. Clusters 4–13 represent the remaining clusters and showed on average more OTUs per cluster and more interrelatedness with one another. Clear shifts in cluster composition and distribution between vaccinated and unvaccinated children were also observed. *Staphylococcus aureus* drifted from cluster 1 in controls toward cluster 8 in vaccinated children. This particular cluster increased in vaccinees because of increased abundance of OTUs already present in that cluster and because of emergence of new OTUs within the cluster. In addition, after vaccination, cluster 3 including *Moraxella catarrhalis* became part of 1 large cluster, which was composed mostly of OTUs in clusters 4–6, 9, and 10. The 10 OTUs that had expanded in vaccinated children all originated from clusters 6 and 8, or were newly emerged, such as *Megasphaera* spp.

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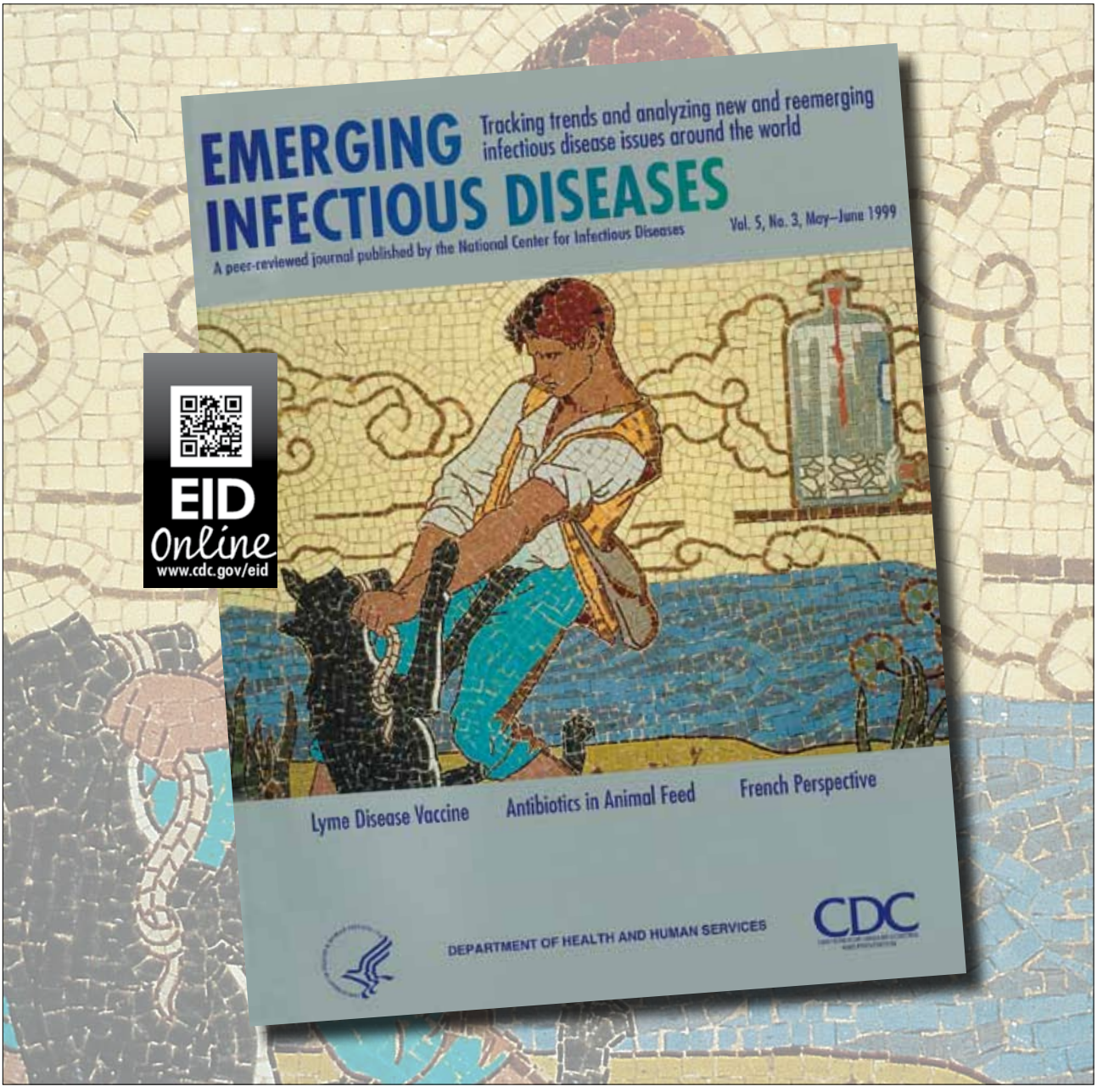
Address for correspondence: Debby Bogaert, Department of Pediatric Immunology and Infectious Diseases, Wilhelmina Children's Hospital, University Medical Center Utrecht, PO Box 85090, Rm KC.03.068.0, 3584 EA Utrecht, the Netherlands; email: dbogaert@umcutrecht.nl



Novel Paramyxovirus Associated with Severe Acute Febrile Disease, South Sudan and Uganda, 2012

César G. Albariño, Michael Foltzer, Jonathan S. Towner, Lory A. Rowe, Shelley Campbell, Carlos M. Jaramillo, Brian H. Bird, DeeAnn M. Reeder, Megan E. Vodzak, Paul Rota, Maureen G. Metcalfe, Christina F. Spiropoulou, Barbara Knust, Joel P. Vincent, Michael A. Frace, Stuart T. Nichol, Pierre E. Rollin, and Ute Ströher

In 2012, a female wildlife biologist experienced fever, malaise, headache, generalized myalgia and arthralgia, neck stiffness, and a sore throat shortly after returning to the United States from a 6-week field expedition to South Sudan and Uganda. She was hospitalized, after which a maculopapular rash developed and became confluent. When the patient was discharged from the hospital on day 14, arthralgia and myalgia had improved, oropharynx ulcerations had healed, the rash had resolved without desquamation, and blood counts and hepatic enzyme levels were returning to reference levels. After several known suspect pathogens were ruled out as the cause of her illness, deep sequencing and metagenomics analysis revealed a novel paramyxovirus related to rubula-like viruses isolated from fruit bats.

Paramyxoviruses comprise a large family of viruses, including pathogens that cause severe disease in humans (*1*). Worldwide, >100 paramyxoviruses have been identified in bats and rodents (*2–4*). Among these, few have been shown to be pathogenic to humans, possibly because of limited host range and/or infrequent exposure. We describe a novel rubula-like virus that was associated with a severe acute febrile illness in a woman. The patient was a wildlife biologist who had participated in a 6-week field expedition to South Sudan and Uganda. During this expedition, she had been exposed to bats and rodents of >20 species while wearing different levels of personal protective equipment.

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (C.G. Albariño, J.S. Towner, L.A. Rowe, S. Campbell, B.H. Bird, P. Rota, M.G. Metcalfe, C.F. Spiropoulou, B. Knust, J.P. Vincent, M.A. Frace, S.T. Nichol, P.E. Rollin, U. Ströher); Geisinger Medical Center, Danville, Pennsylvania, USA (M. Foltzer, C.M. Jaramillo); and Bucknell University, Lewisburg, Pennsylvania, USA (D.M. Reeder, M.E. Vodzak)

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Clinical Presentation

During the summer of 2012, a 25-year-old female wildlife biologist participated in a 6-week field expedition to South Sudan and Uganda, where she traveled to remote rural areas collecting bats and rodents for ecologic research. In the course of her duties, she manipulated animals in traps and mist nets, performed dissections, collected blood and tissues, and visited caves with large bat populations. She received no injuries from sharp objects and no bites or scratches from the animals with which she was working. She occasionally used respiratory protection when working with animals and specimens, and she wore a respirator while in caves. During her trip, she had no known contact with ill members of the field team, no contact with health care facilities, and no sexual contacts. She had been vaccinated against hepatitis A and B, yellow fever, measles, mumps, rubella, rabies, polio, tetanus/diphtheria, and typhoid fever, and she fully complied with a malaria prophylaxis regimen of atovaquone/proguanil. Her medical history included migraines and treatment of presumptive malaria with artemether/lumefantrine during a similar expedition the previous year.

Five days after returning to the United States, the woman was evaluated in the emergency department for a 2-day history of fever, malaise, headache, generalized myalgia and arthralgia, neck stiffness, a metallic taste, and sore throat. Results of rapid malaria test, performed on the day of fever onset, were negative. Other laboratory results and the patient's vital signs at the time of admission are summarized in the Table. The patient seemed to be fatigued but alert and oriented; she was anicteric, and she had no nuchal rigidity or focal neurologic deficits. Mild erythema of the soft palate without ulcerations or exudates was noted. The spleen tip was palpable despite absence of adenopathy.

Examination of heart, abdomen, and lungs (including chest radiographs) revealed no abnormalities. No rash or

Table. Vital signs and laboratory results for patient infected with a novel paramyxovirus related to rubula-like viruses isolated from fruit bats

DSO	DH	Max temp	Max pulse	SBP	WBC	Plate	Creat	AST	ALT	LDH,	TB	PT/INR ratio	TG	Ferr	qRT-PCR, C_t†	IgM ELISA†	IgG ELISA†
2	1	40.1	90	112	1.62	115	0.8	93	19	687	0.2						
3	2	40.1	79	112	1.53	93	0.7	133	20		0.1	17.2/1.45			29.5	<50	<50
4	3	40.4	77	103	1.06	77	0.6	164	28		0.1	15.8/1.29	120				
5	4				1.02	65	0.7	319	134		0.1	13.6/1.06		17,840			
6	5	38.6	77	102	0.95	62	0.6	615	261		0.1	13.7/1.07	127	11,595			
7	6				1.46	79	0.6	589	298		0.2	14.4/1.14		7,309			
8	7				1.64	84	0.5	516	299		0.1	15.5/1.26		3,371			
9	8				0.96	123	0.5	342	259		0.1	16.9/1.41			36.3	≥1,600	≥1,600
10	9				1.20	154	0.4	170	186		0.1	17.4/1.47			36.9	≥1,600	≥1,600
11	10				2.19	222	0.4	185	188		0.1	14.7/1.18			Neg	≥1,600	≥1,600
12	11				2.61	220	0.4	107	149		0.2	13.2/1.02			Neg	≥1,600	≥1,600
13	12				5.62	335	0.4								Neg		
14	13				5.79	387	0.4								Neg		
15	14	36.8	67	98	4.71	437	0.5						212		Neg	≥1,600	≥1,600
30					3.71	221		19	15		0.3						
60					5.44	348								12.4	Neg	≥400	≥1,600

*Shading indicates values outside reference range; blank cells indicate data not obtained. DSO, days from symptom onset; DH, day of hospitalization; max temp, maximum temperature, °C; max pulse; maximum pulse rate, beats/minute; SBP, systolic blood pressure, mm Hg; WBC, leukocytes ,× 1,000/μL; plate, platelets × 1,000/μL; creat, creatinine, mg/dL; AST, aspartate aminotransferase, IU/L; ALT, alanine aminotransferase, IU/L; LDH, lactate dehydrogenase, IU/L; TB, total bilirubin, mg/dL; PT/INR, prothrombin time/international normalized ratio; TG, triglycerides, g/dL; ferr, ferritin, ng/mL; qRT-PCR, quantitative reverse transcription PCR; C_t, cycle threshold; neg, negative.
†Assays were developed after the virus genome was determined.

synovitis was noted. Treatment with intravenous ceftriaxone was begun for possible typhoid fever, and artemether/lumefantrine was continued for presumptive malaria.

On hospital day 2, a maculopapular rash erupted over the patient's trunk (Figure 1, panel A), several small aphthous ulcers appeared on her soft palate, and she had mild diarrhea. As long as the fever persisted, clear pulse/temperature dissociation was present (positive Faget sign); however, hemodynamics, oxygenation, and renal function were stable. Doxycycline was added for the expanded differential diagnosis of a rickettsial illness or plague. On hospital day 3, fever, headache, and myalgia persisted, and the patient experienced bloody emesis, mild diarrhea positive for occult blood but without frank hematochezia or melena, and minimal diffuse abdominal tenderness. Her menstrual period occurred without substantial menorrhagia. The rash became confluent; a centrifugal distribution and prominent petechia appeared at sites of trauma or pressure.

The possibility of hemophagocytosis was considered, and a bone marrow biopsy sample was obtained on day 4. The sample showed a mild increase in macrocytic hemophagocytosis and pancytopenia with a hypocellular marrow with myeloid hyperplasia and erythroid hypoplasia (Figure 1, panel B).

The fever slowly but progressively decreased, and the patient improved over the next few days; the last recorded fever was on hospital day 9. Abdominal pain and diarrhea resolved. Ceftriaxone was discontinued after 8 days. When the patient was discharged on hospital day 14, arthralgia and myalgia had improved, oropharynx ulcerations had healed, the rash had resolved without desquamation, and blood counts and hepatic enzyme levels were returning to reference limits. Considerable sequelae (myalgia, arthralgia, headache, malaise, and fatigue) persisted for several months.

Diagnostic Workup

The initial suspected diagnosis was hematophagocytic syndrome (hemophagocytic lymphohistiocytosis). This clinical syndrome has been associated with a variety of viral, bacterial, fungal, and parasitic infections, as well as collagen–vascular diseases and malignancies. Initial diagnostic testing for various infectious diseases included blood screening for respiratory viruses, HIV, cytomegalovirus, and malaria parasites; all results were negative.

On hospital day 2, a diagnosis of a viral hemorrhagic fever was considered, and blood specimens were sent to the Centers for Disease Control and Prevention (CDC) for testing. Molecular testing results were negative for rickettsiae, filoviruses (Marburgviruses and Ebolaviruses), selected bunyaviruses (Rift Valley fever virus, Crimean Congo hemorrhagic fever virus), arenaviruses (Lassa, Lujo, and lymphocytic choriomeningitis viruses), and several arboviruses (yellow fever, dengue, O'nyong-nyong, chikungunya, and Zika viruses).

A pathogen-discovery deep-sequencing protocol was followed, as described (*5*,*6*). In brief, total RNA was extracted from blood and serum samples obtained 3 days after symptom onset; RNA was nonspecifically amplified with previously described primers (*7*). The cDNA library was sequenced on a 454 FLX Genome Sequencer (Roche Diagnostics, Indianapolis, IN, USA). Unassembled sequences

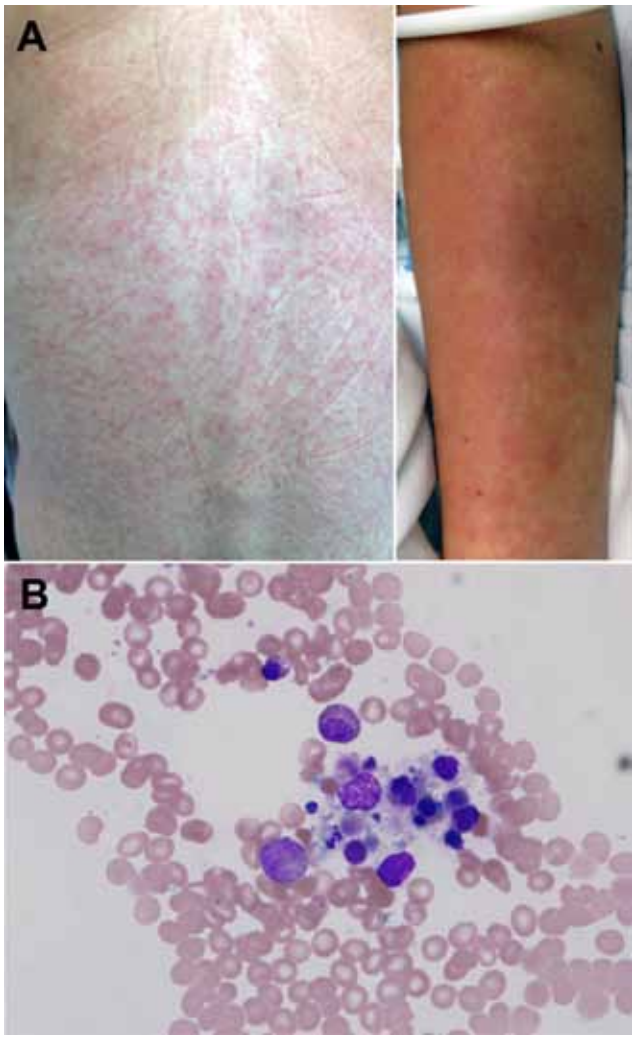

Figure 1. A) Maculopapular eruption observed on the back and arms of 25-year-old female wildlife biologist infected with a novel paramyxovirus related to rubula-like viruses isolated from fruit bats, on hospitalization day 2. B) Bone marrow biopsy sample showing macrocytic hemophagocytosis (possible granulocyte infiltration).

were translated and compared with the nonredundant protein database from the National Center for Biotechnology Information by using a BLASTx algorithm (www.ncbi.nlm.nih.gov/blast/Blast.cgi). The sequence reads linked to the BLASTx results were distributed into taxa by using MEGAN (*8*). Metagenomic analysis revealed a novel paramyxovirus in the patient's blood and serum; the virus was most closely related to Tuhoko virus 3, a rubula-like virus (family *Paramyxoviridae*) recently isolated from *Rousettus leschenaultii* fruit bats in southern China (*4*). The next-generation sequence reads with homology to Tuhoko virus 3 covered ≈25% of the expected complete virus genome. Based on the sequences obtained, a series of primers were designed to amplify overlapping fragments spanning the complete genome of this novel virus. A detailed list of primers is available upon request. Amplicons of different sizes were obtained by standard reverse transcription PCR (RT-PCR) and sequenced by the standard Sanger method (*5*,*6*).

This novel paramyxovirus is provisionally named Sosuga virus in recognition of its probable geographic origin (South Sudan, Uganda). The complete genome of Sosuga virus was 15,480 nt long and conformed to the paramyxovirus rule of 6 (*1*). The genome organization (Figure 2, panels A, B) resembled that of most paramyxoviruses, containing 6 genes, *N*, *V/P*, *M*, *F*, *HN*, and *L*, encoding the 7 viral proteins: nucleocapsid (N), V protein (V), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin–neuraminidase (HN), and polymerase (L). The sequence of the RNA editing site in the *V/P* gene is identical to that of Tuhoko virus 3 (*4*). The faithful transcription of V/P generates the V mRNA, and a GG insertion at the editing site generates the P mRNA. In addition, the terminal 5′ and 3′ sequences of the virus were experimentally determined (Figure 2, panel C) by rapid amplification of cDNA ends as described (*9*).

Pairwise comparison of the full-length sequence of Sosuga virus with the closest related viruses showed 61.6%, 53.1%, and 51.4% identities, respectively, with Tuhoko virus 3, Achimota virus 1, and Achimota virus 2 (Achimota viruses were isolated from the *Eidolon helvum* fruit bat in Ghana) (*3*). When the deduced amino acid sequences of Sosuga virus were compared with those of Tuhoko virus, 3 proteins revealed overall amino acid identities ranging from 57.4% (HN) to 84% (N). Phylogenetic analysis of Sosuga virus and other paramyxoviruses clearly showed that Sosuga virus clusters with other bat-borne rubula-like viruses, which are closely related to rubulaviruses but have not yet been classified as such (Figure 2, panel D).

Virus Isolation

Virus isolation was attempted by inoculating monolayers of Vero-E6, Vero-SLAM, and H292 cells (mucoepidermoid carcinoma cells from human lungs) with patient blood and serum collected 3 days after symptom onset, but no virus isolate was obtained. As an alternative, 10 μL of the blood sample was also inoculated intracranially and intraperitoneally into 2-day-old suckling mice, which were then observed for 28 days for signs of illness. Neurologic signs developed 9–10 days after inoculation in 2 of the 20 mice; these 2 mice were euthanized 2 days later. To confirm the presence of the virus, we extracted total RNA from liver, spleen, and brains of the euthanized animals and used it as input in a specific RT-PCR designed to amplify a 2,188-bp fragment partially spanning the virus *HN* and *L* genes. Consistent with virus replication and observed neurologic signs, viral RNA was found in the brain but not in liver or spleen samples (Figure 3, panel A).

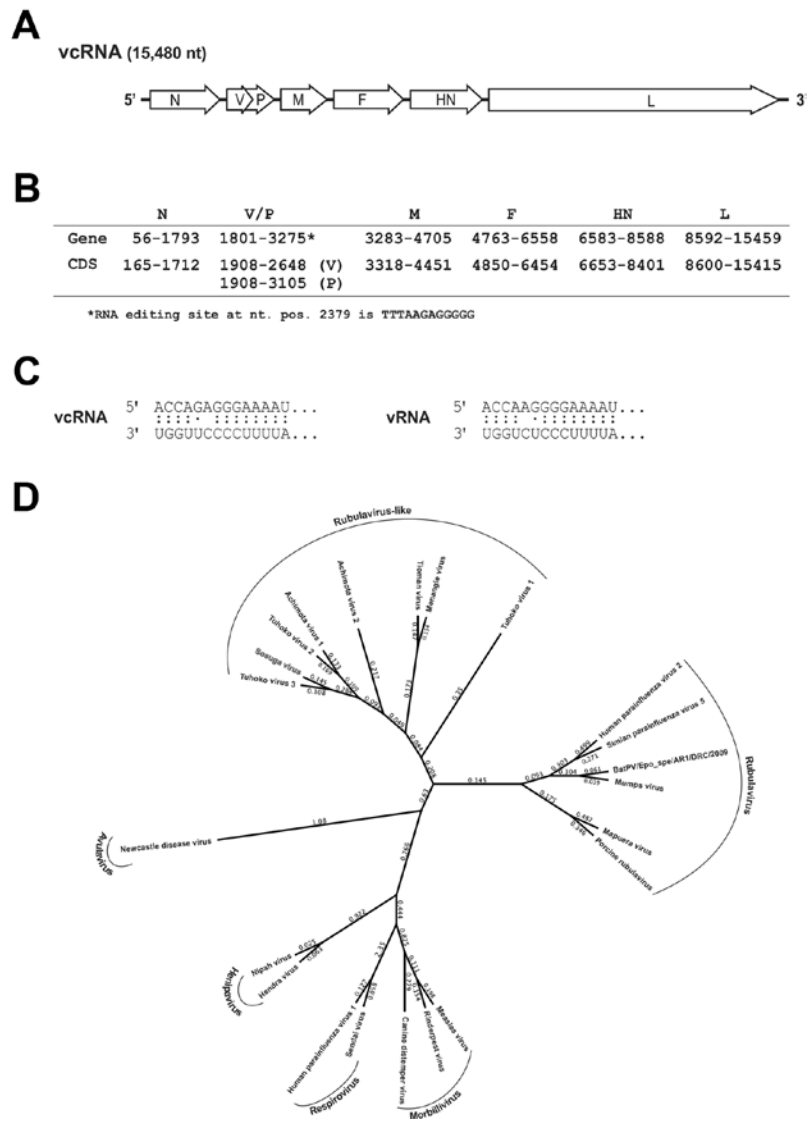

Figure 2. A) Organization of the viral genome of novel paramyxovirus related to rubula-like viruses isolated from fruit bats was determined from the full-length sequence. B) Localization of the predicted viral genes and open reading frames (ORFs). The V/P edition site is predicted from the similarity to Tuhoko virus 3. C) Terminal sequences were determined by standard rapid amplification of cDNA ends (RACE) methods. The complementarity of terminal sequences is shown in vRNA and vcRNA sense. D) Amino acid sequences of the nucleocapsid (N) protein of 22 representative paramyxovirus sequences were aligned by using the MUSCLE algorithm (CLC Genomics Workbench version 6.0.1; CLC bio, Cambridge, MA, USA). The phylogenetic analysis was conducted with a Bayesian algorithm (Mr. Bayes, Geneious version 6.1.5, www.geneious.com/). NP sequences were extracted from the complete genomic sequences in GenBank: KF774436 (Sosuga virus [SosV]), GU128082 (Tuhoko virus 3), GU128081 (Tuhoko virus 2), GU128080 (Tuhoko virus 1), AF298895 (Tioman virus), NC_007620 (Menangle virus), JX051319 (Achimota virus 1), JX051320 (Achimota virus 2), NC_003443 (human parainfluenza virus type 2), AF052755 (simian parainfluenza virus 5), HQ660095 (bat paramyxovirus Epo_spe/AR1/DRC/2009), NC_002200 (mumps virus), NC_009489 (Mapuera virus), NC_009640 (porcine rubulavirus), NC_001498 (measles virus), NC_006296 (rinderpest virus), NC_001921 (canine distemper virus), NC_001552 (Sendai virus), NC_003461 (human parainfluenza virus type 1), NC_002728 (Nipah virus), NC_001906 (Henra virus), NC_002617 (Newcastle disease virus). vcRNA, viral complementary RNA; N, nucleocapsid protein; V/P, V protein; M, matrix protein; F, fusion protein; HN, hemagglutinin-neuraminidase; L, molecular weight DNA ladder; CDS, coding sequence; nt pos. nucleotide position; vRNA, viral RNA.

Brain homogenates from the euthanized mice were inoculated into fresh monolayers of Vero-E6 cells and H292 cells; 12 days after infection, a cytopathic effect, with cell rounding but no syncytia formation, became evident. Virus antigen was detected by immunofluorescence in both cell lines by using patient's convalescent-phase serum, collected 50 days after symptom onset (Figure 3, panel B). Moreover, transmission electron microscopy used to examine virus morphology showed pleomorphic virions, consistent with those of paramyxoviruses (Figure 3, panel C).

Development of New Diagnostic Assays

Because the patient seemed to have acquired the infection during her African research expedition, where she had had extensive contact with rodents and bats, other persons who also come in contact with bats or rodents, such as field biologists, local residents, or ecotourists, might be at risk for infection. This potential public health threat prompted us to develop diagnostic assays for the rapid detection of Sosuga virus.

First, we developed a TaqMan real-time RT-PCR selective for the *N* gene and tested it on all available serum and blood samples from the patient. This test showed that the patient's viremia peaked early in the course of the infection (cycle threshold 29.5 on day 3 after symptom onset), coinciding with the period of high fever and diverse irregularities in blood parameters (Table). By day 9, the viremia had decreased (cycle threshold 36.3); viremia was undetectable 11 days after symptom onset.

Second, we developed a new ELISA specific for Sosuga virus by using the virus recombinant nucleocapsid protein produced and purified from *Escherichia coli*. This assay was tested on all available serum samples from the patient (Table). Although IgG and IgM were not detectable on day

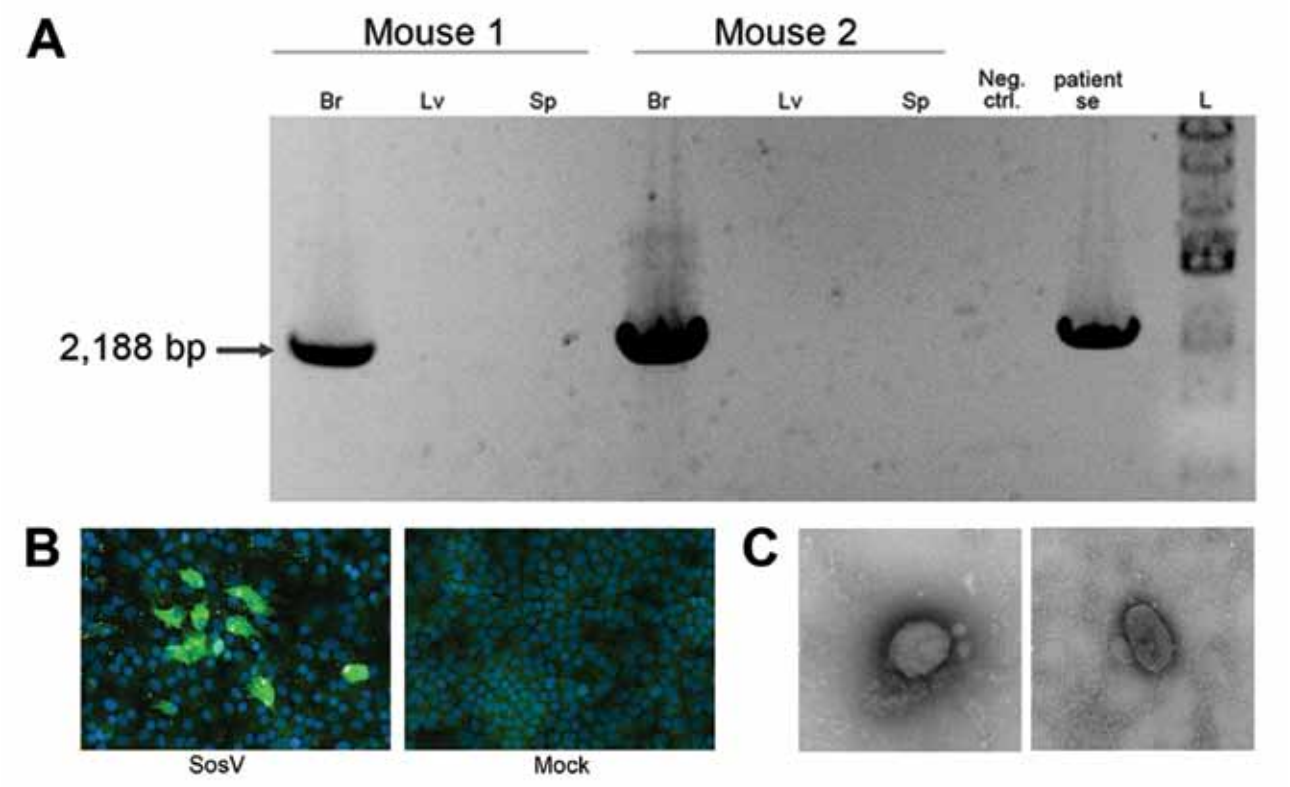

Figure 3. A) Virus isolation confirmed by reverse transcription PCR. SosV was isolated after intracranial and intraperitoneal inoculation into 2-day-old suckling mice. A specific reverse transcription PCR designed to amplify 2,188 bp of the SosV genome was performed by using RNA from brains (Br), liver (Lv), and spleen (Sp) of the euthanized animals. Viral RNA was found only in the brain, not in liver or spleen. B) Propagation of SosV in cell culture. Homogenized tissues (brain, liver, and spleen) were used to infect H292 cells. Fixed monolayers were stained with convalescent-phase serum from the patient and anti-human AlexaFluor 488 antibody (Invitrogen, Grand Island, NY, USA). C) SosV particle. Virus morphologic appearance was examined by taking supernatants from infected Vero-E6 cells, clarifying by slow-speed centrifugation, and depositing on grids for negative staining and examination by transmission electron microscopy. Pleomorphic virions can be observed. Neg.ctrl, negative control; Se, serum; SosV, Sosuga virus; L, molecular mass ladder.

3 after symptom onset (titers <50), seroconversion (IgG and IgM titers ≥1,600) occurred 11 days after symptom onset. As expected, IgM levels later decreased (titer ≥400), and IgG levels remained high 50 days after symptom onset.

In addition, the new ELISA was tested for potential cross-reactivity with some common paramyxoviruses, including mumps and measles viruses. No cross-reactivity was detected on the ELISA plates when control serum from patients with high levels of IgG against mumps and measles viruses was used, a desired feature in a new diagnostic assay because most persons have IgG to these viruses as a result of vaccination or natural infection.

Conclusions

A severe disease affected a wildlife biologist shortly after her return from rural Africa to the United States. Because of the disease characteristics (high fever and blood abnormalities) and travel history, a viral hemorrhagic fever was suspected, and clinical samples were rushed to CDC for investigation of a possible high-risk virus. After molecular and serologic diagnostic assays ruled out several well-known human pathogens (e.g., filoviruses, arenaviruses, phleboviruses, flaviviruses, and rickettsiae) as the cause of the patient's illness, a next-generation sequence approach was followed to detect a possible new infectious agent.

The combination of next-generation sequencing and metagenomic analysis identified a novel paramyxovirus; the virus genome was completely characterized by use of standard sequencing techniques. The complete virus sequence clearly indicated a relationship with other rubula-like viruses isolated from bats. Moreover, the novel virus was isolated from acute-phase serum samples by infecting suckling mice and propagating the virus in cell culture.

The specific molecular and serologic diagnostic assays that we developed will facilitate rapid identification of this novel infectious agent should new cases occur. We used these assays to retrospectively investigate all available

clinical samples from the patient, and the results revealed periods of viremia and seroconversion.

Although the exact source of the patient's infection remains unknown, the sequence similarity with bat-derived rubula-like viruses is highly suggestive. In recent years, a large number of diverse paramyxoviruses have been detected in bats (*2*,*10*), but only Nipah and Hendra viruses (genus *Henipavirus*) are known to cause severe disease in humans (*11*). An investigation to detect Sosuga virus in African bats is currently under way.

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Dr Albariño is a senior research fellow at CDC in Atlanta, Georgia. His research is focused on different aspects of RNA viruses with the goal of developing new diagnostic techniques and evaluating potential vaccines.

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Address for correspondence: Ute Ströher, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop A26, Atlanta, GA 30333, USA; email: ixy8@cdc.gov

Subtyping *Cryptosporidium ubiquitum*, a Zoonotic Pathogen Emerging in Humans

Na Li, Lihua Xiao, Keri Alderisio, Kristin Elwin, Elizabeth Cebelinski, Rachel Chalmers, Monica Santin, Ronald Fayer, Martin Kvac, Una Ryan, Bohumil Sak, Michal Stanko, Yaqiong Guo, Lin Wang, Longxian Zhang, Jinzhong Cai, Dawn Roellig, and Yaoyu Feng

Cryptosporidium ubiquitum is an emerging zoonotic pathogen. In the past, it was not possible to identify an association between cases of human and animal infection. We conducted a genomic survey of the species, developed a subtyping tool targeting the 60-kDa glycoprotein (gp60) gene, and identified 6 subtype families (XIIa–XIIf) of *C. ubiquitum*. Host adaptation was apparent at the gp60 locus; subtype XIIa was found in ruminants worldwide, subtype families XIIb–XIId were found in rodents in the United States, and XIIe and XIIf were found in rodents in the Slovak Republic. Humans in the United States were infected with isolates of subtypes XIIb–XIId, whereas those in other areas were infected primarily with subtype XIIa isolates. In addition, subtype families XIIb and XIId were detected in drinking source water in the United States. Contact with *C. ubiquitum*–infected sheep and drinking water contaminated by infected wildlife could be sources of human infections.

Cryptosporidium infection is a leading cause of diarrhea in humans (*1*). Five *Cryptosporidium* species—*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, and *C. canis*—are responsible for most cases of cryptosporidiosis in humans. Among them, *C. hominis* and *C. parvum* are the most common etiologic agents, and the latter is responsible for most zoonotic infections (*2*). In recent years, *C. ubiquitum,* previously known as the cervine genotype, has been emerging as another major zoonotic species that infects persons. It has been found in humans worldwide, primarily in industrialized nations (*3–11*). In the United Kingdom, more human cases of cryptosporidiosis have been attributed to *C. ubiquitum* than to *C. canis* (*9*).

C. ubiquitum is of public health concern because of its wide geographic distribution and broad host range. Of all *Cryptosporidium* spp. identified by molecular diagnostic tools, it infects the greatest variety of host species (*12*). *C. ubiquitum* has been commonly detected in domestic and wild ruminants (sheep, goats, mouflon sheep, blesboks, nyalas, white-tailed deer, Père David's deer, sika deer, ibexes, buffalos, and yaks), rodents (squirrels, chipmunks, woodchucks, beavers, porcupines, deer mice, house mice, and gerbils), carnivores (raccoons), and primates (lemurs and humans) (*12–16*). It has also been found in drinking source water, storm water runoff, stream sediment, and wastewater in various geographic locations (*17–22*).

Thus far, showing an association between human and animal cases of *C. ubiquitum* infection has not been possible because of the lack of suitable genetic markers for subtyping. For *C. parvum*, *C. hominis*, and some genetically related species, the most commonly used marker for subtyping is the 60-kDa glycoprotein gene (gp60, also called gp40/15). Sequence analysis of the gp60 gene has been used in studies of the genetic diversity, host adaptation, infection sources, and transmission dynamics of these *Cryptosporidium* spp. (*2*). However, it has been suggested that a single locus, such as gp60, is not a reliable marker of *C. parvum* and *C. hominis* population structure because genetic recombination may occur (*23*).

Author affiliations: East China University of Science and Technology, Shanghai, China (N. Li, Y. Guo, L. Wang, Y. Feng); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (N. Li, L. Xiao, Y. Guo, D. Roellig); New York City Department of Environmental Protection, Flushing, New York, USA (K. Alderisio); UK *Cryptosporidium* Reference Unit, Swansea, UK (K. Elwin, R. Chalmers); Minnesota Department of Health, St. Paul, Minnesota, USA (E. Cebelinski); US Department of Agriculture, Beltsville, Maryland, USA (M. Santin, R. Fayer); Academy of Science of Czech Republic, České Budějovice, Czech Republic (M. Kvac, B. Sak); Murdoch University, Perth, Australia (U. Ryan); Slovak Academy of Sciences, Košice, Slovakia (M. Stanko); Henan Agricultural University, Zhengzhou, China (L. Zhang); and Qinghai Academy of Veterinary Medicine and Animal Science, Xining, China (J. Cai)

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Because *C. ubiquitum* is genetically distant from *C. hominis* and *C. parvum*, its homologue of the gp60 gene has thus far not been identified (*24*). In this study, we identified the gp60 gene of *C. ubiquitum* by whole-genome sequencing and used it to develop a subtyping technique to characterize specimens from humans, various animals, and drinking source water.

Materials and Methods

Specimens

DNA extracts from 188 *C. ubiquitum*–positive specimens (collected during 2002–2012) were used, including those from animals, humans, and drinking source water. Animal specimens were obtained from various species of ruminants and rodents, a horse, a raccoon, and a primate, Verreaux's sifaka (*Propithecus verreauxicoquereli*). Animal specimens were collected in the United States, Peru, Brazil, the United Kingdom, Spain, the Czech Republic, the Slovak Republic, Turkey, Algeria, South Africa, China, Nepal, and Australia (Table 1). Human specimens were obtained from the United States, Canada, Peru, the United Kingdom, and Turkey (Table 1). Water samples were collected from storm water and river water in the United States. The storm water samples were collected from a drinking-source watershed in New York where most of the rodent specimens were also collected (*13*,*17*). These specimens were initially found to be positive for *C. ubiquitum* by DNA sequence analysis of an ≈830-bp fragment of the small-subunit rRNA gene (*25*).

Subtyping Marker

To identify a subtyping marker for *C. ubiquitum*, we sequenced the genome of an isolate from a specimen (33496) from a Verreaux's sifaka by 454 technology using a GS FLX+ System (454 Life Sciences, Branford, CT, USA). This specimen was selected for whole-genome sequencing because of the high number of oocysts present, the availability of ample fecal materials for isolation of oocysts by sucrose and cesium chloride gradient flotation

Table 1. Origin and gp60 subtype identity of *Cryptosporidium ubiquitum* specimens from human, animals, and water, 2002–2012

Host group/species	Total no. samples	Source location (no. samples)	Subtype family (no. samples)
Rodent			
Eastern chipmunk	2	USA (2)	XIIb (1), XIId (1)
Eastern gray squirrel	2	USA (2)	XIId (2)
Red squirrel	1	USA (1)	XIId (1)
Woodchuck	1	USA (1)	XIId (1)
Beaver	1	USA (1)	XIId (1)
Ring-tailed porcupine	2	USA (2)	XIIc (2)
Goat through porcupine*	3	USA (3)	XIIc (3)
Yellow-necked field mouse	2	Slovak Republic (2)	XIIe (1), XIIf (1)
Striped field mouse		Slovak Republic (2)	XIIe (2)
Carnivore			
Raccoon	1	USA (1)	XIId (1)
Parissodactyla			
Horse	1	UK (1)	XIIa (1)
Ruminant			
Sheep	56	USA (14), Peru (1), Brazil (3), Spain (9), UK (4), Turkey (1), China (18), Australia (6)	XIIa (56)
Goat	1	Algeria (1)	XIIa (1)
Yak	3	China (3)	XIIa (3)
Buffalo	1	South Africa (1)	XIIa (1)
Alpaca	1	Peru (1)	XIIa (1)
Swamp deer	3	Nepal (3)	XIIa (3)
Impala	1	South. Africa (1)	XIIa (1)
Blesbok	1	Czech Republic (1)	XIIa (1)
Nyala	1	Czech Republic (1)	XIIa (1)
Nonhuman primate			
Verreaux's sifaka	1	USA (1)	XIIb (1)
Human	41	USA (25)	XIIb (9), XIIc (4), XIId (12)
		UK (13)	XIIa (8), XIIb (3), XIId (2)
		Turkey (1)	XIIa (1)
		Peru (1)	XIIa (1)
		Canada (1)	XIIa (1)
Water			
Storm water	15	USA (15)	XIIb (15)
Source water	3	USA (3)	XIIb (2), XIId (1)

*Experimentally infected with specimen from a porcupine.

and immunomagnetic separation, and minor contamination from nontarget organisms in extracted DNA. Of the 3,030 assembled contigs of 11.4 MB nucleotides generated from 1,069,468 sequence reads, 1contig (no. 0067), consisting of 45,014 bp, had a high sequence similarity to the 5′ and 3′ ends of the gp60 gene and the flanking intergenic regions. Alignment of the contig 0067 sequence with the nucleotide sequences of the *C. parvum* gp60 gene (AF203016 and AY048665) led to the identification of sequences conserved between *C. ubiquitum* and *C. parvum*, which were used to design a nested PCR that amplified the entire coding region of the gp60 gene, except for the 54 nt at the 3′ end. The sequences of primers used in primary and secondary PCR were 5′-TTTACCCACACATCTG-TAGCGTCG-3′ (Ubi-18S-F1) and 5′-ACGGACGGAAT-GATGTATCTGA-3′ (Ubi-18S-R1), and 5′-ATAGGT-GATAATTAGTCAGTCTTTAAT-3′ (Ubi-18S-F2) and 5′-TCCAAAAGCGGCTGAGTCAGCATC-3′ (Ubi-18S-R2), which amplified an expected PCR product of 1,044 and 948 bp, respectively.

PCR

The partial *C. ubiquitum* gp60 gene was amplified by nested PCR in a total volume of 50 mL, containing 1 mL of DNA (primary PCR) or 2 mL of the primary PCR product (secondary PCR), primers at a concentration of 0.25 μM (Ubi-18S-F1 and Ubi-18S-R1) or 0.5 μM (Ubi-18S-F2 and Ubi-18S-R2), 0.2 μM deoxyribonucleotide triphosphate mix (Promega, Madison, WI, USA), 3 μM $MgCl_2$ (Promega), 1 × GeneAmp PCR buffer (Applied Biosystems, Foster City, CA, USA), and 1.25 U of Taq DNA polymerase (Promega). The primary PCR reactions also contained 400 ng/mL nonacetylated bovine serum albumin (Sigma, St. Louis, MO, USA) to reduce PCR inhibition. PCR amplification consisted of an initial denaturation at 94°C for 5 min; 35 cycles at 94°C for 45 s, 45 s at 58°C (primary PCR) or at 55°C (secondary PCR), 1 min at 72°C; and a final extension for 7 min at 72°C. Both positive (DNA from the sifaka specimen) and negative (reagent-grade water) controls were used in each PCR run.

DNA Sequence Analysis

Products of the secondary gp60 PCR were sequenced in both directions on an ABI 3130 Genetic Analyzer (Applied Biosystems). The sequences were assembled by using ChromasPro 1.32 (www.technelysium.com.au/ChromasPro.html), edited by using BioEdit 7.04 (www.mbio.ncsu.edu/BioEdit/bioedit.html), and aligned by using ClustalX 2.1 (www.clustal.org/). To assess the genetic relatedness of various subtype families of *C. ubiquitum*, we constructed a neighbor-joining tree by using MEGA 5.05 (www.megasoftware.net/). The reliability of cluster formation was evaluated by the bootstrap method with 1,000 replicates. To assess potential recombination among various subtype families, we used DnaSP 5.10 (www.ub.es/dnasp/) to calculate recombination rates on the basis of segregating sites (excluding insertions and deletions). Unique nucleotide sequences derived from this work were deposited in GenBank under accession nos. JX412915–JX412926 and KC204979–KC204985.

Statistical Analysis

We compared the difference in the distribution of *C. ubiquitum* XIIa and non-XIIa subtype families between rodents and ruminants and between humans in the United States and the United Kingdom by using a χ^2 test implemented in SPSS 20.0 for Windows software (IBM Corp., Armonk, NY, USA). The difference was considered significant when the p value obtained was <0.05.

Results

Features of the gp60 Gene of *C. ubiquitum*

The gp60 gene in contig 0067 in the whole-genome sequencing of specimen 33496 was 945 bp in length, coding for a peptide that consisted of 315 aa. Except for the 5′ and 3′ regions, the gp60 gene of *C. ubiquitum* had no obvious similarity to the gp60 genes of *C. parvum* and *C. hominis* at the nucleotide level. At the amino acid level, the sequences shared 43%–48% sequence identity with those of *C. parvum* and *C. hominis* (Figure 1). The gene has the same structure of gp60 in *C. parvum* and *C. hominis*, with the first 19 aa coding for a signal peptide and the last 20 aa for a transmembrane domain. As in *C. parvum* and *C. hominis*, upstream of the transmembrane domain, the *C. ubiquitum* gp60 gene sequence had a C-terminus hydrophobic region that is likely linked to a glycosylphosphatidylinositol anchor, 2 potential N-linked glycosylation sites, and numerous O-linked glycosylation sites (*26*,*27*). However, no TCA/TCG/TCT trinucleotide repeats were seen in the 5′ region of the gp60 gene of *C. parvum*, *C. hominis,* and related species (*28*), and no N-terminal amino acids, DVSVE, were found between the putative cleavage site of the signal peptide and trinucleotide repeats (*27*). The putative furin cleavage site RSRR or KSISKR between gp40 and gp15 of *C. parvum* and *C. hominis* (*29*) was not found in *C. ubiquitum* (Figure 1).

Amplification of gp60 gene of *C. ubiquitum*

Of the 188 DNA preparations of *C. ubiquitum*, 149 yielded PCR products of the expected size, and 145 were successfully sequenced, including 86 from animals, 41 from humans, and 18 from water (Table 1). Nucleotide sequences of isolates from 31 specimens were identical to those of the *C. ubiquitum* reference sequence (contig 0067 from specimen 33496). The remaining sequences had nucleotide

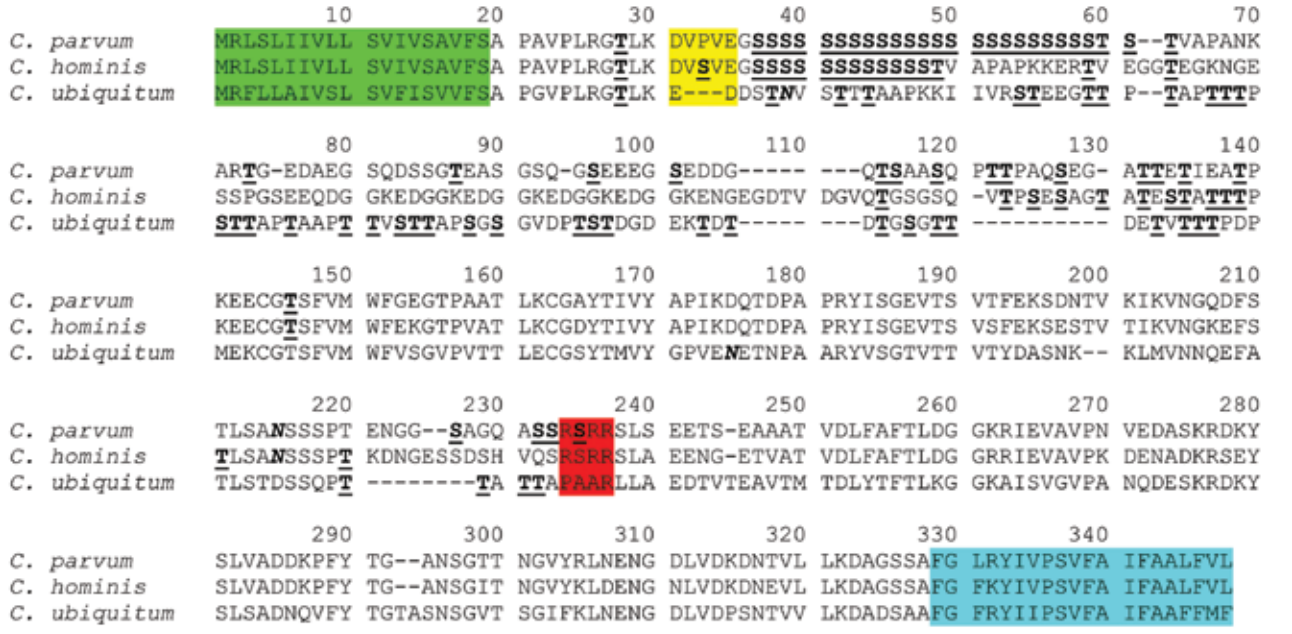

Figure 1. Deduced amino acid sequence of the gp60 gene of *Cryptosporidium ubiquitum* compared with sequences of *C. parvum* and *C. hominis*. gp60 sequences from *C. ubiquitum*, *C. parvum* (GenBank accession no. AF022929), and *C. hominis* (GenBank accession no. ACQ82748) were aligned by using ClustalX (www.clustal.org/). Potential N-linked glycosylation sites are indicated in boldface and italic type, and predicted O-linked glycosylation sites are indicated in boldface and underlined type. The first 19 aa coding for a signal peptide are highlighted in green, and the last 20 aa for a transmembrane domain are highlighted in blue. The signal peptide cleavage site in the N terminal and furin proteolytic cleavage site between gp40 and gp15 of *C. parvum* and *C. hominis* are highlighted in yellow and red, respectively. The furin cleavage site is not present in *C. ubiquitum*. Dashes denote nucleotide deletions.

differences of 8.2%–38.2% from the reference (Table 2). The gp60 nt sequences of *C. ubiquitum* differed from each other in length at most by 171 bp, with all insertions/deletions in trinucleotide, thus maintaining the reading frame (online Technical Appendix Figure; wwwnc.cdc.gov/EID/article/20/2/12-1797-Techapp1.pdf). Most of the length differences were due to 4 sequences generated from rodents in the Slovak Republic. The remaining sequences differed from each other in length by at most 12 bp. Most nucleotide sequence polymorphism occurred in the 3′ half of the gene (online Technical Appendix).

Nomenclature of *C. ubiquitum* Subtype Families

The nucleotide sequences of the gp60 gene of *C. ubiquitum* formed 6 subtype families in a neighbor-joining analysis (Figure 2). They were named XIIa, XIIb, XIIc, XIId, XIIe, and XIIf, in concordance with the established nomenclature of gp60 subtype families (*24*). Subtype families XIIe and XIIf formed a cluster highly divergent from the dominant cluster of the 4 subtype families of XIIa, XIIb, XIIc, and XIId (Figure 2). In the dominant cluster, XIIc and XIId diverged at the 3′ end, and the 3′ end of the gene of XIIc had a high identity to XIIa, an indication of possible genetic recombination at the gp60 locus (online Technical Appendix). Indeed, DnaSP analysis (www.ub.edu/dnasp/) revealed a minimum of 23 potential recombination events among the 6 subtype families (XIIa–XIIf), and 7 recombination events among the 4 subtype families (XIIa–XIId) in the dominant *C. ubiquitum* cluster. Pairwise recombination event comparisons revealed that the genetic recombination was mostly between XIIa and other subtype families (Table 2).

Within the XIIa subtype, minor sequence differences were found among specimens, leading to the formation of 3

Table 2. Nucleotide sequence similarity (lower triangular matrix) and potential genetic recombination events (upper triangular matrix) among *Cryptosporidium ubiquitum* subtype families at the gp60 locus*

	XIIa	XIIb	XIIc	XIId	XIIe	XIIf
XIIa		1	1	1	1	1
XIIb	87.2		0	0	0	0
XIIc	89.5	91.8		0	0	0
XIId	87.2	90.0	94.5		0	0
XIIe	63.1	69.6	62.2	60.6		0
XIIf	71.5	61.8	70.7	69.5	70.9	

*Nucleotide sequence similarity is indicated by the percentage identity between each pair of sequences compared.

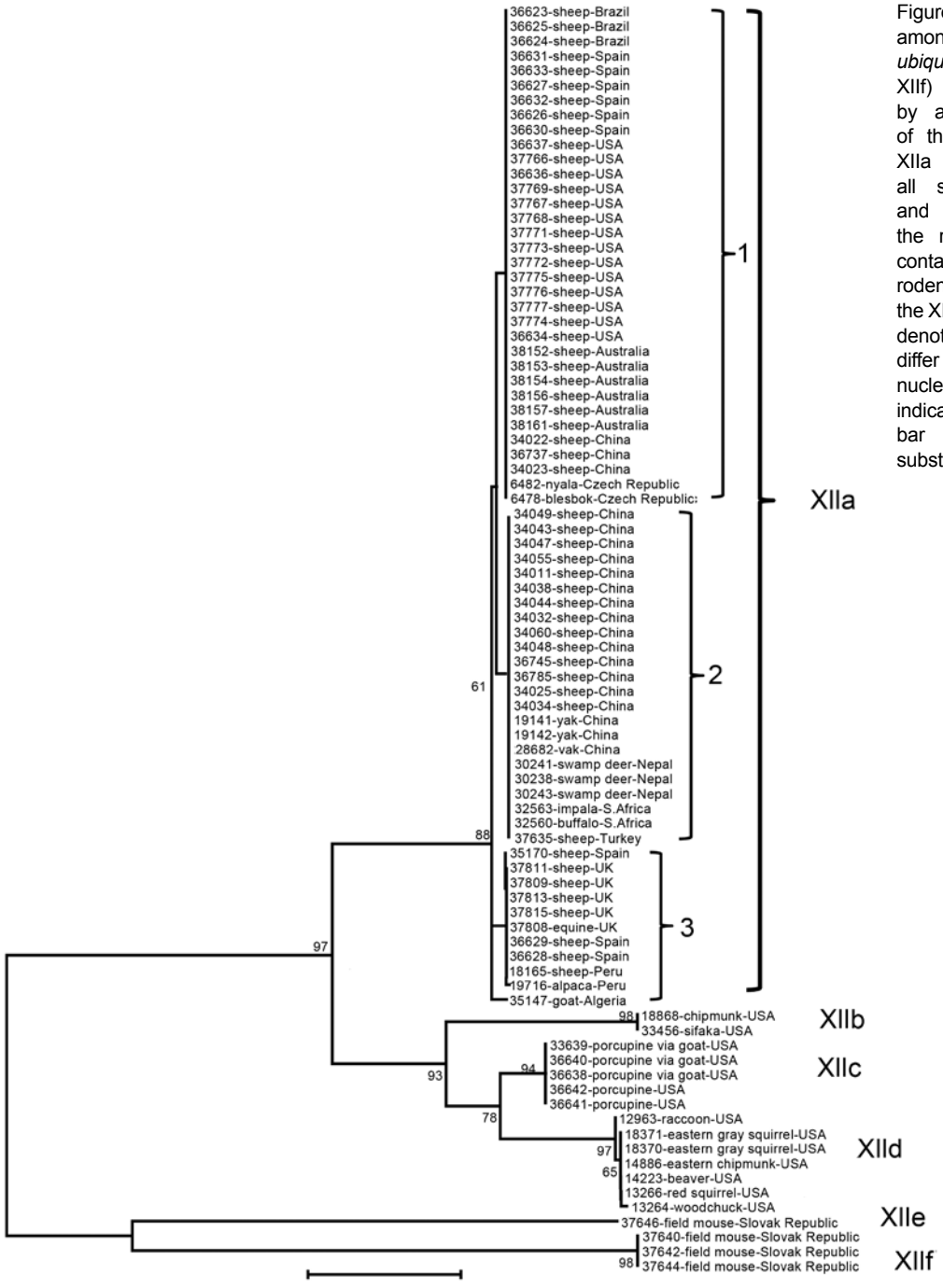

Figure 2. Genetic relationship among 6 *Cryptosporidium ubiquitum* subtype families (XIIa–XIIf) in animals as indicated by a neighbor-joining analysis of the partial gp60 gene. The XIIa subtype family contains all specimens from domestic and wild ruminants, whereas the remaining subtype families contain all specimens from rodents and other wildlife. Within the XIIa subtype family, 1, 2, and 3 denote subtypes 1, 2, and 3, which differ from each other by a few nucleotides. Bootstrap values are indicated along branches. Scale bar indicates 0.02 nucleotide substitutions per site.

common subtypes (subtypes 1–3) and several rare subtypes with a single nucleotide substitution each (for example, 19716, 35147, 37827, 37828, and 37830). A few nucleotide substitutions were also seen in the subtype family XIId (online Technical Appendix). These subtypes within subtype families XIIa and XIId could not be named using the subtype nomenclature based on the number of trinucleotide repeats TCA, TCG, and TGT (*24*) because of the lack of such repeats in the gp60 gene of *C. ubiquitum* (online Technical Appendix).

Subtype Families in Animals

Among 86 isolates from animal specimens characterized, all 68 isolates from Old and New World ruminants belonged to the XIIa subtype family, including 56 from ovine specimens from the United States, Peru,

Brazil, Spain, the United Kingdom, Turkey, China, and Australia; 1 from a goat specimen from Algeria; 3 from yak specimens from China; 1 from an alpaca specimen from Peru; and 7 from 5 species of wild ruminants from Nepal, the Czech Republic, and South Africa. One isolate from an equine specimen from the United Kingdom also belonged to the XIIa subtype family. In contrast, XIIb, XIIc, and XIId subtypes were all seen in specimens from several species of rodents and a few other wildlife species (1 raccoon and 1 Verreaux's sifaka) in the United States, and XIIe and XIIf were seen in specimens from field mice in the Slovak Republic (Table 1, Figure 2). The difference in the distribution of XIIa and non-XIIa subtype families between ruminants and rodents was significant ($\chi^2 = \infty$; $p < 0.001$). This was also the case when the comparison was made with only specimens from the United States ($\chi^2 = 26.00$; $p<0.001$).

Subtype Families in Humans

Among 41 isolates from human specimens successfully subtyped, 25 from the United States belonged to the subtype families of XIIb (*9*), XIIc (*4*), and XIId (*12*); 13 isolates from the United Kingdom belonged to the subtype families of XIIa (*8*), XIIb (*3*), and XIId (*2*); and 3 isolates from Turkey, Peru, and Canada belonged to the XIIa subtype family (Table 1; Figure 3). The difference between the distribution of XIIa and non-XIIa subtype families in humans the United States and United Kingdom was significant ($\chi^2 = 19.49$; $p<0.001$).

Subtype Families in Water

Among 18 water samples from the United States, all 15 storm water samples were collected from a drinking source watershed, and 2 river water samples had the XIIb subtype family. Another river water sample revealed organisms from the XIId subtype family (Table 1).

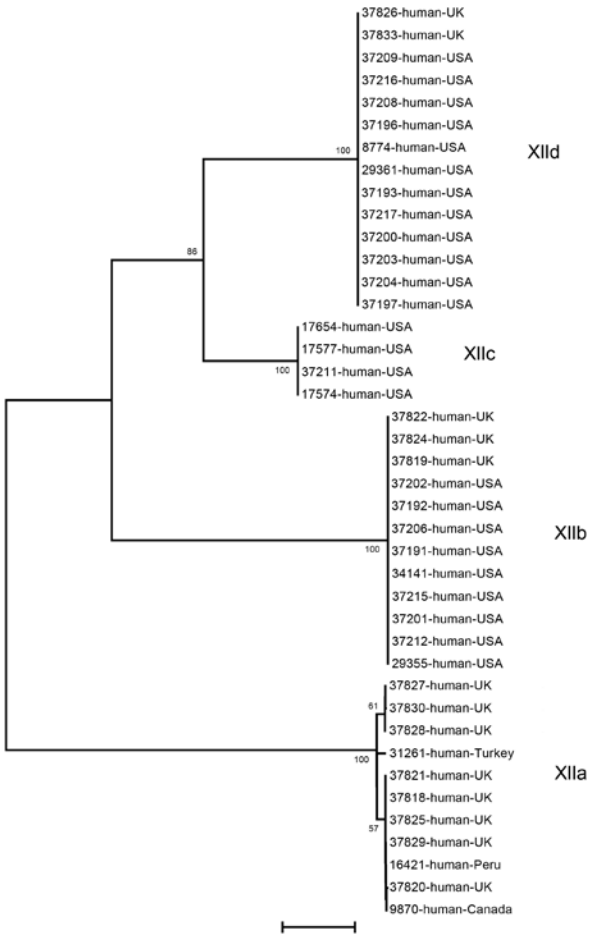

Figure 3.Genetic relationship among 4 *Cryptosporidium ubiquitum* subtype families (XIIa–XIId) in humans as indicated by a neighbor-joining analysis of the partial gp60 gene. The XIIa subtype family is seen in humans in most locations except the United States, where humans are infected with subtype families XIIb, XIIc, and XIId. Bootstrap values are indicated along branches. Scale bar indicates 0.01 nucleotide substitutions per site.

Discussion

The gp60 gene currently is the most commonly used genetic marker for subtyping *Cryptosporidium* spp., including several notable species that are pathogenic to humans and a few pathogenic species/genotypes that are not found in humans. These gp60-based tools have been used effectively in epidemiologic studies of cryptosporidiosis transmission in humans and farm animals (*2*). However, identifying the gp60 gene of the major emerging human-pathogenic species, *C. ubiquitum*, has been challenging (*24*). In this study, we utilized recent developments in next-generation sequencing technology and conducted a genomic survey of 1 *C. ubiquitum* specimen to identify its gp60 gene. On the basis of results from this sequence survey, we developed a gp60-based *C. ubiquitum* subtyping tool. The application of this new tool in the analysis of specimens from humans, animals, and water has shown the existence of host adaptation in *C. ubiquitum* infections, and the findings suggest that sheep and rodents are a key source of *C. ubiquitum* transmission to humans, possibly through direct human contact with infected animals or by contamination of drinking source water.

The gp60 gene of *C. ubiquitum* has extensive sequence differences from the gp60 gene of other *Cryptosporidium* spp. Nucleotide sequences of the near complete gp60 gene obtained from the 6 *C. ubiquitum* subtype families all showed extremely low identity with those of *C. parvum*, *C. hominis*, and related species. Even in the primer regions, substantial nucleotide differences occurred between *C. ubiquitum* and other species/genotypes. These nucleotide

sequence differences could be responsible for the inability of commonly used gp60 primers that were designed on the basis of *C. parvum* and *C. hominis* sequences, to amplify the *C. ubiquitum* gp60 gene (*24*).

The sequence differences between the gp60 gene in *C. ubiquitum* and the gp60 gene in other *Cryptosporidium* spp. may affect its functions. Unlike the gp60 gene of all 11 previously characterized *Cryptosporidium* spp. and genotypes (*24*), the trinucleotide repeats of TCA/TCG/TCT, which code for the polyserine tract at the 5′ end of the gene, commonly used to differentiate subtypes within each subtype family, were not observed in the gp60 gene sequence of *C. ubiquitum*. Previous research suggested that subtypes with short serine repeats may be selectively favored in humans over long repeats (*23*). Other unique features of the gp60 gene in *C. ubiquitum* include the absence of the signal peptide cleavage site in the N terminal and furin proteolytic cleavage site between gp40 and gp15. These differences could affect the processing and transport of the gp60 protein.

Although *C. ubiquitum* has the most broad host range among *Cryptosporidium* spp., the subtype data generated provide evidence that infection with this species has led to host adaptation. All field specimens from domestic and wild ruminants in both the New and Old World belonged to the XIIa subtype family. Despite its common occurrence in both domestic and wild ruminants in many geographic areas, subtype family XIIa has not been found in rodents in the United States and the Slovak Republic. Nevertheless, this host adaptation is not strict host specificity; *C. ubiquitum* of the XIIc subtype family was experimentally transmitted from a ring-tailed porcupine to goats, and the XIIa subtype family was found in an isolate from 1 equine specimen from the United Kingdom (Table 1 and [*12*]).

In contrast to the US findings, subtype family XIIa appears to be more commonly seen in *C. ubiquitum* isolates that infect humans in other areas. Thus, 8 of 13 cases of human infection in the United Kingdom and all 3 cases in humans from 3 other countries were caused by isolates of the subtype family XIIa. The source of *C. ubiquitum* infections in humans is not entirely clear. Because of the common occurrence of the XIIa subtype family in sheep, contact with sheep could be a frequent source of human infection in the United Kingdom and other industrialized nations. Indeed, among the 8 persons infected with XIIa in the United Kingdom, 4 had contact with sheep, 2 with so-called farm animals, and 1 with dogs. The remaining 5 case-patients with infections caused by subtypes XIIb and XIId in the United Kingdom reported no contact with sheep, although 4 reported contact with pets (dogs, cats, or pet birds), which are not known hosts of *C. ubiquitum*. One case-patient who was infected with *C. ubiquitum* of the XIIb subtype reported swimming in pools and had sick family members, indicating potential acquisition of the infection through another transmission route.

Drinking untreated water contaminated by wildlife might be a potential source of *C. ubiquitum* infections in the United States. All US *C. ubiquitum* specimens from humans characterized in this study belonged to the same subtype families found in wild rodents in this country. Because persons in the United States usually have little direct contact with wild rodents, direct zoonotic transmission of *C. ubiquitum* infection is probably less important in this country. This is supported by the absence of the XIIa subtype family in infected humans in the United States. Although XIIa is clearly common in sheep in the United States, the density of sheep is lower than it is in the United Kingdom. *C. ubiquitum* is one of the most common *Cryptosporidium* spp. in drinking source water in the United States (*17–19*). In this study, XIIb and XIId were detected in drinking source water in the United States, and most water samples were collected from the same watershed where US rodent specimens had been collected.

In conclusion, data generated thus far have shown host adaptation in *C. ubiquitum* at the gp60 locus and some potential geographic differences in the epidemiology of *C. ubiquitum* infections in humans. These finding highlight the need for subtype analysis of unusual *Cryptosporidium* species to clarify the sources and transmission dynamics of zoonotic cryptosporidiosis in rural areas.

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Dr Li is a lecturer at East China University of Science and Technology. Her research interest focuses on the molecular epidemiology of zoonotic parasites.

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Address for correspondence: Yaoyu Feng, State Key Laboratory of Bioreactor Engineering, School of Resources and Environmental Engineering, East China University of Science and Technology, Shanghai 200237, People's Republic of China; email: yyfeng@ecust.edu.cn

Monitoring Human Babesiosis Emergence through Vector Surveillance, New England, USA

Maria A. Diuk-Wasser, Yuchen Liu, Tanner K. Steeves, Corrine Folsom-O'Keefe, Kenneth R. Dardick, Timothy Lepore, Stephen J. Bent, Sahar Usmani-Brown, Sam R. Telford III, Durland Fish, and Peter J. Krause

Human babesiosis is an emerging tick-borne disease caused by the intraerythrocytic protozoan *Babesia microti*. Its geographic distribution is more limited than that of Lyme disease, despite sharing the same tick vector and reservoir hosts. The geographic range of *B. microti* is expanding, but knowledge of its range is incomplete and relies exclusively on reports of human cases. We evaluated the utility of tick-based surveillance for monitoring disease expansion by comparing the ratios of the 2 infections in humans and ticks in areas with varying babesiosis endemicity. We found a close association between human disease and tick infection ratios in long-established babesiosis-endemic areas but a lower than expected incidence of human babesiosis on the basis of tick infection rates in new disease-endemic areas. This finding suggests that babesiosis at emerging sites is underreported. Vector-based surveillance can provide an early warning system for the emergence of human babesiosis.

Human babesiosis is an emerging tick-borne disease in the United States, caused primarily by the intraerythrocytic protozoan *Babesia microti* and by other *Babesia* species (*1,2*). *B. microti* also may be transmitted through infected blood and is the most commonly reported transfusion-transmitted pathogen in the United Sates (*3,4*). The outcome of *B. microti* infection varies from asymptomatic or moderate disease in previously healthy persons to severe and sometimes fatal disease in persons who are elderly or immunocompromised (*1*). Fatality rates of 6% to 9% have been reported among hospitalized patients and of ≈20% among those who are immunosuppressed or who experience transfusion-transmitted babesiosis (*3–7*). Although *B. microti* shares the same tick vector (*Ixodes scapularis* ticks) and primary reservoir host (*Peromyscus leucopus* mice) (*1,8*) as the Lyme disease agent (*Borrelia burgdorferi*), the number of Lyme disease cases reported nationally in 2011 was ≈25 times greater than that of babesiosis cases. This disparity probably results from the more limited geographic distribution of *B. microti,* although lower *B. microti* tick infection prevalence in babesiosis-endemic areas or lower tick-to-human transmission rates may also be contributing factors. Babesiosis has expanded in a pattern similar to that of Lyme disease, albeit at a slower pace (*9,10*). Both diseases are following the range expansion of *I. scapularis* ticks over the past 30 years (*9,11*). If *B. microti* continues its current rate of expansion, it may ultimately have the same distribution as *B. burgdorferi*.

Current knowledge of the geographic range of babesiosis is incomplete and relies exclusively on human case reports. Case reports are poor indicators of risk for babesiosis because of the low index of suspicion on the part of many physicians and the lack of distinctive clinical signs such as the erythema migrans rash of Lyme disease. An alternative approach to determining infection risk is tick-based surveillance, which is likely to be a more sensitive method for identifying areas where babesiosis is emerging and can be used to estimate zoonotic prevalence in established areas. The incidence of tick-borne disease is primarily determined by 2 factors: human–tick contact rates and the proportion of the tick population that is infected (*12,13*). Human–tick

Author affiliations: Yale School of Public Health, New Haven, Connecticut, USA (M.A. Diuk-Wasser, Y. Liu, T.K. Steeves, C. Folsom-O'Keefe, S.J. Bent, S. Usmani-Brown, D. Fish, P.J. Krause); Uppsala University, Uppsala, Sweden (Y. Liu); Audubon Connecticut, Southbury, Connecticut, USA (C. Folsom-O'Keefe); Mansfield Family Practice, Storrs, Connecticut, USA (K.R. Dardick); Nantucket Cottage Hospital, Nantucket, Massachusetts, USA (T. Lepore); University of Adelaide, Adelaide, South Australia, Australia (S.J. Bent); Tufts University, Boston, Massachusetts, USA (S.R. Telford III); and L2 Diagnostics, New Haven (S. Usmani-Brown)

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contact rates are difficult to measure accurately because they are highly spatially heterogeneous and are determined by complex interactions between human and tick populations, depending on particular tick densities and human behaviors associated with human exposure.

We proposed an integrative measure of tick-borne disease risk that combines tick infection prevalence and human incidence data for an established disease (Lyme disease) and an emerging disease (babesiosis). We hypothesized that the ratio of human Lyme disease to babesiosis incidence rates (hereafter termed "human ratio") is directly proportional to the prevalence ratio of *I. scapularis* ticks infected with *B. burgdorferi* and that of those infected with *B. microti* (hereafter termed "tick ratio") in babesiosis-and Lyme disease–endemic areas. We based this hypothesis on the fact that the ratio of incidence rates for >1 infection transmitted by the same tick should depend only on the ratio of tick infection prevalence with the respective pathogens because human–tick contact rates are the same for both. We further hypothesized that the human ratio would be relatively higher than the tick ratio in areas where babesiosis is newly emerging because of underreporting of emerging disease. Accordingly, we determined the relationship between the human ratio and the tick ratio in regions in southern New England where the disease is endemic and emerging.

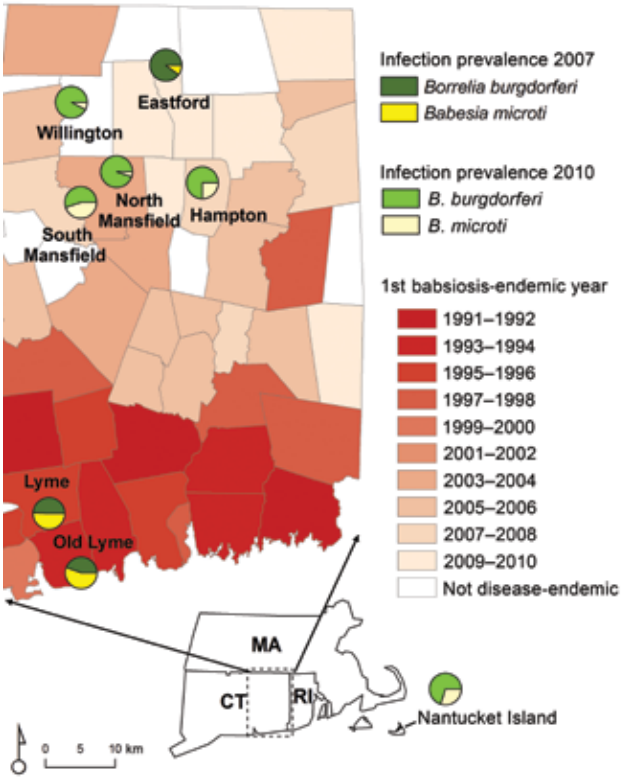

Figure 1. *Borrelia burgdorferi* and *Babesia microti* infection prevalence among humans and *Ixodes scapularis* ticks, eastern Connecticut (CT) and Nantucket, Massachusetts (MA). Shading indicates human babesiosis incidence in study towns by year in which the disease became endemic in the town (defined as the first year babesiosis cases were reported for 2 consecutive years). *I. scapularis* nymphal infection prevalence is shown for *B. microti* and *B. burgdorferi* in Lyme/Old Lyme in 2007 and for Nantucket and northeastern CT in 2010, represented as a pie chart for each sampled location. RI, Rhode Island.

Methods

Study Sites

We established study sites in towns in Lyme disease–endemic regions in Connecticut and Massachusetts on the basis of when human babesiosis became endemic. A town was defined as babesiosis-endemic the first time the state public health department reported human babesiosis cases for 2 consecutive years. We identified 2 sites in southeastern Connecticut (Lyme and Old Lyme) and 1 site in Massachusetts (Nantucket) where babesiosis has been endemic for at least a decade. Nantucket was the first area where human babesiosis was identified as endemic in the United States (*14*), and Lyme disease and babesiosis have been highly endemic there for 4 decades. Lyme disease and babesiosis have been endemic in Lyme and Old Lyme for 2 decades (Figure 1, Table 1). We also established 5 study sites in northeastern Connecticut (Eastford, Hampton, North Mansfield, South Mansfield, and Willington), where babesiosis has become endemic within the past 10 years or where it is not yet considered endemic (Figure 1, Table 1).

Prevalence *of B. burgdorferi* and *B. microti* Infection in *I. scapularis* Nymphs

Host-seeking *I. scapularis* nymphs were collected at the study sites in 2007 and 2010 (Table 1). Sampling was conducted once or twice at each site during the peak nymphal host-seeking period from late May to late June. We focused on the nymphal stage of *I. scapularis* because it is the only tick stage with a significant role as a vector for *B. burgdorferi* in North America (*15*). Because of their small size, these nymphs often escape detection long enough to transmit *B. burgdorferi* (*16*). To sample *I. scapularis* nymphs, researchers dragged a 1-m^2 corduroy cloth over leaf litter along a variable number of 100-m transects and conducted supplemental nonquantitative drags to provide additional ticks (*17*). A total of 1,170 nymphal ticks (87 on Nantucket, 247 in southeastern Connecticut, 836 in northeastern Connecticut) were collected in forested areas and peridomestic habitats adjacent to forests (Table 1). The cloth was inspected for ticks at 20-m intervals, and ticks were preserved in vials containing 70% ethanol. The geographic coordinates of all transects relative to the map datum WGS84 were recorded by using a handheld global positioning system receiver (Garmin, Olathe, KS, USA).

Table 1. Summary of nymphal *Ixodes scapularis* infection with *Babesia microti* and/or *Borrelia burgdorferi* on Nantucket Island, Massuchusetts, and in Connecticut, 2007 and 2010

Region	Year became babesiosis endemic	Year tick sample collected	*B. burgdorferi* infection prevalence (no. positive/no. tested)	*B. microti* infection prevalence (no. positive/no. tested)	Tick ratio*	Co-infection prevalence (no. positive/no. tested)
Nantucket, MA	1969	2010	0.21 (18/87)	0.09 (8/87)	2.33	0.01 (1/87)
Southeastern CT						
Lyme	1996	2007	0.13 (23/182)	0.15 (28/182)	0.82	0.04 (8/182)
Old Lyme	1992	2007	0.20 (13/65)	0.20 (13/65)	1.00	0.06 (4/65)
Combined sites			0.15 (36/247)	0.17 (41/247)	0.88	0.05 (12/247)
Northeastern CT						
Hampton	2007	2010	0.29(43/147)	0.10 14/147)	2.90	0.05 (8/147)
South Mansfield	2002	2010	0.11 (12/111)	0.09 (10/111)	1.22	0.01 (1/111)
North Mansfield	2002	2010	0.13 (18/139)	0.01 (1/138)	13.00	0.00 (0/138)
Willington	Not endemic	2010	0.36 (51/142)	0.03 (4/142)	12.00	0.02 (3/142)
Eastford	Not endemic	2007	0.31 (93/298)	0.03 (10/298)	10.33	0.03 (10/298)
Combined sites			0.26 (217/836)	0.05 (39/836)	5.20	0.03 (24/828)

*Ratio of *I. scapularis* ticks infection prevalence with *B. burgdorferi* and infection prevalence with *B. microti*.

All ticks were identified by using a dissecting microscope and taxonomic keys (*18*). DNA was extracted from all *I. scapularis* nymphs with QIAGEN DNeasy blood and tissue kit (QIAGEN Inc., Valencia, CA, USA) by using a modified protocol (*19*). A nested PCR was performed to amplify the 16S-23S rRNA intergenic spacer region of *B. burgdorferi* by using the primers and protocols developed by Liveris et al. (*20*). Amplicons were visualized on 1% agarose gel by using ethidium bromide. All positive samples were sequenced bi-directionally. *B. burgdorferi* amplicons were typed by comparing them with known genotypes by using BLAST (*21*). Ticks were tested for *B. microti* infection by using a reverse transcription PCR that targets a sequence of the *B. microti* 18S rRNA gene (GenBank accession no. AY144696.1) (*22*). Forward and reverse primers and probe sequences were (5′ to 3′) AACAGGCATTCGCCTTGAAT,CCAACTGCTCCTATTAAC CATTACTCT, and 6FAM-CTACAGCATGGAATAATGA-MGBNFQ, respectively. The assay was performed by using Applied Biosystems 7500 Real-Time PCR machine (Foster City, CA, USA). The PCR reaction consisted of TaqMan Universal PCR Master Mix (2X) (with AmpErase, Applied Biosystems), 0.9 mM forward and reverse primers, 0.2 mM Probe, and 5 mL DNA template in a total reaction volume of 25 mL. Ticks were considered positive if they displayed amplification at or before a cycle threshold value of 35.

Incidence Rates for Babesiosis and Lyme Disease in Humans

We calculated annual incidence rates for babesiosis and Lyme disease in humans for the towns and years in which tick assessments were conducted by using reported cases and mid-year population estimates from the Connecticut Department of Public Health (*23*) and the Massachusetts Department of Public Health (*24*). We also calculated annual incidence rates for 2010 by using clinical diagnosis data from a private physician practice on Nantucket Island with ≈10,000 patients and a practice in Mansfield, Connecticut. The Mansfield practice services a large catchment area that includes 4,000 patients from the towns where ticks were collected for this study (Eastford, Hampton, Mansfield, and Willington).

Data Analyses

Logistic regression analyses were performed to assess differences in prevalence of infection with *B. burgdorferi* and *B. microti* in *I. scapularis* nymphs and to compare infection prevalence of each of these pathogens among study sites. We derived a tick-infection prevalence ratio (or tick ratio) by dividing the prevalence of *B. burgdorferi* infection by the prevalence of *B. microti* infection from samples from each of the 3 study regions (Nantucket, southeastern Connecticut, and northeastern Connecticut). We derived a disease incidence-rate ratio (or human ratio) by dividing Lyme disease incidence rates by babesiosis incidence rates for state-reported cases by town and for each medical practice. For the combined estimates of the southeastern and northeastern Connecticut regions, the state-reported annual incidence rates were calculated by adding all cases and dividing by the respective mid-year population estimates for the towns where tick samples were collected.

We evaluated the association between the human ratio and tick ratio by fitting a linear regression model. The model included state-reported case data from babesiosis-endemic sites and case diagnoses from both medical practices. These datasets were selected with the assumption that Lyme disease and babesiosis would probably be diagnosed and reported in a similar manner at these sites. Both diseases are well known by the study physicians on Nantucket and in Mansfield and at the study sites where Lyme disease and babesiosis have been endemic for ≥2 decades (Lyme and Old Lyme). We then compared the state-reported human ratio in areas where babesiosis is emerging to its expected value, on the basis of the tick ratio. In areas where babesiosis was

emerging, if the value of the human ratio was higher than expected from the tick ratio, underreporting of babesiosis (in comparison to Lyme disease) was probable. Statistics were performed by using Stata 12 Statistical Software (StataCorp. LP, College Station, TX, USA).

Results

Prevalence of *B. burgdorferi and B. microti* Infection in *I. scapularis* Nymphs

Mean tick infection prevalence for *B. burgdorferi* was 0.23 ± 0.42 and for *B. microti* was 0.08 ± 0.26 for all sites and years combined. *B. burgdorferi* tick infection prevalence was higher than *B. microti* tick infection prevalence when all sites were combined but varied significantly by region. *B. burgdorferi* infection was 5.2 times more prevalent than *B. microti* infection in northeastern Connecticut, 2.3 times more prevalent than *B. microti* infection on Nantucket, and was not significantly different in prevalence from *B. microti* infection in southeastern Connecticut (Tables 1, 2). The tick ratio varied from 0.88 to 5.20 across regions (Tables 1, 3). Rates of co-infection with both pathogens varied from 0 to 0.06 across regions.

Incidence Rates for Lyme Disease and Babesiosis in Humans

Incidence rates of Lyme disease in humans ranged from 191 to 211 cases per 100,000 population, based on state-reported data; 660, based on data from the medical practice in northeastern Connecticut; and 3,750, based on data from the medical practice on Nantucket. Incidence rates for human babesiosis ranged from 7 to 85 cases per 100,000 population, based on state-reported data; 70, based on data from the northeastern Connecticut medical practice, and 1,250, based on data from the Nantucket medical practice. Lyme disease incidence rates were higher than babesiosis incidence rates in all regions, and the human ratio varied from 2.3 in southeastern Connecticut (state reported) to 30.0 in northeastern Connecticut (state reported) (Table 3).

Association between Human and Tick Ratios

The human ratio was higher than the corresponding tick ratio for both private practice and state-reported case data in all regions/datasets (Table 3). A linear regression of the human ratio (combining medical practice and state-reported cases in babesiosis-and Lyme disease–endemic regions) on the tick ratio yielded the following (Figure 2):

$HR = 1.95 \times TR + 0.34$

HR = human ratio or human incidence rate ratio

TR = tick ratio or tick infection prevalence ratio

Ninety percent of the variance in the human ratio was explained by the tick ratio ($R^2 = 0.9$). The 1.95 regression coefficient indicates that exposure to *B. burgdorferi*–infected ticks was nearly twice as likely to result in a case diagnosis as was exposure to *B. microti*–infected ticks. The reported human ratio from state-reported case data in the babesiosis-emergent area in northeastern Connecticut was higher than expected by the regression model (Figure 2), suggesting underreporting of babesiosis.

Discussion

We examined the ratio of *B. burgdorferi* infection to *B. microti* infection in humans and ticks and found that in areas where the 2 infections have been endemic for at least 20 years, the human ratio was consistently 2 times higher than the tick ratio. We found a strong positive association between the human and tick ratios, using either state health department data from long-time babesiosis–endemic states or data from private practices, indicating that the tick ratio can be used to estimate the relative risk for humans of acquiring *B. burgdorferi* and *B. microti* infection. In contrast, the human ratio in *B. microti*–emerging areas in northeastern Connecticut calculated from state-reported data was about 6 times higher than the tick ratio. These larger human ratios in emerging areas probably result from physician underreporting of babesiosis in comparison to Lyme disease cases. The nationally reported Lyme disease and babesiosis case ratio (25:1) is even greater and is probably caused by a combination of underreporting of babesiosis and the wider geographic distribution of *B. burgdorferi*. We conclude that the ratio of ticks infected with *B. burgdorferi* to those infected with *B. microti* may be used as a sensitive surveillance tool to identify new areas where human babesiosis is endemic but has not yet been reported or is underreported.

We found areas in Connecticut where Lyme disease is endemic but babesiosis is absent, or present at very low prevalence. In contrast, no areas where babesiosis is endemic and Lyme disease is not present have been reported. Babesiosis is increasingly being reported from areas where Lyme disease alone was previously endemic. Originally reported from Nantucket Island, Massachusetts, in 1969 (*14*), babesiosis has expanded its geographic range

Table 2. Logistic regression comparing the prevalence of *Borrelia burgdorferi* infection with *Babesia microti* infection among *Ixodes scapularis* nymphal ticks, New England*

Prevalence	Odds ratio	SE	*z* score	p value (95% CI)
Nantucket, MA	2.57	1.17	2.08	0.038 (1.050–6.290)
Northeastern CT	7.16	1.30	10.82	0.00 (5.01–10.23
Southeastern CT	0.86	0.21	–0.62	0.53 (0.52–1.39)
All sites combined	3.71	4.8	10.02	0.00 (2.86–4.78)

*Results are shown stratified by region and for all sites combined.

Table 3. Comparison between the ratio of Lyme disease and babesiosis incidence in humans (human ratio) and the ratio of *Borrelia burgdo*rferi and *Babesia microti* infection in ticks (tick ratio), New England, 2007 and 2010*

Data source, y	Lyme disease†	Babesiosis†	Human ratio‡	*B. burgdorferi*§	*B. microti*§	Tick ratio¶	Human/tick ratio
Private practice							
Nantucket, 2010	7,500	1,250	6.0	0.21	0.09	2.33	2.6
Northeastern CT, 2010	660	70	9.4	0.26	0.05	5.20	1.8
Reported to CT DPH or MA DPH							
Nantucket, 2010	194	56	3.5	0.21	0.09	2.33	1.5
Northeastern CT, 2010	211	7	30.0	0.26	0.05	5.20	5.8
Southeastern CT, 2007	191	85	2.3	0.15	0.17	0.88	2.6

*CT DPH, Connecticut Department of Public Health; MA DPH, Massachusetts Department of Public Health.
†Annual incidence rates of Lyme disease and human babesiosis calculated for the towns and years for which tick assessments were conducted by using private practice or state-reported cases and mid-year population estimates from CT DPH (*23*) and MA DPH (*24*).
‡Ratio of human Lyme disease incidence rates to babesiosis incidence rates.
§Nymphal *Ixodes scapularis* tick infection prevalence with *Borrelia burgdorferi* and *Babesia microti*.
¶ Ratio of *I. scapularis* ticks infection prevalence with *B. burgdorferi* and infection prevalence with *B. microti*.

to mainland Connecticut (*9,25*), Maine (*26*), Massachusetts (*27,28*), New Jersey (*29,30*), New York (*7*,*31*), and Rhode Island (*32,33*). A similar range expansion is being reported from the Upper Midwest (*34*). As with Lyme disease (*12*,*35*), the emergence of *B. microti* in ticks has resulted in dramatic increases in the number of reported human cases of babesiosis, with a 3-fold increase in Connecticut from 1998 to 2008 (*23*) and a 20-fold increase in the lower Hudson Valley in New York from 2001 to 2008 (*31*). Our finding of a 2.3–3.5 ratio of babesiosis to Lyme disease in babesiosis-endemic areas is consistent with the 1.7 ratio reported in a 10-year prospective study on Block Island, Rhode Island, where babesiosis and Lyme disease have been endemic for >2 decades (*33*) This finding suggests that as *B. microti* expands into areas previously enzootic for *B. burgdorferi* alone, the national 25:1 human ratio is likely to narrow.

The lower incidence of babesiosis in areas where both diseases are endemic is due in part to the lower prevalence of *B. microti* infection in *I. scapularis* nymphs. Less efficient transmission of *B. microti* from infectious hosts to ticks may account for this difference. Mather et al. (*36*) found that 45% of nymphs derived from larvae that fed on *B. microti*–infected *P. leucopus* mice were infected with *B. microti* sporozoites, whereas 92% of nymphs derived from larvae that fed on a *B. burgdorferi*–infected mouse were infected with the spirochete. The decreased ability of *B. microti* to survive in overwintering nymphs compared with that of *B. burgdorferi* appears to further reduce infection prevalence in *I. scapularis* ticks (*37*). Although these laboratory findings are consistent with the higher average prevalence of *B. burgdorferi*, they are not sufficient to explain the large variability observed in prevalence ratios for *B. burgdorferi*/*B. microti* infection in *I.*

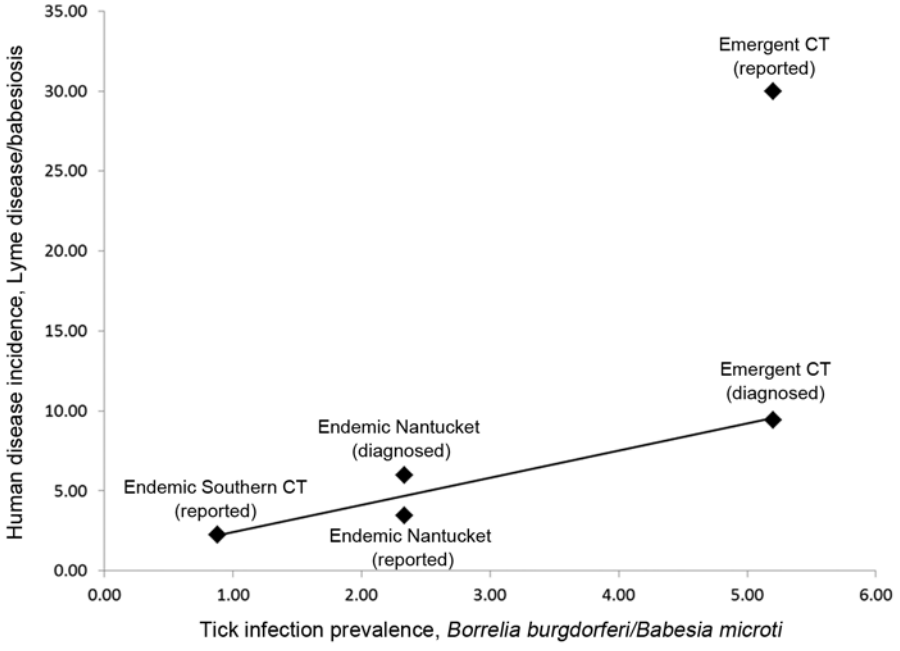

Figure 2. Linear regression model of the human ratio (disease incidence rate ratio) and the tick ratio (tick infection prevalence ratio). The regression model includes state-reported case data from disease-endemic sites and case diagnoses from both medical practices. The human ratio derived from state-reported case data in the emerging area (northeastern Connecticut) is also displayed.

scapularis nymphs, which ranged from 0.9 to 5.2. Local differences in *I. scapularis* nymphal abundance (*32*,*38*) or the composition of the enzootic host community (*8*) may help explain this variation.

Differences in *B. burgdorferi* and *B. microti* infection prevalence in ticks were ≈2-fold, whereas incidence of human Lyme disease was ≈4-fold higher than that of babesiosis in human babesiosis–endemic areas. Thus, the disparity in Lyme disease/babesiosis human case reporting was about twice that of tick-infection prevalence. This finding may be caused by a combination of decreased ability of nymphs to transmit *B. microti* efficiently to humans (*39*); greater difficulty in diagnosing babesiosis than Lyme disease; and more frequent asymptomatic *Babesia* infection, particularly in the young, immunocompetent population. The rates of asymptomatic infection in adults were 25% for *B. microti* infection and 10% for *B. burgdorferi* infection in previous studies (*33*,*40*). Longitudinal studies of Lyme disease and babesiosis in humans and infection prevalence in ticks are necessary for assessing the relative importance of these factors in determining the ratio of human Lyme disease to babesiosis.

The proposed tick surveillance method can be used to monitor the emergence of babesiosis and other *I. scapularis* tick–borne emerging infections in the eastern United States, such as human granulocytic anaplasmosis (*Anaplasma phagocytophilum*), Powassan virus disease (Powassan virus), and hard tick relapsing fever (*Borrelia miyamotoi*). Vector-based surveillance that compares the incidence of emerging infections to that of Lyme disease can serve as a powerful tool for identifying new disease-endemic areas, leading to early case recognition and reduction in disease risk.

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Dr Diuk-Wasser is an assistant professor in the Department of Epidemiology of Microbial Diseases, Yale School of Public Health. Her work focuses on the eco-epidemiology of vector-borne and zoonotic diseases such as Lyme disease, West Nile virus infection, and dengue.

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Address for correspondence: Maria Diuk-Wasser, Yale School of Public Health, PO Box 208034, 60 College St, New Haven, CT 06520, USA; email: maria.diuk@yale.edu

Genomic Variability of Monkeypox Virus among Humans, Democratic Republic of the Congo

Jeffrey R. Kugelman,[1] Sara C. Johnston,[1] Prime M. Mulembakani, Neville Kisalu, Michael S. Lee, Galina Koroleva, Sarah E. McCarthy, Marie C. Gestole, Nathan D. Wolfe, Joseph N. Fair, Bradley S. Schneider, Linda L. Wright, John Huggins, Chris A. Whitehouse, Emile Okitolonda Wemakoy, Jean Jacques Muyembe-Tamfum, Lisa E. Hensley, Gustavo F. Palacios,[2] and Anne W. Rimoin[2]

Monkeypox virus is a zoonotic virus endemic to Central Africa. Although active disease surveillance has assessed monkeypox disease prevalence and geographic range, information about virus diversity is lacking. We therefore assessed genome diversity of viruses in 60 samples obtained from humans with primary and secondary cases of infection from 2005 through 2007. We detected 4 distinct lineages and a deletion that resulted in gene loss in 10 (16.7%) samples and that seemed to correlate with human-to-human transmission (p = 0.0544). The data suggest a high frequency of spillover events from the pool of viruses in nonhuman animals, active selection through genomic destabilization and gene loss, and increased disease transmissibility and severity. The potential for accelerated adaptation to humans should be monitored through improved surveillance.

Viruses in the family *Poxviridae,* genus *Orthopoxvirus*, consist of numerous pathogens known to infect humans, including variola virus (VARV), monkeypox virus (MPXV), cowpox virus (CPXV), and vaccinia virus (VACV). Genomes of these viruses are ≈200 kb long, have highly conserved central regions coding for replication and assembly machinery, and have more variable terminal ends that contain genes involved in host range determination and pathogenesis (*1*).

Although orthopoxviruses are antigenically and genetically similar, they have diverse host range and virulence properties (*1*–*6*). Comparative genomics studies have shown that the evolution of orthopoxviruses is ongoing and can be driven by selective pressure from a host species (*4*,*7*). It has been postulated that progressive gene loss, primarily at the terminal ends of the genome, has been a driving force behind the evolution of these viruses (*7*). CPXV, which causes only mild infection in humans, contains the largest genome of all sequenced orthopoxviruses (≈220 kb), encodes 223 open reading frames (ORFs), and has a broad host range that includes rodents, humans, felids, bovids, and voles (*7*–*10*). Conversely, VARV, the causative agent of smallpox, is highly pathogenic (case-fatality rate ≈30%) (*11*), has the smallest genome of all naturally occurring orthopoxviruses (≈186 kb), is predicted to encode 20% fewer functional proteins than CPXV, and has a host range restricted to humans (*7*).

Similar to VARV, MPXV is a virulent orthopoxvirus that causes high levels of illness and death (case-fatality rate ≈10%) (*12*–*15*). Unlike natural VARV infections, which were declared eradicated in 1979, MPXV infections occur naturally in MPXV-endemic regions of Africa, such as the Democratic Republic of the Congo (DRC) and Republic of the Congo. Human exposure to animal reservoirs, including squirrels of the genera *Funisciurus* and *Heliosciurus*, poses high risk for MPXV infection (*16*–*19*). Recent reports suggest that human-to-human transmission

Author affiliations: United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland, USA (J.R. Kugelman, S.C. Johnston, M.S. Lee, G. Koroleva, S.E. McCarthy, M.C. Gestole, J. Huggins, C.A. Whitehouse, G.F. Palacios); Kinshasa School of Public Health, Kinshasa, Democratic Republic of the Congo (P.M. Mulembakani, E.O. Wemakoy); University of California, Los Angeles, California, USA (N. Kisalu, A.W. Rimoin); Global Viral Forecasting (now known as Metabiota), San Francisco, California, USA (N.D. Wolfe, J.N, Fair, B.S. Schneider); The Eunice Kennedy Shriver National Institute of Child Health and Human Development, Bethesda, Maryland, USA (L.L. Wright); National Institute of Biomedical Research, Kinshasa (J.J. Muyembe-Tamfum); and US Food and Drug Administration, Silver Spring, Maryland, USA (L.E. Hensley)

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[1]These authors are co–first authors.

[2]These authors contributed equally to this work

of MPXV is increasing (*20*,*21*). In 2003 in the DRC, 7 generations of uninterrupted spread among humans were reported (*21*). Increasing susceptibility coincident with decreasing herd immunity is expected to profoundly increase the introduction and spread of MPXV among humans. In 2003, an outbreak of monkeypox in the midwestern United States revealed the propensity for transmission to MPXV-naive populations (*22*). The strain of MPXV responsible belonged to the Western African clade; these strains tend to be less pathogenic than the Central African strains that circulate in the DRC (*2*).

MPXV is a linear DNA genome of ≈197 kb and contains ≈190 nonoverlapping ORFs >180 nt long (*1*,*7*,*26*). Like all orthopoxviruses, the central coding region sequence (CRS) at MPXV nucleotide positions ≈56000–120000 is highly conserved and flanked by variable ends that contain inverted terminal repeats (ITRs) (*1*). VACV homologs to genes found in the terminal ends of the MPXV genome are predominantly involved in immunomodulation, and most are either predicted or known to influence host range determination and pathogenicity (*27*). Unlike VARV, which lacks ORFs in the ITR region, MPXV contains at least 4 ORFs in the ITR region (*7*,*26*).

The evolution of short-read, high-throughput sequencing technologies has made genomic surveys of large populations readily attainable (*23*,*24*). It has been suggested that the progressive loss of genes that were not essential for pathogenesis in humans is the mechanism that led to the emergence of a highly adapted virus that causes serious disease and is capable of efficient and rapid human-to-human transmission (*7*). A recent study demonstrated that gene copy number variation might be a crucial factor for modulating virus fitness (*25*). In the study reported here, we investigated the genomic plasticity of MPXV strains obtained from patients in the DRC during active surveillance for monkeypox disease (*20*).

Materials and Methods

Sample Selection

From November 2005 through November 2007, a total of 760 cases of human monkeypox in the Sankuru District, DRC, were identified by active disease surveillance and confirmed by quantitative real-time PCR (*20*). From these, we selected 60 cases on the basis of 3 epidemiologic measures: severity, transmission, and geography. The samples chosen represented the entire set of samples and included samples from every category of severity and transmission observed and every geographic location sampled. Of the 60 cases, we sequenced 29 representative scab or vesicle fluid samples from patients whose samples met the criteria for concentration and quality of genetic material (≥500 ng total DNA and ≥1.8 260/230 and 260/280 absorbance ratio). We used the Genome Analyzer IIx and MiSeq sequencers (Illumina, Inc., San Diego, CA, USA) to conduct 76-bp or 100-bp, paired-end sequencing. To avoid laboratory-induced mutation events, we sequenced these samples directly from scab or vesicle material without virus isolation or propagation.

Sample Collection and Processing

Techniques for sample collection, processing, nucleotide isolation, and real-time PCR have been described (*20*,*28*,*29*). The World Health Organization scoring system used during the smallpox eradication program was used to generate severity designations: presence of <25 lesions on the body was considered mild disease; 25–99 lesions, moderate; 100–250 lesions, severe, and >250 lesions, serious. Libraries with an average insert size of ≈300 bp were processed by using the TruSeq (Illumina, Inc.) sequencing kit version 5 and sequenced for either 76 or 100 cycles from each end on an Illumina Genome Analyzer IIx or MiSeq instrument.

Genome Assembly

All raw Illumina sequence data were processed through an in-house–developed filtering program that removes or trims reads with known artifacts. Specifically, all reads containing adaptor sequence, low-complexity reads, or PCR duplicates were removed or trimmed. Host read exclusion was performed by removing reads that aligned to the human genome (*Homo sapiens* chromosome 10, GRCh37 primary reference assembly) by using the CASAVA pipeline 1.8 (Illumina, Inc.) with default parameters. De novo assemblies were performed by using SeqMan Pro NGen (DNAStar, Inc., Madison, WI, USA) with default parameters. Genome size was set to 200 kb. The optimal *k*-mer was determined by using an iterative assembly process. We closed gaps in coverage and resolved large repeats by using Sanger sequencing for the 23 genomes described in this work. Sequences were submitted to GenBank with accession nos. JX878407–JX878429.

Phylogenetic Analysis

We conducted the phylogenetic analysis by using methods identical to those reported for VARV diversity (*4*). In brief, we aligned the CRS (MPXV nucleotide positions ≈56000 120000) of samples with sufficient sequence information with 11 MXPV reference sequences available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/), GenBank nos. KC257459, KC257460, DQ011154, DQ011155, HQ857562, HQ857563, NC_003310, AY603973, AY753185, AY741551, DQ011156, DQ011153, and DQ011157 in SeqMan Pro NGen (DNAStar, Inc.). Manual editing of gaps and ambiguous base calls of the alignment were performed to include the draft genomes and provide a more robust

phylogenetic narrative. The resulting alignment was analyzed by using Bayesian methods of phylogenetic reconstruction and the GTR+Γ evolutionary model. MrBayes version 3.1.2 (*30*) was run by using 1,000,000 generations with 4 parallel runs. Markov chain Monte Carlo convergence was assessed by checking the average standard deviation of split frequencies (<0.01) during >10,000 generations. Figure 1 shows the unrooted tree based on alignment of the 11 MPXV reference sequences and the viral genomes from the 23 clinical samples in this study.

PCR Screening

All primers were developed by using Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) with default parameters. This software uses Primer3 to design the primers and then BLAST to determine specificity. We designed qualitative PCRs to detect the left and right short tandem repeats (STRs) at MPXV nucleotide positions 72–486 and 196710–197124, a region spanning the 625-bp deletion between bases 189820 and 190444 in the right ITR, and a 410-bp region of the CRS that contains 4 single-nucleotide polymorphism locations positively typing the lineages described in this article between nucleotide positions 89273 and 89944. All PCRs were run by using standard methods and optimized annealing temperatures of 55°C through 65°C. The PCR product Sanger sequences were aligned to the reference in SeqMan Pro software (DNAStar) for validation by using default parameters. Primer sequences are available on request.

Results

Referenced and de novo alignments were performed by using SeqMan Pro NGen to determine the scaffold of the viral genome. Genomes were finished by using PCR and traditional dideoxynucleotide sequencing (Sanger) to resolve gaps and repetitive and low coverage areas. We obtained 23 MPXV genomes, expanding the available completed genomes for Central African clade MPXV strains

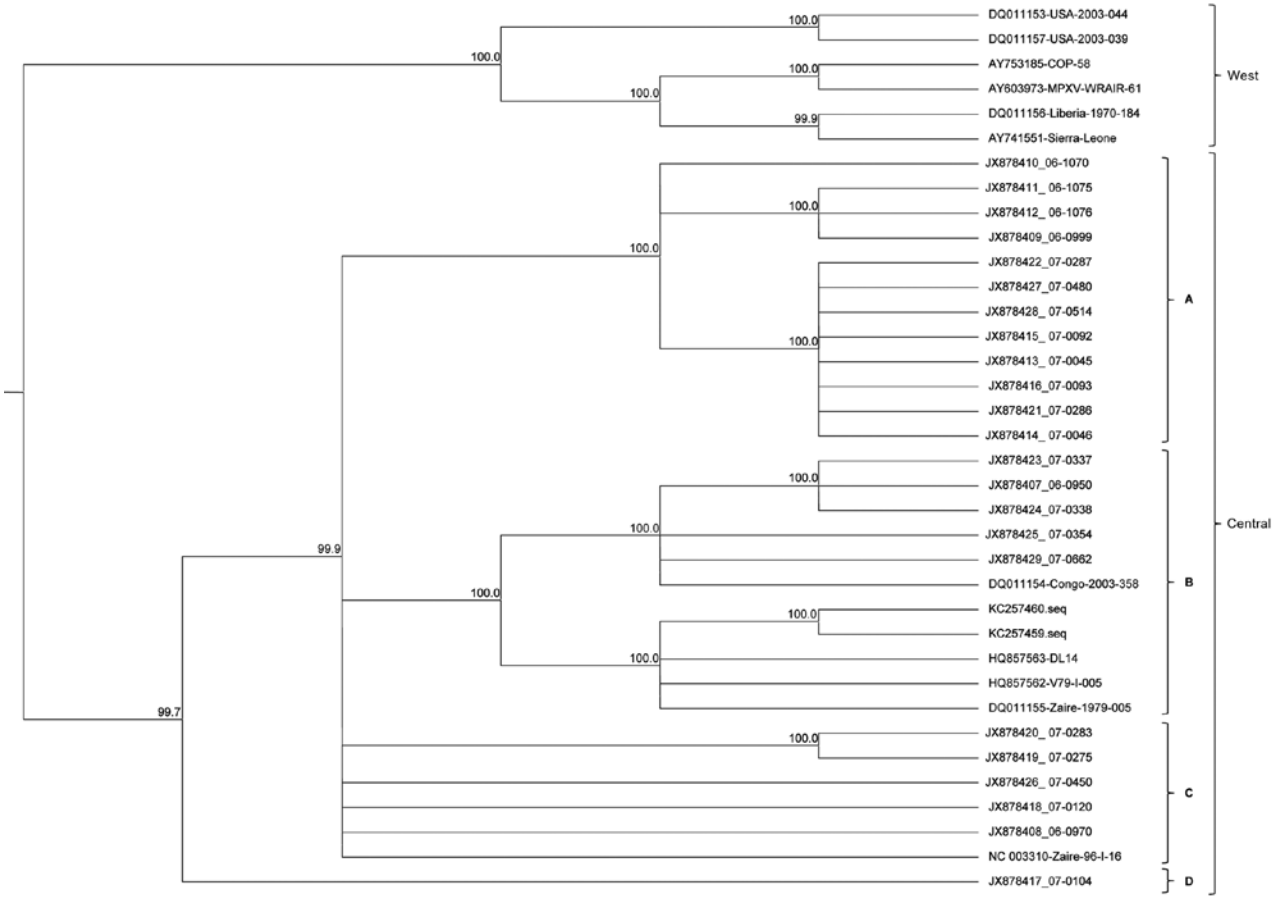

Figure 1. Phylogenetic analysis of whole-genome direct sequencing. Evolutionary relationships between sequenced samples and archived monkeypox virus (MPXV) sequences were determined for the central coding region sequence (MPXV nucleotide positions ≈56000–120000). A cladogram representing the topology of an unrooted Bayesian phylogenetic reconstruction is shown for samples identified by sample number and/or GenBank accession number. The Central and Western African clades and the 4 distinct lineages are indicated. Confidence values for branching events were computed by Markov chain Monte Carlo convergence. Numbers at nodes represent Bayesian posterior probabilities computed by using MrBayes 3.1.2 (*30*).

by ≈6-fold. To assess the potential genomic diversity of MPXV strains circulating in the DRC, we performed a phylogenetic analysis of the CRS. The resulting cladogram suggested the emergence of 4 distinct lineages within the Central African clade.

Genomic Reduction

Among the MPXV alignments, we detected a polymorphism in the noncoding region of the ITR with 12 variants. Of the 23 (17.4%) complete genome sequences, 4 showed substantial genomic instability directly upstream of the right ITR. This subset of samples contained a 625-bp deletion between bases 189820 and 190444 (genome positions based on MPXV-COG_2003_358). This deletion completely removes MPV-Z-N2R and eliminates the first 103 bp of the orthopoxvirus major histocompatibility complex class I–like protein (OMCP, MPV-Z-N3R) (Figure 2, panel A). The function of the protein expressed from MPV-Z-N2R is unknown, and no homologous gene is in the VARV or West African MPXV genomes. OMCP is a secreted protein that binds to NKG2D and interferes with NKG2D-mediated cell killing by natural killer cells (*31*). A conventional PCR created to characterize the deletion enabled us to detect this deletion in 6 additional genomes (online Table, wwwnc.cdc.gov/EID/article/20/2/13-0118-T1.htm.) We found no evidence of a mixed population within the sequence information for the isolates containing the deletion or in the gel electrophoresis of the Amplicon Sanger sequencing products for samples tested (data not shown).

This genetic polymorphism was absent from all MPXV sequences available through GenBank. Moreover, although 1 full-length copy and 1 truncated copy of the OMCP gene are present in all genomes of the Western African clade MPXV, only the full-length copy of the gene is present in genomes of the Central African MPXV clade (Figure 2, panel A). No homologous gene is present in the VARV genome (*26*) (www.poxvirus.org). The deletion identified in our clinical samples completely removes the region of OMCP containing the leader sequence (Figure 2, panel B) and substantially alters protein folding (data not shown), suggesting that the truncated protein is nonfunctional. The deletion was identified in genomes belonging to lineage B only (online Table). Data regarding route of infection were available for 44 patients; transmission for 24 cases was classified as human-to-human (secondary) and for 20 as animal-to-human (primary) (online Table). Metadata were available for 8 of the samples containing the deletion; 7 (87.5%) of the 8 were from cases resulting from human-to-human transmission ($p = 0.0544$, Fisher exact test), suggesting that this deletion might be associated with increased fitness in humans. Further work, however, is needed to elucidate the role of OMCP in transmissibility.

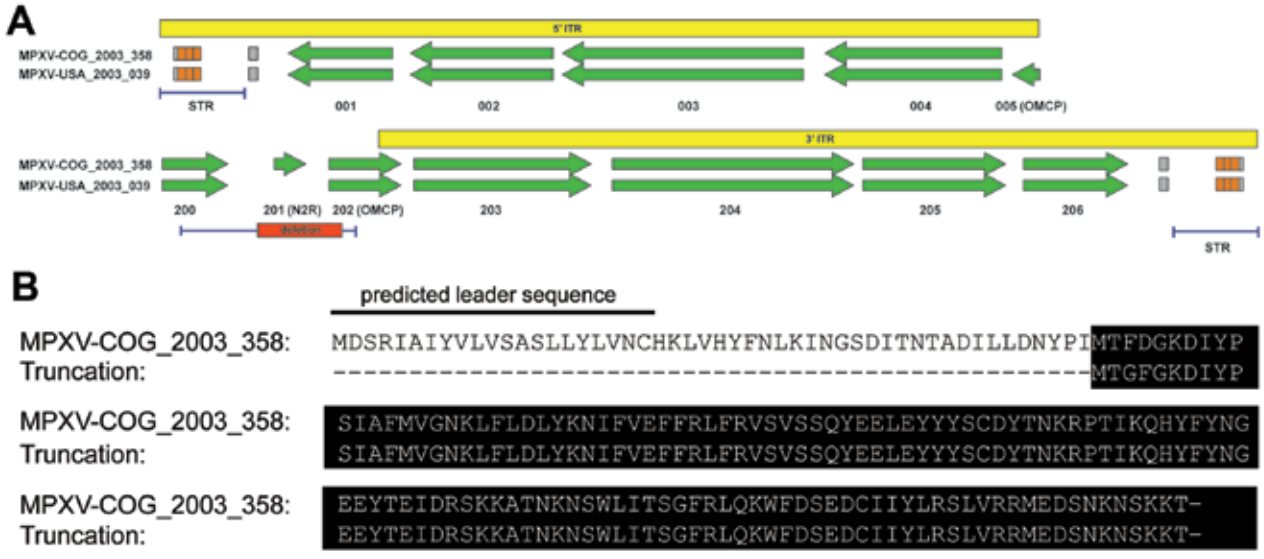

Figure 2. Truncation of OMCP. A) Whole-genome deep sequencing revealed a 625-bp deletion directly upstream of the right ITR (red box), which completely removed MPV-Z-N2R (locus 201) and truncated OMCP (MPV-Z-N3R, locus 202). A Western African clade virus, MPXV-USA_2003_039, is shown for comparison with OMCP and N2R copy number. PCR amplification regions for the large deletion and the STRs (right and left) are indicated by the blue bar below each ITR diagram. The yellow box represents the ITR region, and all open reading frames are identified by their MPXV-COG_2003_358 locus number with genes of interest in parenthesis. B) Nucleotide alignment of the large deletion with MPXV-COG_2003_358 reference sequence. The truncated protein created by the deletion is indicated by the black background. ITR, inverted terminal repeat; MPXV, monkeypox virus; COG, Congo; STR, short terminal repeat; OMCP, orthopoxvirus major histocompatibility complex class I–like protein.

STR Analysis

Substantial variations from other orthopoxviruses were detected in the 70-bp STR region (bases 72–486 and 196710–197124 in MPXV-COG_2003_358) (*4*,*32*–*34*). A traditional PCR designed against the left and right STR regions enabled us to evaluate this polymorphism in all 60 samples. We did not quantitatively test the purity of the repeat population beyond visualization of the PCR product by gel electrophoresis. A small percentage of the samples (10%) had faint secondary products that could not be resolved. The STRs described here should be considered to be the dominant population. In total, we identified 12 unique STR combinations (online Table). STR variations have been described for VARV isolates obtained during 1944–1974 (*34*); however, no studies have addressed the possibility of STR variations in VARV isolates belonging to the same geographic cluster. Samples collected during episodes of VARV activity in Bangladesh, South Africa, or the Far East showed limited variation (<3 left STR patterns); whereas, we observed 12 patterns for MPXV samples collected during a 2-year period in the DRC. Given that the poxvirus genomes in our study were characterized directly from clinical samples, it is plausible that this smaller range of diversity for VARV is only the result of a genetic bottleneck that occurred during cell culture isolation. Rapid copy number variation of the K3L gene in the VACV ITR has recently been described as a mechanism for host immunomodulation (*25*). However, none of the STR copy number variations observed among the MPXV strains involved a known coding region. In contrast to some VARV isolates, all 60 MPXV samples had identical right and left STRs (online Table).

Phylogenetic Analysis of the Entire Dataset

Because only 23 samples contained enough material for complete genome characterization, to expand our analysis to all 60 samples, we performed PCR amplification of a 410-bp variable region of the CRS corresponding to the H4L gene (VACV-COP gene notation). When selecting this region, we used the phylogenetic relationships of the CRS regions of the Central African clade by using 72 segregating sites over 61 kb of sequence. The selected region contained 4 segregating sites that are phylogenetically relevant to Central African clade MPXV (MPXV accession no. DQ01154: 89505, 89545, 89674, and 89914). No evidence of additional diversity was detected at these sites among the samples from the DRC except at the fourth site (89914) and only among the members of lineage B. Phylogenetic reconstruction of this region among the samples resulted in a topologic distribution for DRC isolates that correlates with the whole-genome tree (bootstrap values, as a measure for confidence measures, were lineage A (n = 12, 82%), lineage B (n = 11, 81%), lineage C (n = 6, 80%), and lineage D (n = 1). Moreover, this region contains 7 sites that differentiate between the Central African (n = 29) and Western African (n = 6) clades with a bootstrap value of 82%, which can be evaluated as another measure of the phylogenetically informative character of this genomic area. In this region, we identified 1 nonsynonymous and 3 synonymous changes. An additional nonsynonymous change was identified in all lineages ≈800 bp downstream of this region, for a total of 5 polymorphic sites in H4L within the Central African clade.

Overall, 11.6% of the genetic variations identified in this study were located in an ≈1.2-kb conserved region of the CRS. H4L is reported to be tightly associated with the DNA-dependent RNA polymerase, aiding in early gene transcription before initiation and termination (*35*,*36*). Increased mutation rates in a tightly constrained area of a genome can be a marker of increased selective pressure. The effect of the genetic polymorphisms on the function of H4L is unknown, and further studies to evaluate their effects on early gene expression are needed.

The phylogenetically informative single-nucleotide polymorphisms were assessed by PCR amplification and dideoxynucleotide sequencing for all 60 samples. This information was then used to extrapolate the lineage of each sample (online Table).

Lineage A contains the widest range of STR repeats (*1*–*15*) and represents 33 (55.0%) of the samples analyzed. The lineage seems to have arisen in the Lomela health zone (forest) where it continued to circulate throughout 2007. The remaining members of the lineage are a cluster of strains with 13–15 STR repeats that were geographically restricted to the Vangakete health zone (savannah). According to the World Health Organization scoring system for severity used during smallpox eradication, the virus strains belonging to lineage A caused predominantly moderate disease (66.7%, p = 0.2816). No significant differences in animal-acquired versus human-acquired infection were associated with this lineage.

Lineage B contains 1–8 STRs and represents 16 (26.7%) of the samples analyzed. These strains were predominantly isolated from the Kole (forest) and Lomela health zones and were associated with moderate disease (80.0%, p = 0.2179). Although no statistically significant association with secondary (human) transmission was observed among all lineage B strains, the MPV-Z-N2R/OMCP deletion was identified only in genomes belonging to this lineage and, as stated above, was significantly associated with human-to-human transmission.

Lineage C showed high homogeneity with only 1 STR and represented 10 (6.7%) of the samples analyzed. These strains were predominantly obtained from the Djalo-Ndjeka (ecotone) and Vangakete health zones and were associated with severe/serious disease (70.0%, p = 0.0234).

Only 1 representative of lineage D was identified and contained 10 STRs. The sample was collected in Bene Dibele (forest), was associated with severe disease, and was acquired from a human source.

Although the data do not support causality linking the number of STR repeats to the severity of any lineage, the composition of the STR region merits further investigation for effects on virulence and transmission and as a marker of passaging effects. Our data support a wide diversity of STR composition that varies according to lineage; 1 population exhibited only a dominating single STR repeat and others had variants between 1 and 15 repeats. On the basis of our data and the information previously reported (*25*) depicting an accordion-like expansion of gene-copy in response to antiviral systems, there is precedence for a copy number variation mechanism used by the virus. Considering that there is no coding element in this region, the most likely need to vary this region is structural; therefore, we predict that the STR region may be under selective pressure to provide stability to this region of the genome.

Discussion

Our data demonstrate that a combination of genomic destabilization and genetic polymorphism are influencing the evolution of MPXV strains actively circulating in the Sankuru District of the DRC. Patterns of genomic destabilization and gene loss were consistent with models of orthopoxvirus genomic evolution (*7*). A recently submitted genomic sequence derived from 2 isolates obtained in Sudan in 2005 contains a large indel in the right flanking region. This indel simultaneously inserts a 10.8-kb inverted repeat of several immune modulatory coding regions from the left flanking region and deletes a 2.1-kb region that surrounds the region that we identified as reduced. The authors who reported the sequence posit that this insertion and deletion resulted from a single event, and the identifying sequence is the only evidence of either the insertion or the exact deletion in any *Poxviridae* isolate (*37*). The 2 reported changes cannot be functionally compared in this study; however, additional evidence supports genetic variability in this region. Genomic reduction might have played a role in the emergence of VARV as a highly adapted human pathogen capable of efficient human-to-human spread (*7*). Although it has been suggested that gene loss was associated with a restricted host range for VARV, no link has been demonstrated between gene loss and increased severity of disease. VARV is thought to have diverged from its rodent-borne ancestor 3,000–4,000 years ago (*38*). The strains of VARV isolated during periods of high activity in the 20th century were already well adapted to their evolutionary niche (humans), and, as a result, their genetic sequences were highly conserved. Conversely, the level of variability for MPXV isolates might suggest an active pattern of change; however, longer-term surveillance is required.

Although genomic changes are predicted by models of host transition, the correlation of the gene loss pattern with secondary transmission might indicate that MPXV is adapting for efficient replication in a novel ecologic niche: humans. It is also plausible that the association of OMCP gene loss and transmissibility is coincidental because numerous factors, including vaccination status and human encroachment on reservoir habitats, could also explain increased human-to-human transmission and variant introduction frequency. The scarcity of information regarding historical orthopoxvirus emergence and the absence of sequencing data for MPXV reservoir isolates precludes our ability to pinpoint the exact source of the observed variability; however, we predict that the 4 lineages are circulating in the reservoir population and are introduced into the human population after direct contact with those reservoir hosts. Further genomic surveillance that includes reservoir species might help explain the variant emergence described herein.

Active surveillance during November 2005–November 2007 in Sankuru District, DRC, suggested a strong correlation between smallpox vaccination and protection from MPXV (*20*). However, as the number of unvaccinated persons continues to increase, so does the size of the susceptible population. Other factors in the DRC, such as malnutrition, disease, and an inadequate health care system, have provided an ideal environment for MPXV. It is possible that increased circulation of MPXV in the human population of the DRC is a driving force in the evolution of the virus. Our data also indicate that certain genetic changes might affect disease severity or human-to-human transmissibility, suggesting an active period of adaptation that could result in virus strains with increased fitness in humans. However, we have no evidence to directly link the genetic changes to increased severity or transmissibility in vivo.

The global effects of the emergence of MPXV strains that are highly adapted to humans could be devastating. Importation of MPXV by infected vertebrates is of concern because of the potential for establishment of new reservoirs outside Africa. In fact, American ground squirrels have been found to be susceptible to infection (*39*), suggesting that other rodent species worldwide might also be susceptible. Small genetic changes could favor adaptation to a human host, and this potential is greatest for pathogens with moderate transmission rates (such as MPXV) (*40*). The ability to spread rapidly and efficiently from human to human could enhance spread by travelers to new regions. Therefore, active disease surveillance should continue to be used monitor MPXV for changes that are consistent with increasing adaptation to humans. Continued active surveillance of the Sankuru District, and

expansion to all other regions where the virus is known or predicted to circulate, would help determine the true geographic range of this virus. Given the apparent rapid evolution of this virus, when suspected or confirmed cases in humans are observed, health authorities in presently unaffected areas should become vigilant and actively prepare to take immediate action.

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Dr Kugelman is a biodefense research scientist and bioinformatician in the Center for Genome Sciences at United States Army Medicjal Research Institute of Infectious Diseases. His research interest is the genomic study of filovirus and orthopoxvirus infections.

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Addresses for correspondence: For questions regarding the clinical and epidemiological aspects of the article, contact Anne W. Rimoin, Epidemiology Department, UCLA Fielding School of Public Health, 640 Charles E. Young Dr S, Los Angeles, CA 90024, USA; email: arimoin@ucla.edu. For questions regarding the molecular epidemiology and genomics aspects of the article, contact Gustavo F. Palacios, Center for Genome Sciences, 1425 Porter St, Frederick, MD 21702, USA; email: gustavo.f.palacios.ctr@us.army.mil

Lymphocytic Choriomeningitis Virus in Employees and Mice at Multipremises Feeder-Rodent Operation, United States, 2012

Barbara Knust, Ute Ströher, Laura Edison, César G. Albariño, Jodi Lovejoy, Emilian Armeanu, Jennifer House, Denise Cory, Clayton Horton, Kathy L. Fowler, Jessica Austin, John Poe, Kraig E. Humbaugh, Lisa Guerrero, Shelley Campbell, Aridth Gibbons, Zachary Reed, Deborah Cannon, Craig Manning, Brett Petersen, Douglas Metcalf, Bret Marsh, Stuart T. Nichol, and Pierre E. Rollin

We investigated the extent of lymphocytic choriomeningitis virus (LCMV) infection in employees and rodents at 3 commercial breeding facilities. Of 97 employees tested, 31 (32%) had IgM and/or IgG to LCMV, and aseptic meningitis was diagnosed in 4 employees. Of 1,820 rodents tested in 1 facility, 382 (21%) mice (*Mus musculus*) had detectable IgG, and 13 (0.7%) were positive by reverse transcription PCR; LCMV was isolated from 8. Rats (*Rattus norvegicus*) were not found to be infected. S-segment RNA sequence was similar to strains previously isolated in North America. Contact by wild mice with colony mice was the likely source for LCMV, and shipments of infected mice among facilities spread the infection. The breeding colonies were depopulated to prevent further human infections. Future outbreaks can be prevented with monitoring and management, and employees should be made aware of LCMV risks and prevention.

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (B. Knust, U. Ströher, L. Edison, C.G. Albarino, L. Guerrero, S. Campbell, A. Gibbons, Z. Reed, D. Cannon, C. Manning, B. Petersen, S.T. Nichol, P.E. Rollin); Georgia Department of Public Health, Atlanta (L. Edison); Indiana Board of Animal Health, Indianapolis, Indiana, USA (J. Lovejoy, D. Metcalf, B. Marsh); Deaconess Hospital, Evansville, Indiana, USA (E. Armeanu); Indiana State Department of Health, Indianapolis (J. House); Vanderburgh County Health Department, Evansville (D. Cory); Green River District Health Department, Owensboro, Kentucky, USA (C. Horton, J. Austin); and Kentucky Department for Public Health, Frankfort, Kentucky, USA (K.L. Fowler, J. Poe, K.E. Humbaugh)

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Lymphocytic choriomeningitis virus (LCMV), a rodent-borne arenavirus, is a rare, zoonotic cause of aseptic meningitis in Europe and North America. It is carried by the common house mouse (*Mus musculus),* but other rodent species, such as hamsters and guinea pigs, can become infected and transmit infection to humans (*1*). Infected rodents shed the virus in urine, saliva, and droppings. Transplacental infection in mice results in persistently infected offspring, that shed virus throughout life (*2*). Humans become infected through close contact with infected rodents, through transplantation of infected organs, or by vertical transmission. In immunocompetent adults, infections range from mild febrile illness to aseptic meningitis; in immunosuppressed organ recipients, infections are highly fatal, and congenitally infected infants can have a range of severe birth defects (*3*).

In late April 2012, an infectious disease physician contacted the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA) about a 27-year-old woman (patient 1) who sought hospital care for fever, severe headache, photophobia, and vomiting. Cerebrospinal fluid (CSF) had elevated leukocytes (>1,000/mm^3 [reference <5]), elevated protein (153 mg/dL [reference 12–80 mg/dL]), and negative bacterial culture. Patient 1 reported working at an Indiana rodent breeding facility (facility A). In April 2012, aseptic meningitis had been diagnosed in patient 2, who was patient 1's domestic partner and co-worker (E. Armeanu, unpub. data). LCMV infection was suspected, and specimens were submitted to CDC for diagnostic confirmation. Serum samples from patients 1 and 2 and CSF

from patient 1 were positive for LCMV IgM by ELISA, indicating recent LCMV infection. Reverse transcription PCR (RT-PCR) for LCMV was negative, indicating that viremia was no longer present.

The Vanderburgh (Indiana) County Health Department, in conjunction with the Indiana State Department of Health, Indiana Board of Animal Health, and CDC, initiated an outbreak investigation to determine the extent of LCMV infection in the staff and rodents in facility A (*4*). Trace-back investigations also identified a distributor (facility B) where live rats (*Rattus norvegicus*) and mice (*M. musculus*) from facility A were handled and packaged for sale as live and frozen animal food in 21 states (*5*). An additional mouse breeding facility in Kentucky (facility C) had shipped live mice to facility B, which redistributed them to facility A shortly before the outbreak. We describe the diagnostic and epidemiologic aspects of this investigation and the response taken to control the outbreak at these facilities.

Materials and Methods

Employee Serosurvey

As part of the investigation, we provided all current and former employees of facilities A, B, and C within the previous 6 months the opportunity to be interviewed and tested for LCMV as part of the investigation. County health officials administered a questionnaire that collected information about recent clinical illness and work habits at the facilities, including hygiene measures. A blood sample was collected from each interviewee and refrigerated for transport to CDC, where IgG and IgM ELISA were performed (online Technical Appendix, wwwnc.cdc.gov/eid/article/20/2/pdfs/13-0860.pdf). We considered a current or former employee to be recently infected (within the past 2–3 months) with LCMV if he or she had IgM with or without IgG. An employee for whom only IgG was detectible by ELISA was considered to have had a previous infection. If employee had evidence of recent LCMV infection and had sought medical care because of illness, we reviewed his or her medical records. All employees signed an informed consent form.

Rodent Testing

Adult breeding rodents (mice and rats) from facility A were sampled to determine LCMV infection status for each room (online Technical Appendix). In accordance with the facility's standard operating procedures for processing feeder rodents, we euthanized mice and rats with carbon dioxide gas, and the carcasses were frozen and shipped to CDC on dry ice. Carcasses were thawed, and animals were dissected under Biosafety Level 3 conditions. Heart blood and small sections of kidney and spleen were collected. Rodent specimens were tested for LCMV RNA by RT-PCR and for LCMV IgG by ELISA. RT-PCR–positive specimens were inoculated onto cell culture to isolate virus, and viral RNA was sequenced and compared with other LCMV strains (online Technical Appendix).

Statistical Analysis

All data were collected by using a computerized spreadsheet (Microsoft Excel, Microsoft, Redmond, WA, USA) and analyzed by using statistical analysis software (SAS 9.3, SAS Institute, Cary, NC, USA). Rodent location information was combined with diagnostic test results to calculate an observed prevalence of IgG seropositivity and RT-PCR positivity per room sample. Ninety-five percent CIs for the prevalence of mice with LCMV antibodies and apparent viremia per room population were estimated by using a binomial distribution equation, and estimated ranges of antibody-positive and viremic rodents per room were calculated from these ranges.

Employee questionnaire data were combined with diagnostic test results, and a case–control comparison was performed. We defined an LCMV case-patient as an employee who had detectable LCMV IgM and/or IgG and controls as employees of the same facility who had negative test results. Statistics for each facility were examined separately. Univariate analysis was conducted, and $p<0.05$ was considered statistically significant.

Results

Human LCMV Investigation

All 52 employees of facility A were tested by ELISA. Fifteen (29%) had detectable anti-LCMV antibodies; 13 had IgM and IgG, indicating recent infection. Nine IgM/IgG-positive employees reported recent clinical illness, including 5 who had sought medical treatment. Symptoms described most frequently were headache, nausea and vomiting, subjective fever, decreased appetite, diarrhea, muscle ache, and stiff neck (Table 1). Aseptic meningitis was diagnosed in 4 employees from facility A after lumbar punctures demonstrated lymphocytic CSF (Table 2). Dates of onset for laboratory-confirmed aseptic meningitis ranged from April 7 through May 14, 2012. All case-patients recovered fully.

All 13 employees of facility B were tested; 1 (8%) had evidence of previous infection (LCMV IgG only). This 38-year-old woman did not recall any recent distinct clinical illness that fit the clinical profile for LCMV infection. She had not directly handled any live mice but had handled and labeled shipping packages of live mice from facilities A and C in the months before being tested.

Thirty-two of 36 facility C employees were tested; 15 (47%) had detectable LCMV antibodies, and 11 (34%) had evidence of recent LCMV infection. In facility C, 1 of the

Table 1. Symptoms reported by 9 employees of facility A who tested positive for LCMV IgM and reported recent illness, Indiana, USA, 2012*

Symptom or sign	Present no. (%)	Absent, no. (%)	Unknown, no.
Headache	9 (100)	0	0
Nausea/vomiting	9 (100)	0	0
Fever	8 (89)	1 (11)	0
Decreased appetite	8 (89)	1 (11)	0
Diarrhea	6 (67)	3 (33)	0
Muscle ache	6 (67)	3 (33)	0
Stiff neck	6 (67)	3 (33)	0
Joint pain	5 (56)	4 (44)	0
Malaise	5 (56)	2 (22)	2 (22)
Cough	4 (44)	5 (56)	0
Drowsiness	3 (33)	6 (67)	0
Sensory disturbance	2 (22)	6 (67)	1 (11)
Parotid pain	1 (11)	7 (78)	1 (11)
Confusion	1 (11)	8 (89)	0
Increased blood leukocytes	3 (33)	1 (11)	5 (56)
Increased CSF leukocytes	4 (44)	0	5 (56)
Increased CSF protein	4 (44)	0	5 (56)

*LCMV, lymphocytic choriomeningitis virus; CSF, cerebrospinal fluid.

tested employees was pregnant and negative for LCMV antibodies, and a 29-year-old man who was IgG/IgM positive had visited the emergency department in May 2012 because of chest pain and headaches. Electrocardiogram, serum chemistry, and complete blood count did not show any abnormalities. Altogether, 97 employees of facilities A, B, and C were tested, and 31 (32%) had LCMV antibodies.

Job duties of employees at facilities A, B, and C ranged from administrative/managerial duties to direct handling of live and euthanized rodents to cleaning. Most employees conducted multiple duties in multiple buildings. No particular job duty was associated with LCMV infection (data not shown). Employees had worked at the facilities for 2 days–20 years, and 1 case-patient had worked at facility A for only 5 days before becoming ill. We found no association between length of time employed and previous infection, suggesting relatively recent LCMV introduction into the facilities (Table 3, Appendix, wwwnc.cdc.gov/EID/article/20/2/13-0860-T3.htm).

For facility A, working in building 2 and smoking were independently associated with having recent or previous LCMV infection (Table 3, Appendix). For facilities B and C, no specific factors evaluated were associated with LCMV infection in employees tested (Table 3, Appendix). At the time of the investigation, all 97 employees reported washing hands after handling the rodents, and 90%–100% of employees reported wearing masks and gloves, although many admitted having begun using these items only when the LCMV outbreak was suspected.

Rodent LCMV Investigation

Facility A

Facility A, located in Indiana, bred and raised mice and rats exclusively for sale as feeder animals for reptiles and birds of prey. Most rodents were euthanized and frozen on-site for sale as frozen feeder rodents; however, live rodents also were shipped. Live and frozen rodents were transported to facility B, also in Indiana, for storage, sale, and distribution. Facility A had 4 buildings that housed breeding rodents; each building was subdivided into rooms by species (Table 3, Appendix). At the time of the investigation, facility A housed ≈155,000 adult mice and ≈14,000 adult rats. In accordance with standard procedures, baited rodent traps were set outside and inside buildings at regular intervals throughout the facility; any mice that were caught were promptly euthanized. Rodent feed was stored indoors on pallets.

In May 2012, a total of 1,820 mice and rats from facility A were tested for LCMV IgG by ELISA and for LCMV RNA by RT-PCR. Of 1,421 mice tested, 296 (20.8%) had detectable IgG, and 10 (0.7%) had detectable RNA; apparent prevalence varied by room (Table 4). No RT-PCR–positive mice had detectable LCMV IgG. Only 1 mouse room tested (building 3, room 2) contained no IgG- or RT-PCR–positive mice, indicating that LCMV infection was widespread. The estimated number of viremic mice in a

Table 2. Demographic and clinical features of laboratory-confirmed illness in 5 facility A employees who sought health care after exposure to LCMV-infected mice, Indiana, USA, 2012*

Patient no.	Age, y/sex	Time employed	Onset date	Symptoms	Clinical findings†
1	27/F	2 y	Apr 20	Fever, muscle ache, nausea, vomiting, abdominal pain, diarrhea, malaise, headache	Lymphocytic CSF, leukocytosis (13.8), elevated protein in CSF, appendicitis, UTI
2	34/M	1.5 y	Apr 11	Fever, nausea, vomiting, malaise, headache, stiff neck	Lymphocytic CSF (1,311/μL), elevated protein in CSF
3	28/F	1.5 y	Apr 7	Fever, muscle ache, nausea, vomiting, cough, joint pain, photophobia, headache, diarrhea	UTI; no blood or CSF collected during medical evaluation
4	48/M	5 d	Apr 27	Fever, muscle ache, nausea, vomiting, abdominal pain, cough, headache, diarrhea	Lymphocytic CSF, elevated protein in CSF, mild anemia (34.9%), CT normal
5	38/M	2 mo	May 14	Fever, muscle ache, nausea, vomiting, joint pain, headache, diarrhea	Lymphocytic CSF (320/μL), elevated protein in CSF (77), leukocytosis (15.1), anemia (Hgb 11.5), CT normal

*LCMV, lymphocytic choriomeningitis virus; CSF, cerebrospinal fluid; UTI, urinary tract infection; CT, computed tomographic scan; Hgb, hemoglobin.
†Lymphocytic CSF is defined as >5 lymphocytes/μL; leukocytosis is >11,000 erythrocytes/μL; and elevated protein in CSF is >60 mg/dL.

Table 4. Test results for LCMV from mice and rats in facility A, Indiana, USA, 2012*

Building/ room	Species	No. adult rodents in room	Sample size	IgG positive		RT-PCR positive	
				No. (%)	Estimated no. per room (95% CI)†	No. (%)‡	Estimated no. per room (95% CI) †
1							
1	Rat	1,512	110	0	0 (0–41)	0	0 (0–41)
2	Rat	2,058	83	0	0 (0–72)	0	0 (0–72)
2							
1	Mouse	13,104	110	28 (25)	3,342 (2,306–4,534)	4 (4)	472 (131–1,179)
2	Mouse	10,368	110	25 (23)	2,354 (1,586–3,287)	2 (2)	187 (21–664)
3	Mouse	13,104	102	36 (35)	4,626 (3,420–5,949)	0	0 (0–380)
4	Mouse	10,368	110	42 (38)	3,961 (3,017–4,966)	1 (1)	93 (2–508)
3							
1	Mouse	14,400	110	3 (3)	389 (86–1,123)	1 (1)	130 (3–706)
2	Mouse	14,400	110	0	0 (0–389)	0	0 (0–389)
3	Mouse	14,400	110	19 (17)	2,491 (1,541–3,701)	0	0 (0–389)
4	Mouse	5,760	109	25 (23)	1,319 (887–1,843)	0	0 (0–156)
5	Mouse	14,400	110	20 (18)	2,621 (1,656–3,845)	0	0 (0–389)
6	Mouse	12,384	110	36 (33)	4,050 (2,984–5,238)	0	0 (0–334)
7	Mouse	4,320	110	1 (1)	39 (1–212)	0	0 (0–117)
4							
1	Mouse	11,520	98	37 (38)	4,355 (3,248–5,541)	1 (1)	115 (2–645)
2	Mouse	17,280	108	24 (22)	3,836 (2,557–5,391)	1 (1)	155 (3–881)
3	Rat	5,460	110	0	0 (0–147)	0	0 (0–147)
4	Rat	4,704	110	0	0 (0–127)	0	0 (0–127)

*LCMV, lymphocytic choriomeningitis virus; RT-PCR, reverse transcription PCR.
†Binomial estimators were used to calculate 95% CIs for the entire adult population for each room, based on the number of test-positive animals detected with the samples obtained.
‡All RT-PCR–positive animals were LCMV IgG-negative as measured by ELISA.

given room at the time of sampling varied from 0 to 472 (as estimated by the number of RT-PCR–positive mice) (95% CI 0–1,180 viremic mice per room) (Table 4). None of 399 rats tested were positive by ELISA or RT-PCR.

LCMV was successfully isolated from 8 of 10 RT-PCR–positive mice. The sequence analysis of a 630-bp amplicon from the S-segment from the 8 isolates showed a high degree of similarity (98.6%–99.7% identity; data not shown), suggesting a single origin of LCMV. The complete S segment sequence of the prototype outbreak strain (201202467 Indiana, GenBank accession no. KF732824) was aligned and compared with 34 sequences available in GenBank, corresponding to representative strains of LCMV. The phylogenetic analysis (Figure) shows that strain 201202467 belongs to lineage I, a heavily populated LCMV clade with numerous isolates from Europe and North America (*6*).

After evidence of LCMV infection was detected, the Indiana Board of Animal Health (Indianapolis, IN, USA) issued quarantines and stop-movement orders on all live and frozen rodents from facility A. After animal testing was done, regulatory restrictions were lifted on rats but were retained on mice. More than 400,000 mice were euthanized and buried on site. All rodent feed and bedding was incinerated, and the facility was disinfected by using a 0.1% bleach solution. Employees were provided N95 respirators, gloves, boots, and water-resistant coveralls to wear when handling the mice and possibly contaminated equipment, and they were instructed about how to properly use the equipment and disinfect appropriately to limit their risk for infection. Once these measures were fully implemented, no additional employees became ill throughout the depopulation process, which ended on July 11, 2012.

All frozen rodents from facility A that were remaining in storage at facility B were destroyed. Live rodents originating from facility A were sold to >500 different purchasers in 21 different states (*5*).

Facility C

Facility C exclusively produced frozen feeder mice that were euthanized on site in Kentucky and then transported to facility B for storage and distribution. In March 2012, ≈90,000 live mice were shipped from facility C to facility A via facility B to replace breeding stock. Other than this instance, live shipments were not common. Management was similar to that of facility A. After the LCMV outbreak among facility A employees, the owners of facility C had 50 mice tested at a commercial laboratory (IDEXX RADIL, Columbia, MO, USA); 33 (66%) were antibody-positive by immunofluorescent antibody testing. No mice were tested by CDC. The owners of facility C reported this finding to state public health authorities and voluntarily depopulated all mice. Approximately 380,000 mice from facility C were euthanized, and 810,000 frozen mice stored at facility B were destroyed. Bedding was buried, and the facility was disinfected. The owners of facility C reported that 6 months before the outbreak, wild mice had infested the feed storage areas, and the owners noted occasional litters of mice born

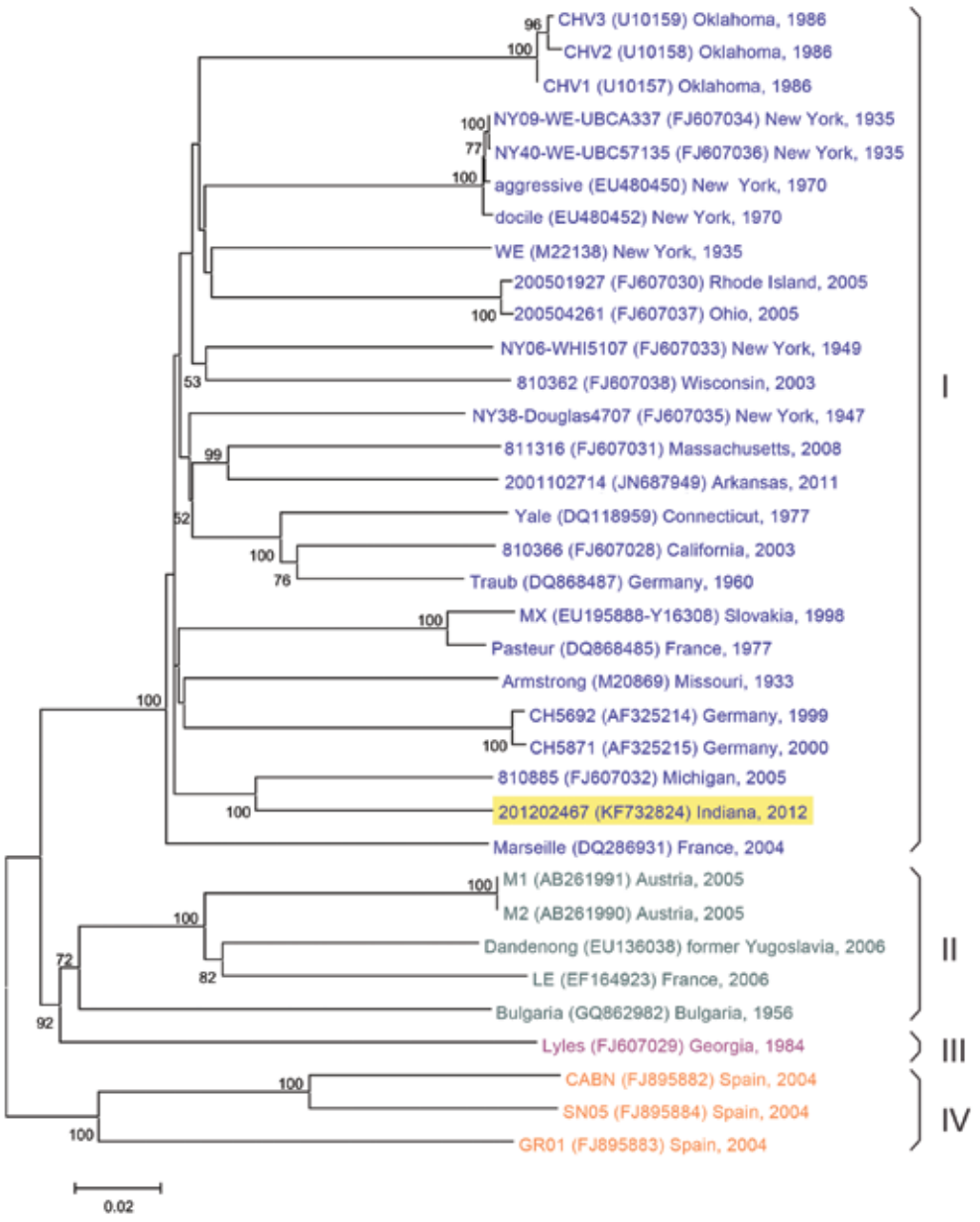

Figure. Phylogenetic tree comparing S RNA genomes of most representative lymphocytic choriomeningitis virus (LCMV) strains. Evolutionary analysis was conducted in MEGA5 (online Technical Appendix, wwwnc.cdc.gov/eid/article/20/2/pdfs/13-0860.pdf) by using the neighbor-joining method. Bootstrap values listed at the nodes provide statistical support for 1,000 replicates. Branches corresponding to partitions reproduced in <50% bootstrap replicates are collapsed. Scale bar indicates substitutions per site. The main LCMV lineages are indicated with roman numerals (I–IV). After the strain denomination, the location and date it was isolated are noted. A color version of this figure is available online (www.cdc.gov/EID/ content/20/2/130860-F.htm

with black eyes (colony mice are albino), indicating that wild mice had interbred with the colony mice.

Discussion

LCMV is endemic in wild *M. musculus* populations across the United States and throughout the world. Sporadic human LCMV cases occur after contact with infected wild house mice, but the virus has the potential to cause large epidemics when it enters high-density rodent populations, as in the outbreak of this report. Previous outbreaks of human disease have been linked to contact with pet hamsters and laboratory animals (*7*–*11*). In the current outbreak investigation, we found that nearly one third of rodent facility employees tested had LCMV antibodies. This overall attack rate is consistent with previous outbreaks of LCMV in hamsters and nude mice at research facilities (*10*,*12*,*13*).

Employees of rodent breeding facilities of all kinds should be aware of the risks posed by exposure to rodents infected with LCMV, and monitoring programs should be in place to detect and control infections in rodents. Commercial laboratory rodent breeding colonies have developed management practices to avoid contact between wild mice and colony animals; the US Department of Health and Human Services and the Federation of European Laboratory Animal Science Associations recommend routine virologic and serologic monitoring to detect pathogens, including LCMV (*2*,*14*). Facilities producing rodents for the pet and feeder-rodent industries should adapt these

practices to avoid such outbreaks. When LCMV or LCMV antibodies are detected in rodents or employees of a rodent breeding operation, all animal-handling personnel should wear protective equipment, including a respirator. After personal protective gear and training in its proper use were provided to facility employees, LCMV infection was not laboratory confirmed in any additional employees of facility A throughout the depopulation process, which implies that use of such equipment can reduce the risk for infection.

In facility A, many mice had LCMV antibodies, and several were viremic at the time of the investigation. The rodent testing results fit with the human testing results; that is, working in building 2, which had the highest prevalence of LCMV antibody-positive and RT-PCR–positive mice, was significantly associated with employee infections. Of the ≈13,000 adult mice in room 1 of building 2, ≈131–1,179 were viremic. In such a high-density environment, the virus can be present in aerosol form (*15*), which explains why many employees of facilities A and C had detectable antibodies. In facility B, which only transiently held live mice for further distribution, only 1 (8%) of 13 employees had detectable LCMV antibodies, suggesting that less intensive exposure to mice put employees at lower risk for infection. Smoking, a risk factor for many bacterial and viral infections, including tuberculosis, pneumococcal pneumonia, and influenza (*16*), was associated with LCMV infection in facility A employees. The structural changes to the respiratory epithelium and modulation of immune function hypothetically impair the smoker's immune response. The physical act of smoking also might facilitate transfer of pathogens from the hands to the mouth.

The proportion of infected employees in whom clinical illness developed varied by facility. In facility A, 69% of employees who had recent infection reported illness, and 33% had symptoms severe enough to cause them to seek medical care. Conversely, all facility C employees appeared to have asymptomatic infections, except for 1 with nonspecific illness. Previous LCMV outbreak investigations have found various rates of clinical illness (*9*,*10*,*13*), with asymptomatic infections of 25%–55%. Because employee age and sex did not differ among facilities, the reason for the difference in disease manifestation remains unclear. The longevity of LCMV IgM detectable in peripheral blood is not well-established for humans; thus, some employees with IgM who did not develop symptoms might have been infected previously.

In immunocompetent adults, the neurologic form of LCMV infection classically has biphasic features consisting of a nonspecific initial phase, with fever, myalgia, and headache most commonly observed, and can include nausea and/or vomiting and retroorbital pain (*8*,*17*). Symptoms may subside after several days to be followed by a neurologic phase comprising meningeal symptoms with fever, headache, nuchal rigidity, vomiting, and light sensitivity. In LCMV patients in whom aseptic meningitis is diagnosed, CSF characteristically shows a lymphocytic pleocytosis, and elevated protein and decreased glucose also might be present (*1*,*3*); in this report, all 4 case-patients on whom lumbar punctures were performed had lymphocytic CSF with elevated protein. Symptoms frequently reported by case-patients in this outbreak included headache; fever; and abdominal symptoms, such as nausea, vomiting, and diarrhea. Such nonspecific symptoms obscure the clinical diagnosis. A thorough clinical history covering relevant animal contacts remains vital to determining the source and appropriate etiologies to test for in work-ups of aseptic meningitis.

Although all case-patients in this LCMV outbreak recovered from their illness, the specter of more severe disease manifestations remains a cause for concern. Infections during pregnancy can result in spontaneous abortion or characteristic congenital defects, such as chorioretinitis, microencephaly or macroencephaly, and hydrocephalus (*18*). Mental retardation, vision deficits, cerebral palsy, and epilepsy are potential lifelong manifestations (*19*). Since 2005, five outbreaks of LCMV have occurred after organ transplantation, resulting in the death of 14 (82%) of 17 organ recipients (*20*–*23*). Concern about these severe forms of LCMV infection led the outbreak response team to recommend depopulating the mouse breeding facilities and to conduct a trace-forward investigation of live mice that were sold and shipped from facility B (*5*).

LCMV is transmitted horizontally and vertically in affected rodents (*17*,*24*). Horizontal infections, acquired through direct contact with infected rodents or indirect contact with contaminated fomites, can lead rodents to shed infectious virus for a few weeks to a few months. When mice are exposed in utero to LCMV, they become persistently infected and shed the virus throughout their lives, including to all offspring, which also will be persistently infected (*2*,*25*,*26*). Infections in rodents are inapparent. As a result, when LCMV is introduced to a high-density environment, such as the breeding colonies of this outbreak, the number of infected rodents can silently reach very high numbers and thus pose a risk to the humans coming in contact with them.

The primary goal of the rodent sampling scheme was to detect LCMV infection in the colony; only adult mice were sampled because we assumed that they would have the greatest likelihood of having detectable antibodies. If younger mice had been tested, the seroprevalence is likely to have been lower and the proportion of viremic animals higher. In this investigation, 21% of adult mice tested had antibodies; this prevalence is higher than that found in wild mouse populations not associated with human infections (2%–9%) (*27*–*29*).

These molecular investigations demonstrated a unique strain of LCMV, 201202467, in facility A mice, which suggests a single introduction and transmission event throughout the breeding facility. Although we were unable to sample mice from facility C, it is likely that the outbreak strain was the same as in facility A and was introduced during the wild mouse infestation that had occurred. Molecular analysis also shows the close relationship of strain 201202467 with another LCMV strain isolated from a mouse found in an infested house in Michigan in 2005 (*30*). The geographic distribution of this LCMV strain throughout North America is not known (*7*).

As in previous outbreaks, interactions between wild mice and colony rodents frequently introduce LCMV into breeding colonies (*17*). In the absence of control measures and monitoring, movements of live mice among breeder mice can contribute to the spread of potentially infected mice. No human or rodent LCMV vaccine is available, and no treatment exists. Therefore, prevention measures are necessary and must rely on wild rodent exclusion, infection control, and microbiological monitoring (*2*). When LCMV antibodies are detected in colony mice, transmission must be assumed to be ongoing, and all possibly infected or exposed rodents should be removed from the colony by euthanasia and disposal to mitigate human risk (*24*,*31*).

In conclusion, laboratory and epidemiologic investigations effectively identified a large outbreak of LCMV in 3 commercial mouse breeding facilities and associated infections in several employees. The presumptive source of virus introduction was contact between wild mice and colony mice, and the outbreak spread among facilities when mice were transported for use as breeding stock. The breeding colonies were depopulated to prevent further human infections. Future outbreaks can be prevented with strict biosecurity and microbiological monitoring, and employees should be made aware of the symptoms of LCMV infection and prevention measures.

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Dr Knust is an epidemiologist and veterinarian in CDC's Viral Special Pathogens Branch. Her primary research interests include epidemiology and prevention of zoonotic diseases.

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Address for correspondence: Barbara Knust, Viral Special Pathogens Branch, Centers for Disease Control and Prevention, 1600 Clifton Rd, NE, Mailstop G14, Atlanta, GA 30333, USA; email: bknust@cdc.gov

Fungal Endophthalmitis Associated with Compounded Products

Christina A. Mikosz,[1] Rachel M. Smith,[1] Moon Kim, Clara Tyson, Ellen H. Lee, Eleanor Adams, Susanne Straif-Bourgeois, Rick Sowadsky, Shannon Arroyo, Yoran Grant-Greene, Julie Duran, Yvonne Vasquez, Byron F. Robinson, Julie R. Harris, Shawn R. Lockhart, Thomas J. Török, Laurene Mascola, and Benjamin J. Park; for the Fungal Endophthalmitis Outbreak Response Team[2]

Fungal endophthalmitis is a rare but serious infection. In March 2012, several cases of probable and laboratory-confirmed fungal endophthalmitis occurring after invasive ocular procedures were reported nationwide. We identified 47 cases in 9 states: 21 patients had been exposed to the intraocular dye Brilliant Blue G (BBG) during retinal surgery, and the other 26 had received an intravitreal injection containing triamcinolone acetonide. Both drugs were produced by Franck's Compounding Lab (Ocala, FL, USA). *Fusarium incarnatum-equiseti* species complex mold was identified in specimens from BBG-exposed case-patients and an unopened BBG vial. *Bipolaris hawaiiensis* mold was identified in specimens from triamcinolone-exposed case-patients. Exposure to either product was the only factor associated with case status. Of 40 case-patients for whom data were available, 39 (98%) lost vision. These concurrent outbreaks, associated with 1 compounding pharmacy, resulted in a product recall. Ensuring safety and integrity of compounded medications is critical for preventing further outbreaks associated with compounded products.

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (C.A. Mikosz, R.M. Smith, Y. Grant-Greene, B.F. Robinson, J.R. Harris, S.R. Lockhart, T.J. Török, and B.J. Park); Los Angeles County Department of Public Health, Los Angeles, California, USA (C.A. Mikosz, M. Kim, C. Tyson, and L. Mascola); New York City Department of Health and Mental Hygiene, New York City, New York, USA (E.H. Lee); New York State Department of Health, Albany, New York, USA (E. Adams); Louisiana Office of Public Health, New Orleans, Louisiana, USA (S. Straif-Bourgeois); Nevada Division of Public and Behavioral Health, Carson City, Nevada, USA (R. Sowadsky); Indiana State Department of Health, Indianapolis, Indiana, USA (S.Arroyo); Illinois Department of Public Health, Springfield, Illinois, USA (Y. Grant-Greene); Colorado Department of Public Health and Environment, Denver, Colorado, USA (J. Duran); and City of El Paso Department of Public Health, El Paso, Texas, USA (Y. Vasquez)

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Endophthalmitis is inflammation of the intraocular cavities and is often caused by infection (*1*). Exogenous endophthalmitis is a complication for ≈2–4 per 10,000 intravitreal injections (*2*,*3*) or pars plana vitrectomies (*4*). Most infections are bacterial; fungal infections are rare (*5*–*7*). The clinical course of fungal endophthalmitis is frequently prolonged and is associated with poor outcomes; vision loss is not uncommon (*5*,*8*–*10*). We describe 2 concurrent multistate outbreaks of fungal endophthalmitis associated with intraocular use of contaminated products labeled as sterile from a single compounding pharmacy.

Methods

Initial Epidemiologic Investigation

On March 5, 2012, the Healthcare Associated Infections Program of the California Department of Public Health was alerted to a cluster of 9 cases of fungal endophthalmitis, all among patients at the same Los Angeles County ambulatory surgical center who had undergone pars plana vitrectomies during October–December 2011. Two patients had histopathologic evidence of fungal hyphae in intraocular eye specimens; the others had a clinical diagnosis of fungal endophthalmitis. The initial investigation, led by the Los Angeles County Department of Public Health, demonstrated that all 9 patients had been exposed to a single lot of Brilliant Blue G dye (BBG), used to stain epiretinal membranes during vitrectomies. Although BBG is not approved by the Food and Drug Administration (FDA) for human use, it is increasingly being used in certain ocular procedures (P. Dugel, pers. comm.). The BBG implicated in the cases reported here was produced on

[1]These authors contributed equally to this article and are co–first authors.

[2]Members of the Fungal Endophthalmitis Outbreak Response Team are listed at the end of this article.

August 23, 2011, at Franck's Compounding Lab (Franck's), a compounding pharmacy in Ocala, Florida, USA, which distributes products throughout the United States.

During the investigation, cases were defined as laboratory-confirmed or suspected fungal endophthalmitis among patients who had undergone vitrectomy at the Los Angeles County ambulatory surgical center during October 2011–January 2012, when the implicated BBG lot was in use. No additional cases were identified. A case–control study was conducted among 42 vitrectomy patients (the 9 case-patients and 33 control participants) at the same ambulatory surgical center (*11*). Information was obtained with regard to the operating surgeon, patient sex, and preoperative and intraoperative medications commonly used for vitrectomies at the Los Angeles County ambulatory surgical center. The only factor significantly associated with case status was Franck's BBG (odds ratio ∞, $p<0.001$). Microbiological testing, conducted at a commercial laboratory, of an unopened BBG vial from the same lot yielded the environmental mold *Fusarium.* These data collectively turned the investigation focus on Franck's products.

Multistate Investigation

Franck's records indicated that BBG from the contaminated lot was shipped to 22 facilities in 15 states. On March 9, 2012, Franck's recalled all lots of BBG. That same day, the Centers for Disease Control and Prevention (CDC) and state and local health departments initiated a multistate investigation to identify additional cases of fungal endophthalmitis associated with invasive ocular procedures, identify the outbreak source, and prevent further exposures and illness.

The multistate investigation defined a probable case as ophthalmologist-diagnosed fungal endophthalmitis occurring after an invasive ophthalmic procedure performed on or after August 23, 2011; each affected eye was counted separately as a case. A case was considered confirmed after laboratory identification of fungi from eye specimens by culture, genetic sequencing, or histopathologic examination, at local hospitals, public health laboratories, or CDC.

On March 26, 2012, CDC was notified of a patient in whom fungal endophthalmitis had developed during February 2012 after an intravitreal injection of triamcinolone acetonide (triamcinolone) at a New York ophthalmology practice. Triamcinolone is a corticosteroid used to treat a variety of ophthalmic conditions. Three additional patients with suspected fungal endophthalmitis were identified at the practice; all 4 had received intravitreal injections of triamcinolone from a single lot manufactured at Franck's on November 4, 2011. Preliminary CDC laboratory testing identified *Bipolaris hawaiiensis*, a rare environmental mold infrequently described as a human pathogen, in ocular specimens from these case-patients. Because Franck's invoices indicated that triamcinolone from the same lot had been shipped to 5 ophthalmology practices in 4 states, the investigation was expanded. On March 31, 2012, Franck's recalled this lot of triamcinolone.

Case Finding

Case finding was conducted through postings to Epi-X (www.cdc.gov/epix/), a secure notification network for public health professionals. Email messages were sent to ClinMicroNet (www.asm.org/index.php/online-community-groups/listservs), a network of clinical microbiology laboratories; to academic microbiology laboratories known from previous ophthalmic disease outbreaks (*12–14*); and to members of 2 major ophthalmology professional societies. CDC also reviewed available Franck's internal adverse event logs and sales records and contacted physicians listed in these reports.

Laboratory Testing

CDC performed fungal cultures on case-patient specimens, including vitreous fluid, intraocular lenses, and intraocular swabs. CDC also performed confirmatory testing on fungal specimens from other laboratories, including a fungal isolate recovered by FDA from a BBG vial and fungal DNA isolated by an outside laboratory from a vitreous cassette specimen. Fungal isolates were identified by morphologic and DNA sequence analysis (*15*). *Fusarium* isolates were compared by multilocus sequence analysis (*16*); species of *Bipolaris* isolates were identified by examination of the ribosomal internal transcribed spacer region (*15*).

Case–Control Analyses

To confirm that Franck's products were associated with cases identified outside California and to evaluate the hypothesis that there were 2 separate outbreaks, both associated with Franck's, we conducted 2 case–control studies. One case–control study was conducted for each fungal species outbreak (*Fusarium* infections associated with Franck's BBG administered during vitrectomies and *Bipolaris* infections associated with Franck's triamcinolone injected intravitreally); only species-confirmed cases (i.e., cases confirmed by culture) were included. A third case–control analysis separately examined probable cases. Control participants were well patients from the same clinical practice, matched 3:1 on case-patient procedure type and week of the procedure during which case-patient exposure to recalled Franck's BBG or triamcinolone first occurred. Medical charts were abstracted by using a standardized form. Case-patients were followed up after exposure to either of the 2 Franck's products for either 6 months or until documented resolution of infection, whichever occurred first; vision loss in this analysis reflects documentation of reduced visual

acuity by the treating ophthalmologist at any point during this 6-month period and not necessarily permanent vision loss. Statistical analysis was conducted by using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA); categorical variables were assessed by the Cochran-Mantel-Haenszel test, by using the matched quartets as a stratification factor; and 95% CIs and p values were calculated by using exact methods.

This investigation was considered to be an urgent public health response and thus was not considered to be research that required approval by an institutional review board or informed consent from involved patients. All patient names and protected health information were kept confidential.

Results

As of March 22, 2013, a total of 47 cases among 45 patients had been identified in 9 states. Twenty-three cases were confirmed through histopathologic examination or microbiological testing; of these, infection with *F. incarnatum-equiseti* species complex mold was confirmed by culture for 7, and a different mold, *B. hawaiiensis*, was confirmed by culture for 9. The rest of the cases were confirmed only by histopathology. The definition of a probable case was met by 24 cases. Two case-patients were hospitalized; none died.

CDC confirmed *F. incarnatum-equiseti* species complex in an isolate derived from an unopened BBG vial from the implicated lot. *Fusarium* isolates from case-patients and the BBG vial were indistinguishable by multilocus DNA sequencing at 4 loci. *B. hawaiiensis* was confirmed in 10 isolates from 9 case-patients in 3 states.

Case–Control Studies

Preliminary evidence indicated that there were 2 separate, concurrent outbreaks associated with 2 Franck's products: *Fusarium* infections associated with BBG and *Bipolaris* infections associated with triamcinolone. Results of the several case–control analyses are as follows.

Fusarium Case Cluster

This analysis included 6 confirmed case-patients and 18 control participants (Table 1). Only Franck's BBG was significantly associated with *Fusarium*-confirmed fungal endophthalmitis (p = 0.002). All *Fusarium*-confirmed case-patients had been exposed to BBG. Female sex was also associated with case status (p = 0.05).

Bipolaris Case Cluster

Among 7 confirmed case-patients and 18 control participants in the study, Franck's triamcinolone was significantly associated with *Bipolaris*-confirmed infection (p = 0.001); all *Bipolaris*-confirmed case-patients had received this product (Table 1). Diabetes mellitus was also statistically associated with case status (p = 0.04).

Probable Case Cluster

All probable case-patients had been exposed to either Franck's BBG or triamcinolone; among 17 enrolled probable case-patients and 51 control participants, exposure to either product was the only factor significantly associated with case status (p<0.001; Table 2). No case-patients were exposed to both products.

Case Summaries

With epidemiologic and laboratory evidence demonstrating 2 distinct outbreaks associated with 2 Franck's products, all 47 cases could be sorted into 1 of 2 outbreak cohorts. Overall, 21 cases of fungal endophthalmitis were associated with BBG exposure and *Fusarium* mold and 26 cases were associated with Franck's triamcinolone exposure and *Bipolaris* mold (Figure 1).

Dates of symptom onset were difficult to ascertain because of the subacute onset of disease; Figure 2 reports case counts by date of procedure during which exposure to BBG or triamcinolone first occurred. In this outbreak, exposure to Franck's BBG occurred primarily during late October–late December 2011, whereas exposure to Franck's triamcinolone occurred during December 2011–March 2012.

BBG-associated Outbreak

Data were available for all 21 BBG-exposed case-patients (Table 3). Among these case-patients, 16 (76%) were women, median age was 69 years (range 58–86), 11 (52%) had a history of diabetes mellitus, 11 (52%) had a history of hypertension, and 14 (67%) had undergone previous eye surgeries or procedures. All patients in this cluster reported unusual vision loss. Vitreous debris was noted during examination of 12 (57%) case-patients; hypopyon, eye inflammation, and fibrin were observed in 9 (43%), 10 (48%), and 12 (57%) case-patients, respectively. Eye pain was reported by 16 (76%) case-patients and floaters by 4 (19%).

Antifungal therapy was received by 17 (81%) case-patients. Among all treated case-patients, 10 (59%) received combination antifungal therapy. The most common treatment was intravitreal voriconazole, received by all 17 (100%) treated case-patients (Table 3). Oral voriconazole was the next most common treatment, received by 6 (35%) treated case-patients, followed by intravitreal amphotericin B, received by 2 (12%) treated case-patients. The median time from exposure to diagnosis in this cluster was 78 days (range 60–125); 95% of patients required additional surgeries (e.g., vitrectomies) to treat their infections (Table 3). After 6 months, 3 (14%) case-patients did not have documentation of resolved infection.

Table 1. Case–control results for species-confirmed fungal endophthalmitis cases, United States, 2012*

Variable	*Fusarium* spp. cluster			*Bipolaris* spp. cluster		
	Case-patients, no. (%), n = 6	Controls, no. (%), n = 18	mOR (95% CI)	Case-patients, no. (%), n = 7	Controls, no. (%), n = 18	mOR (95% CI)
Patient characteristic						
Female sex	5 (83)	4 (22)	12.0 (0.99–472.73), p = 0.05	3 (43)	11 (61)	0.61 (0.05–5.71), p = 0.94
Diabetes	3 (50)	7 (39)	1.50 (0.18–11.43), p = 0.98	6 (86)	4 (22)	13.33 (1.12–492.51), p = 0.04
Hypertension	3 (50)	7 (39)	1.40 (0.15–24.76), p>0.99	7 (100)	15 (83)	Undef (0.33–∞), p = 0.50
History of eye surgery or procedures	4 (67)	12 (67)	1.00 (0.11–12.77), p>0.99	4 (57)	16 (89)	0.25 (0.003–2.440), p = 0.28
Medications received						
Cyclopentolate	4 (67)	15 (83)	0 (0–6.33), p = 0.50	0	0	NC
Phenylephrine	3 (50)	15 (83)	0 (0–1.16), p = 0.13	0	0	NC
Tropicamide	0	0	NC	0	0	NC
Bupivacaine	0	4 (22)	0 (0–3.47), p = 0.50	0	0	NC
Atropine	0	4 (22)	0 (0–3.00), p = 0.38	0	0	NC
Lidocaine	1 (17)	7 (39)	0.20 (0.005–4.32), p = 0.59	2 (29)	5 (28)	1.00 (0.008–130.300), p>0.99
Tetracaine	2 (33)	10 (56)	0 (0–3.40), p = 0.56	3 (43)	3 (17)	Undef (0.16–∞), p = 0.50
Brilliant blue G dye†	6 (100)	2 (11)	Undef (3.47–∞), p = 0.002	0	0	NC
Cefazolin	0	1 (6)	0 (0–57.00), p>0.99	0	0	NC
Antimicrobial ophthalmic ointment	0	3 (17)	0 (0–5.81), p = 0.75	1 (14)	0	Undef (0.16–∞), p = 0.50
Vancomycin	2 (33)	6 (33)	1.00 (0.06–16.26), p>0.99	0	0	NC
Moxifloxacin	4 (67)	11 (61)	1.50 (0.07–91.71), p>0.99	0	3 (17)	0 (0–3.87), p = 0.50
Triamcinolone†	0	0	NC	7 (100)	2 (11)	Undef (3.68–∞), p = 0.001
Bevacizumab	0	0	NC	5 (71)	14 (78)	1.00 (0.008–130.30), p>0.99
Dexamethasone	0	3 (17)	0 (0–5.14), p = 0.84	0	10 (56)	0 (0–0.52), p = 0.02

*mOR, median odds ratio; Undef, undefined; NC, not calculated.
†Manufactured by Franck's Compounding Lab, Ocala, Florida, USA.

Triamcinolone-associated Outbreak

Detailed data were available for 19 (73%) of 26 triamcinolone-exposed case-patients (Table 3). Among these case-patients, 8 (42%) were women, median age was 67 years (range 53–77), 15 (79%) had a history of diabetes mellitus, and 15 (79%) had a history of hypertension. Most case-patients had a history of previous eye surgery or procedures. Virtually all (18 [95%]) case-patients experienced vision loss. Vitreous debris was commonly noted on examination. Hypopyon, inflammation, and fibrin were also observed during examination of the affected eye, although with lower frequency than for patients in the BBG cluster (Table 3). Pain was reported by 4 (21%) case-patients and floaters by 6 (32%). Two patients had received bilateral injections with triamcinolone, and for each of these patients, infection subsequently developed in both eyes.

All case-patients in this cluster received antifungal therapy, most commonly with intravitreal (11 [58%]) or oral (11 [58%]) voriconazole (Table 3). Most case-patients received a combination of antifungal therapies. Intravitreal amphotericin B was received by 8 (42%) case-patients. Median time from exposure to diagnosis in this cluster was 80 days (range 35–185); 84% of case-patients required additional surgeries (Table 3). After 6 months, documentation of resolved infection was unavailable for approximately half (42%) of these case-patients; 2 required enucleation because of severe infection. Both enucleations were performed after the 6-month follow-up

Table 2. Case–control study results for probable fungal endophthalmitis cases, United States, 2012*

Variable	Case-patients, no. (%), n = 17	Controls, no. (%), n = 51	mOR (95% CI)	p value
Patient characteristic				
Female	10 (59)	25 (49)	1.45 (0.43–5.12)	0.68
Diabetes	10 (59)	20 (39)	1.77 (0.63–6.82)	0.28
Hypertension	10 (59)	30 (59)	1.00 (0.30–3.45)	>0.99
History of eye surgery or procedures	10 (59)	39 (76)	0.47 (0.14–1.71)	0.30
Medication received				
Cyclopentolate	0	8 (16)	0 (0–0.83)	0.07
Phenylephrine	8 (47)	28 (55)	0.33 (0.02–4.60)	0.52
Tropicamide	8 (47)	22 (43)	Undef (0.10–∞)	>0.99
Bupivacaine	8 (47)	25 (49)	0.83 (0.08–10.28)	>0.99
Atropine	5 (29)	7 (14)	3.67 (0.56–44.41)	0.22
Lidocaine	11 (65)	33 (65)	1.00 (0.21–5.61)	>0.99
Tetracaine	8 (47)	24 (47)	1.00 (0.17–5.33)	>0.99
Brilliant blue G dye or triamcinolone†	17 (100)	7 (14)	Undef (11.90–∞)	< 0.001
Cefazolin	5 (29)	19 (37)	0.33 (0.02–4.60)	0.52
Antimicrobial ointment	8 (47)	17 (33)	3.33 (0.48–262.41)	0.27
Vancomycin	2 (12)	3 (6)	Undef (0.16–∞)	0.50
Moxifloxacin	3 (18)	6 (12)	4.00 (0.14–196.39)	0.75
Bevacizumab	5 (29)	20 (39)	0.17 (0.003–3.20)	0.31
Dexamethasone	5 (29)	24 (47)	0.25 (0.04–1.48)	0.14

*mOR, median odds ratio; Undef, undefined.
†Manufactured by Franck's Compounding Lab, Ocala, Florida, USA.

period and were not systematically reported. Additional data for these 2 case-patients are not available, and more enucleations or other unreported complications among case-patients are possible.

Public Health and Regulatory Action

On May 3, 2012, CDC and state and local public health officials reported the outbreaks (*17*), publishing the name of Franck's and advising against use of any of Franck's sterile compounded products while the investigation was ongoing. On May 25, Franck's suspended all sterile compounding services and announced a recall of all sterile compounded products distributed during November 21, 2011–May 21, 2012, in response to an FDA investigation that revealed fungal growth in Franck's clean room, where sterile compounds were produced (*18*). Further details of the FDA findings are not publicly available.

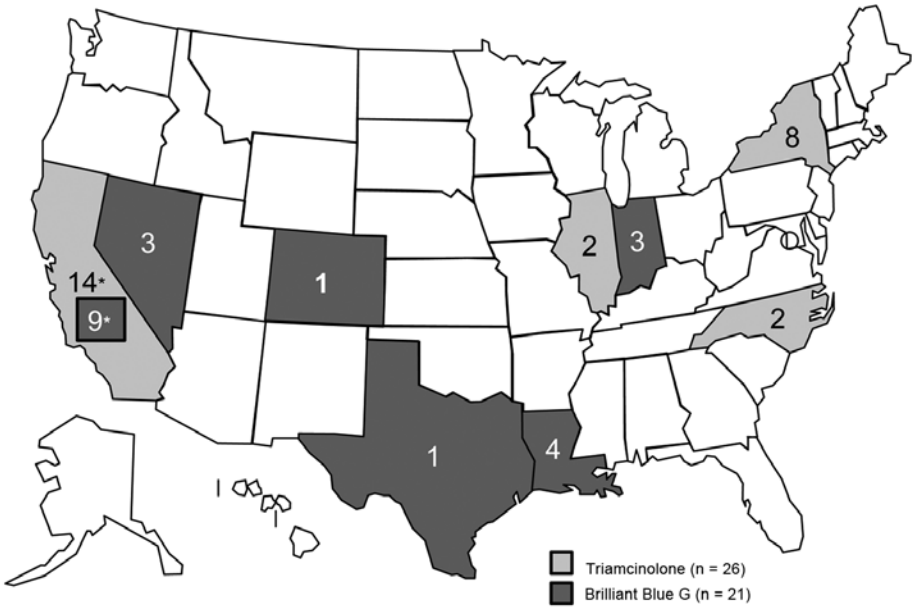

Figure 1. Confirmed and probable cases of postprocedural fungal endophthalmitis, by state, United States, 2011–2012. Infections occurred after exposure to a product from Franck's Compounding Lab (Ocala, FL, USA), through March 2012, when the implicated product was recalled. *In California, cases were associated with exposure to each product.

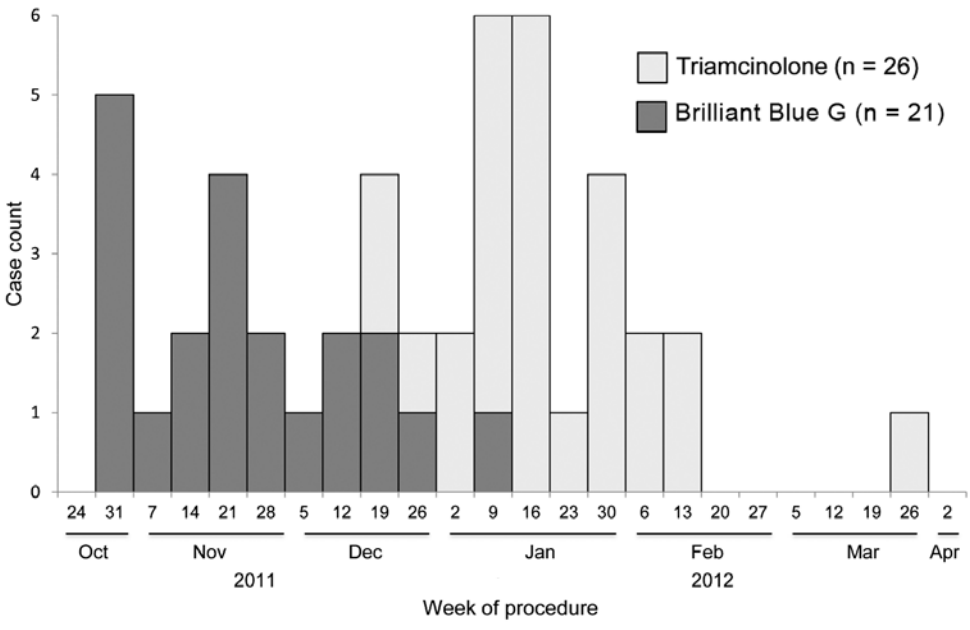

Figure 2. Epidemic curve of confirmed and probable cases of postprocedural fungal endophthalmitis, by week of procedure, United States.

Discussion

Two concurrent outbreaks of fungal endophthalmitis were associated with 2 environmental molds contaminating 2 compounded medications labeled as sterile from the same compounding pharmacy. Together, these 2 outbreaks represent the largest reported outbreak of infectious endophthalmitis and one of the largest outbreaks in the United States attributed to contamination of a compounded medication.

Infections among case-patients were characterized by poor outcomes, including vision loss, as has been described for fungal endophthalmitis (*5*,*9*,*10*). Prolonged time to diagnosis probably contributed to these poor outcomes; diagnostic delay might be explained by the rarity of fungal endophthalmitis and the resulting unfamiliarity of some physicians with this diagnosis, the subacute nature of early infection, or the difficulty of distinguishing true infection from the occasionally observed postprocedural sterile inflammation (*19*). Female sex was associated with case status among patients in the BBG cluster; however, sex differences have not been reported for prior clusters of *Fusarium* endophthalmitis patients (*20*,*21*). The association of diabetes mellitus with case status among patients in the triamcinolone cluster probably reflects the fact that multiple patients received these injections for complications associated with diabetes or that diabetes might have predisposed the patients to infection after exposure.

Compounded medications are a vital part of the health care delivery system, but the role of BBG in this outbreak merits focused discussion. BBG is a triphenylmethane dye in the Coomassie dye family, commonly used for protein staining in biochemical analysis (*22*–*24*). BBG became popular among ophthalmologists for ocular surgery requiring dyes (P. Dugel, pers. comm.) because of its superior safety profile (*25*,*26*) and staining capabilities, compared with FDA-approved products (*27*,*28*). Although BBG is licensed and manufactured in several European countries, it is not FDA approved for any human use in the United States and cannot be manufactured by US pharmaceutical companies for clinical indications. Instead, BBG for clinical use in the United States is provided by compounding pharmacies. Whether providers who administered BBG or the patients who received it were aware that it was not FDA approved for human use is unknown. Regardless, greater transparency and improved education among involved parties as to BBG's regulatory status (as a medication not approved by FDA for human use), particularly at the time of purchase, might have affected the extent of its use and, subsequently, the number of infections in these outbreaks.

This investigation had certain limitations. First, although the epidemiologic association between case status and triamcinolone exposure was strong, triamcinolone from the implicated Franck's lot was unavailable for testing (all vials had been either opened or used in their entirety when the investigation began). Second, follow-up data through 6 months after the procedure or resolution of infection, whichever occurred first, was not available for all case-patients. Third, whether a lack of documented resolution of infection at the end of the 6-month follow-up period truly represented ongoing infection for all case-patients is unclear. Detailed treatment data, including dosages, were not systematically available, and some case-patients were still receiving treatment at the end of the 6-month follow-up period, which limited our ability to correlate treatment

Table 3. Clinical characteristics of fungal endophthalmitis case-patients, United States, 2012*

Characteristic	Exposure cluster†	
	Brilliant Blue G dye, n = 21	Triamcinolone, n = 19
Demographics		
Median age, y (range)	69 (58–86)	67 (53–77)
Female sex	16 (76)	8 (42)
Concurrent medical conditions		
Diabetes mellitus	11 (52)	15 (79)
Hypertension	11 (52)	15 (79)
History of prior eye surgery or procedures	14 (67)	13 (68)
Signs and symptoms		
Vision loss	21 (100)	18 (95)
Vitreous debris	12 (57)	10 (53)
Floaters	4 (19)	6 (32)
Inflammation	10 (48)	6 (32)
Pain	16 (76)	4 (21)
Hypopyon	9 (43)	3 (16)
Fibrin	12 (57)	1 (5)
Treatment		
Any antifungal treatment	17 (81)	19 (100)
Intravitreal amphotericin B	2 (12)‡	8 (42)
Intravitreal voriconazole	17 (100)‡	11 (58)
Oral voriconazole	6 (35)‡	11 (58)
Intravenous voriconazole	1 (6)‡	0
Topical voriconazole	1 (6)‡	0
Oral fluconazole	1 (6)‡	0
Combination of antifungal therapies	10 (59)‡	9 (47)
Outcome		
Median no. days from exposure to diagnosis (range)	78 (60–125)§	80 (35–185)
Additional surgeries required (range)	20 (95) (1–5)	16 (84) (1–6)
No documentation of resolved infection within follow-up period	3 (14)	8 (42)
Enucleations	0	2 (11)

*Data available for 40 case-patients and are presented as no. (%) unless otherwise indicated.
†Manufactured by Franck's Compounding Lab, Ocala, Florida, USA.
‡Calculated percentage out of 17 treated case-patients.
§n = 18.

strategy with patient outcomes. Last, objective data on visual acuity (e.g., 20/20 scale measurements) were not available for all case-patients; because some degree of temporary, minor vision loss is not unusual after any invasive ocular procedure (P. Dugel, pers. comm.), we relied on the clinical judgment of treating ophthalmologists to define atypical postprocedural vision loss.

These outbreaks of fungal infections are not the first associated with contaminated compounded products; they preceded (by only a few months) a large multistate outbreak of *Exserohilum rostratum* infections associated with compounded methylprednisolone acetate (*29*). A 2002 outbreak of meningitis and sacroiliitis attributed to compounded corticosteroid injections contaminated with *Exophiala dermatitidis* was also reported (*30*). Unlike outbreaks associated with bacteria in compounded medications, those associated with fungi present unique challenges, because the insidious, prolonged nature of certain fungal infections might obscure an association with an earlier medication exposure. In addition, unlike bacteria, fungi are often difficult to isolate by culture; molecular diagnostic techniques, such as PCR, might be more useful, but their availability to the average clinician is limited. Furthermore, when directly inoculated into sterile spaces, these fungi can cause infections that are challenging to recognize, as we observed in these outbreaks, not only because they often involve environmental agents that are exceedingly rare causes of human illness but also because once instilled into a sterile space, they can cause disease in an uncommon anatomic area or in an unusual way.

Bipolaris spp. are a common cause of chronic sinusitis among immunocompetent persons (*31*); however, before this outbreak, endophthalmitis with *Bipolaris* spp. had been described in limited case reports only (*32*,*33*). Similarly, fusariosis usually occurs in immunocompromised hosts; endophthalmitis has been reported but usually as a sequela of *Fusarium* keratitis (*10*,*34*) and not de novo, as in this outbreak. The rarity of the infections in these outbreaks provided an additional clue to clinicians that an atypical event had occurred, underscoring the crucial role of clinicians in recognizing unusual disease clusters and promptly reporting them to public health authorities for rapid epidemiologic investigation necessary to rule out widespread exposure.

Oversight of compounding pharmacies lies primarily with state pharmacy boards because compounding pharmacies are regulated as pharmacies despite the fact that some (e.g., Franck's) produce large quantities

of medications and distribute them across state lines. Federal oversight of compounding pharmacies has been limited and often contested. FDA had investigated Franck's twice before these outbreaks. During 2004–2005, FDA inspected Franck's for improper compounding practices, including compounding medications without a valid patient prescription (*35*), and during 2009, Franck's veterinary compounding unit incorrectly compounded a nutritional supplement that resulted in the deaths of 21 polo horses (*36*). These investigations did not preclude Franck's from continuing its operations. During 2010, FDA sought a federal injunction to compel Franck's to cease veterinary compounding from bulk ingredients (*37*) but ultimately lost the court case (*38*). Enhanced regulatory authority toward compounding pharmacies with multiple infractions should be considered a part of efforts to improve compounded medication safety. Furthermore, greater transparency surrounding ongoing disciplinary action against a compounding pharmacy might empower clinicians to make more informed purchasing decisions. Enhanced oversight of compounding pharmacies that produce large quantities of medications, especially sterile products that are distributed nationwide, should be considered to ensure that mass-produced sterile compounded products are safe.

Clinicians should be aware that the availability of a compounded medication in the United States is not a guarantee of its quality or of FDA approval. Disclosure of a medication's FDA approval status should be encouraged at all stages of purchase and use. This information might enable clinicians to make informed decisions about the medications they purchase for patient use and to educate patients about the status of medications to which they are exposed. Maintenance of the safety and integrity of sterile compounded drugs in the United States demands a thorough review and improvement of compounding pharmacy regulatory practices.

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Dr Mikosz is an Epidemic Intelligence Service officer assigned to the Acute Communicable Disease Control program of the Los Angeles County Department of Public Health. Her research interests include health care–associated and emerging infections.

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Address for correspondence: Benjamin J. Park, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop C09, Atlanta, GA 30333; email: bpark1@cdc.gov

Andes Hantavirus Variant in Rodents, Southern Amazon Basin, Peru

Hugo Razuri,[1,2] Rafal Tokarz,[2] Bruno M. Ghersi, Gabriela Salmon-Mulanovich, M. Claudia Guezala, Christian Albujar, A. Patricia Mendoza, Yeny O. Tinoco, Christopher Cruz, Maria Silva, Alicia Vasquez, Víctor Pacheco, Ute Ströher, Lisa Wiggleton Guerrero, Deborah Cannon, Stuart T. Nichol, David L. Hirschberg, W. Ian Lipkin, Daniel G. Bausch,[3] and Joel M. Montgomery[3]

We investigated hantaviruses in rodents in the southern Amazon Basin of Peru and identified an Andes virus variant from *Neacomys spinosus* mice. This finding extends the known range of this virus in South America and the range of recognized hantaviruses in Peru. Further studies of the epizoology of hantaviruses in this region are warranted.

Hantaviruses are enveloped, tripartite, single-stranded, negative-sense RNA viruses belonging to the genus *Hantavirus*, family *Bunyaviridae*. More than 15 hantaviruses have been recognized in the Americas, most in South America (*1*). Hantaviruses are maintained in rodents and shrews, usually with a tight pairing between the specific virus and host species. On the American continents, hantaviruses can evoke a severe acute disease known as hantavirus pulmonary syndrome (HPS), which carries typical case-fatality rates of 35%–40%, depending on the particular virus (*1*). Hantavirus host–reservoir pairs continue to be discovered, and details of hantavirus epidemiology and the risk for transmission of hantaviruses to humans continue to emerge.

Author affiliations: United States Naval Medical Research Unit No. 6, Lima, Peru (H. Razuri, B.M. Ghersi, G. Salmon-Mulanovich, M.C. Guezala, C. Albujar, A.P. Mendoza, Y.O. Tinoco, C. Cruz, M. Silva, D.G. Bausch, J.M. Montgomery); Columbia University, New York, New York, USA (R. Tokarz, D.L. Hirschberg, W.I. Lipkin); Universidad Nacional Mayor de San Marcos, Lima (A. Vasquez, V. Pacheco); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (U. Ströher, L. Wiggleton Guerrero, D. Cannon, S.T. Nichol); and Tulane School of Public Health and Tropical Medicine, New Orleans, Louisiana, USA (D.G. Bausch)

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Data on hantaviruses in Peru are sparse and confined to the Loreto Region in the northern Amazon Basin (Figure 1), where a 1996 study of rodents showed a 20% prevalence of IgG to hantaviruses and the identification of a Rio Mamoré–like hantavirus from *Oligoryzomys microtis* rodents (*2*). The IgG prevalence in humans in Loreto tested during 2007–2010 was low (1.7%) (*3*). Nevertheless, 4 cases (3 fatal) of human hantavirus infection were reported in 2011 in this region, 2 from Rio Mamoré and 2 from Seoul virus (*4*,*5*). To expand the knowledge base on hantaviruses in Peru, we conducted an investigation of rodents in a previously unexplored area of Peru's southern Amazon Basin.

The Study

In 2003, Peru began construction on an interoceanic highway through the southern Amazon Basin in the Peruvian administrative regions of Madre de Dios and Puno; the highway is intended to link Amazon sites with Pacific ports (Figure 1). During October 2009–October 2010, we collected small mammals at 6 sites in the region of the highway construction as part of a study exploring the effects of this construction on the region's fauna and flora, including the risk for rodent-borne disease. This study was approved by the US Naval Medical Research Unit No. 6 Institutional Animal Care and Use Committee, and collection permits were obtained from the Peruvian Ministry of Agriculture Office of Forestry and Wildlife. Animals were collected in 6 trapping sessions by using live traps (H.B. Sherman Traps, Tallahassee, FL, USA; Tomahawk Live Trap, Hazelhurst, WI, USA) and standard methods (*6*). We also collected animals at a relatively undisturbed site near the Tambopata National Reserve (Figure 1). Animals were anesthetized and euthanized before morphometric measurements were taken and necropsies performed. Samples of blood, heart, lung, spleen, liver, and kidney were collected (*7*); tissues were stored in liquid nitrogen for transport to the laboratory. A section of liver was also preserved in 95% ethanol for molecular identification of rodent species. Rodent carcasses were fixed in 10% formalin and preserved in 70% ethanol as permanent voucher specimens.

Rodent blood was tested by ELISA for IgG to New World hantaviruses at the Centers for Disease Control and Prevention Special Pathogens Branch in Atlanta, Georgia, USA, by using nucleocapsid antigen derived from Sin Nombre virus, which is broadly cross-reactive among New World hantaviruses (*6*). Nested reverse transcription PCR (RT-PCR) was performed by Naval Medical Research Unit No. 6 on homogenized lung samples from IgG-positive

[1]Current affiliation: McGill University Health Centre, Montreal, Quebec, Canada.

[2]These authors contributed equally to this article.

[3]These authors jointly supervised this work.

Figure 1. Regions of Peru, indicating areas of previous hantavirus study (Loreto [*2*]) and the study of hantaviruses described in this article (Madre de Dios and Puno). Capital cities of the Loreto and Madre de Dios Regions are indicated by black dots.

rodents. PCR targetED a 434-bp region of the hantavirus small (S) segment and 296- and 242-bp regions of the medium (M) segment G1 and G2 regions, respectively (*8*). Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA, USA) high-throughput sequencing and additional consensus RT-PCR on ELISA-positive rodents were performed at Columbia University in New York, NY, USA. Rodent species was determined on the basis of morphometric assessment by experienced mammalogists at the San Marcos University Natural History Museum in Lima, Peru; identification of IgG-positive animals was confirmed by DNA extraction from the liver followed by PCR amplification and sequencing of the cytochrome *b* gene (*9*).

During a total of 15,145 trap-nights, 362 rodents (trapping success rate 2.4%) belonging to 14 species were captured. The most frequently trapped rodents were 155 (42.8%) *Oligoryzomys microtis* mice, 49 (13.5%) *Necromys lenguarum* mice, 41 (11.3%) *Hylaeamys* spp. rodents, 35 (9.7%) *Euryoryzomys nitidus* rats, and 34 (9.4%) *Neacomys spinosus* mice. Six (1.7%) rodents had positive results for hantavirus IgG: 2 *N. spinosus* mice (both adult males), 2 *N. lenguarum* mice (1 adult and 1 juvenile, both females), 1 *H. yunganus* rat (juvenile female), and 1 *E. nitidus* rat (adult male). IgG-positive rodents were trapped at 5 times from 3 sites at distances 43–145 km from Puerto Maldonado, the capital city of Madre de Dios.

Results of RT-PCR testing were positive for 2 of 6 IgG-positive rodents, the 2 *N. spinosus* mice, which were trapped on consecutive days at the same site, 80 km from Puerto Maldonado. However, a hantavirus sequence was identified in only 1 of these rodents by both consensus RT-PCR and high-throughput sequencing. Assembly of hantavirus reads obtained by high-throughput sequencing resulted in the generation of 1,068 bp of the S, 1,673 bp of the M, and 572 bp of the large (L) segments for the virus. Phylogenetic analysis showed the virus to be an Andes virus clade variant most similar to viruses within the Castelo dos Sonhos (CASV) group, which consists of CASV and CASV-2, found in Brazil, and Tunari virus (TUNV), found in Bolivia (Figure 2) (*10*). The nucleotide sequences of the S and M segments were 91% and 90%, respectively, identical to TUNV (98% and 97% aa identity, respectively). The L segment sequence was most similar to CASV-2 (85% nt and 98% aa identity). No TUNV L segment sequence data are available for comparison. The S, M, and L nucleotide sequences for the virus we identified were deposited in GenBank under accession nos. KF581134, KF581135, and KF581136, respectively.

Conclusions

Our data confirm the presence of an Andes virus variant similar to TUNV and CASV in the southern Amazon Basin of Peru; this finding extends the known range of this virus in South America and the range of recognized hantaviruses in Peru. HPS has been associated with CASV in Brazil (*11*,*12*) and with TUNV in Bolivia (*13*). However, no cases have been reported in the southern Amazon Basin of Peru, nor is formal surveillance for the disease conducted in the area, and no human antibody prevalence studies have been conducted. The lack of reported human cases could mean that the frequency of infected rodents, or of human contact with them, is low; that human infection occurs but is asymptomatic or too mild to be recognized; or that HPS is misdiagnosed by clinicians who are unfamiliar with the condition and lack access to diagnostic testing. In some areas of South America, including Bolivia, Paraguay, Argentina, and Chile, a high prevalence of hantavirus IgG in humans has been reported, but reports of cases of HPS have been infrequent (*14*).

Oligoryzomys spp. rodents appear to be the principal reservoirs for most Andes viruses, including CASV (*12*,*15*); no reservoir has been associated with TUNV. Our data suggest that *N. spinosus* mice may be the reservoir for the Andes virus variant found in Madre de Dios and Puno, although spillover infection from an alternate

reservoir cannot be excluded. If these mice are the reservoir for this virus, the human population at risk for hantavirus infection by transmission from *N. spinosus* mice could be large, because the range of this species encompasses considerable portions of Brazil, Colombia, Ecuador, Peru, and Bolivia. *N. spinosus* mice have not been associated with other hantaviruses. Further investigation of the epizoology of hantaviruses and possible risks to humans in the southern Amazon Basin of Peru is warranted.

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The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the 1996 edition of the National Research Council's Guide for the Care and Use of Laboratory Animals (www.nap.edu/openbook.php?record_id=5140). Animal

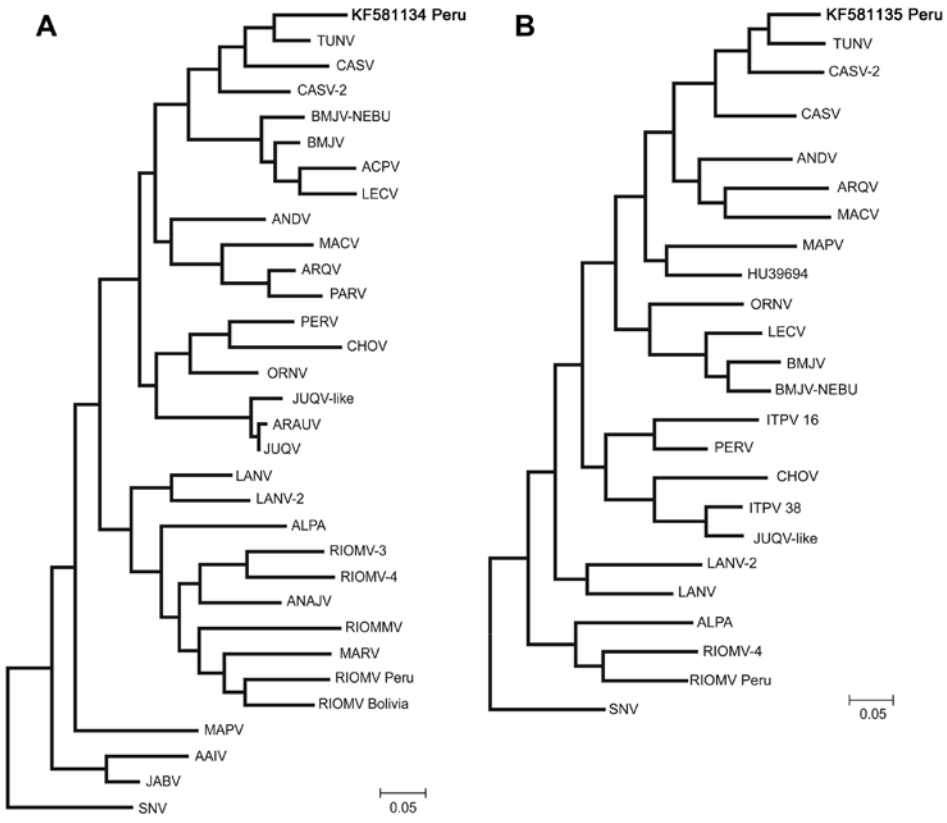

Figure 2. Maximum likelihood phylogenetic trees of the small (S; panel A) and medium (M; panel B) segments of the hantavirus identified in this study in Peru (boldface) compared with segments of hantaviruses from throughout South America. Trees were generated by using MEGA5.2 software (www.megasoftware.net) with 2,000 bootstrap replicates. All viruses are shown relative to North American Sin Nombre virus (SNV; GenBank accession nos. L25784, L25783 [for M segment]). Hu39694 (AF028023 [M]) is also shown. TUNV, Tunari virus (GenBank accession nos. JF750417 [S], JF750420 [M]); CASV, Castelo dos Sonhos virus (AF307324, HQ719471 [S]; JX443698, JX443699 [M]); BMJV-NEBU, Bermejo-Neembucu virus (DQ345763 [S], AY515603 [M]); BMJV, Bermejo virus (AF482713 [S], AF028025 [M]); ACPV, Andes Central Plata virus (EU564715 [S]); LECV, Lechiguanas virus (AF482714 [S], AF028022 [M]); ANDV, Andes virus (AF004660 [S], AF324901 [M]); MACV, Maciel virus (AF482716 [S], AF028027 [M]); ARQV, Araraquara virus (AF307325 [S], AY970821 [M]); PARV, Paranoa virus (EU643620 [S]); PERV, Pergamino (AF482717 [S], AF028028 [M]); CHOV, Choclo virus (DQ285046 [S], DQ285047 [M]); ORNV, Oran virus (AF482715 [S], AF028024 [M]); JUQV-like, Juquitiba-like (GU213197 [S], AY963900 [M]); ARAUV, Araucaria (AY740628 [S]); JUQV, Juquitiba (EF492472 [S]); LANV, Laguna Negra virus (AF005727 [S], FJ816031 [S], AF005728 [M], JX443703 [M]); ALPA, Alto Paraguay virus (DQ345762 [S], AY515602 [M]); RIOMV, Rio Mamore virus (FJ532244, U52136, JX443667, JX443679 [S]; FJ608550 [M], JX443701 [M]); ANAJV, Anajatuba virus (JX443690 [S]); RIOMMV, Rio Mearim virus (DQ451828 [S]); MARV, Maripa virus (GQ179973 [S]); MAPV, Maporal virus (FJ008979 [S], AY363179 [M]); AAIV, Ape Aime Itapua virus (GU205340 [S]); JABV, Jabora virus (JN232079 [S]); and ITPV, Itapua virus (16, AY515606 [M]; 38, AY515604 [M]). Scale bars indicate nucleotide substitutions per site.

collection was authorized by the Peru Office of Forestry and Wildlife through permit nos. RJ 002-2010-SERNANP-DGANP-PNBS, RD 383-2009-AG-DGFFS-DGEFFS, and RD 425-2010-AG-DGFFS-DGEFFS. Exportation authorization of serum samples was issued by the same office, permits no. 004546-AG-DGFFS and 412-2013-MINAGRI 004546-AG-DGFFS/DGEFSS.

Dr Razuri is a physician from Peru and is now working as a research coordinator at the Division of Clinical Epidemiology, McGill University Health Centre, Montreal, Quebec, Canada. His area of focus is the epidemiology of infectious diseases, including HIV/AIDS, influenza, and zoonotic diseases.

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Address for correspondence: Hugo Razuri, McGill University Health Centre, Royal Victoria Hospital Campus, 687 Pine Avenue West, V-Building, Rm V208, Montreal, QC H3A1A1, Canada; email: hugo.razuri@clinepi.mcgill.ca

Human Cutaneous Anthrax, Georgia 2010–2012

Ian Kracalik,[1] Lile Malania,[1] Nikoloz Tsertsvadze, Julietta Manvelyan, Lela Bakanidze, Paata Imnadze, Shota Tsanava, and Jason K. Blackburn

We assessed the occurrence of human cutaneous anthrax in Georgia during 2010–2012 by examining demographic and spatial characteristics of reported cases. Reporting increased substantially, as did clustering of cases near urban centers. Control efforts, including education about anthrax and livestock vaccination, can be directed at areas of high risk.

Anthrax, a zoonosis caused by the bacterium *Bacillus anthracis*, is associated with rural areas or agricultural production (*1*–*3*). Human infections often result directly from contact with infected livestock or contaminated animal materials (*2*). Although several countries have implemented successful control strategies, anthrax remains a reemerging threat to public health in areas with weak health systems. After the Soviet Union collapsed, the country of Georgia underwent funding cuts to public health and animal health infrastructure, limiting disease management (*2*). Human anthrax cases more than tripled in Georgia after Soviet governance discontinued; 118 cases were reported during 1991–1996, compared with 36 during 1985–1990 (*4*,*5*). Recent reports indicating a worsening anthrax situation in Georgia have raised concern about spread of anthrax to new areas (*5*,*6*). To assess this situation, we analyzed demographic risk factors and characterized spatial patterns of human cutaneous anthrax (HCA) in Georgia.

The Study

Anthrax is nationally reportable in Georgia. We analyzed passive surveillance data on HCA cases reported to the National Center for Disease Control and Public Health (Tbilisi, Georgia) during 2010–2012. Case investigation forms included patients' community of residence, age, and sex. Self-reported source of infection was ascertained from categorical responses that included butchering/slaughtering cattle, handling/preparing meat, performing field work/harvesting crops, processing hair/wool, receiving an insect bite, unknown, or other. We also obtained information about regional-level anthrax cases in livstock (www.oie.int/wahis_2/public/wahid.php/disease information/statusdetail). HCA cases and incidence per million persons were plotted over time with livestock cases. To estimate changes in HCA risk over time, we compared data for the study period with data for 2007–2009 by using a cumulative incidence ratio (total cases/median year population). We calculated incidence risk ratios for demographic characteristics by using a negative binomial regression (online Technical Appendix, wwwnc.cdc.gov/eid/article/20/2/pdfs/13-0522-Techapp1.pdf).

Data were anonymized and aggregated to each case-patient's community of residence and mapped in a geographic information system. We extrapolated current population from the 2002 census using the United Nations medium variant population growth projections (http://data.un.org/). Cumulative incidences per 10,000 population were calculated for each community (total cases/median year population). National and community population data were obtained from GeoStat (http://geostat.ge).

We adjusted for community population heterogeneity using Empirical Bayes smoothing estimates of the cumulative incidence (online Technical Appendix). Kernel density estimation was used to map the cumulative incidence risk/km^2 (online Technical Appendix). To identify community-level HCA spatial clustering, we used the Poisson model in SaTScan (*7*) with a maximum cluster size of 25% of the population at risk. We compared the proportion of urban and rural communities inside and outside clusters using a χ^2 analysis (PROC FREQ, SAS Institute, Cary, NC, USA).

During 2010–2012, a total of 251 HCA cases and 74 livestock cases (38 cattle, 1 pig, 4 sheep/goats, 1 horse) were reported (Figure 1). HCA cases peaked in 2012 (142 cases), corresponding to a national incidence of 31.7 per million population (95% CI 28.4–36.1). Compared with 2007–2009 (133 cases), the period studied had a higher risk for HCA (cumulative incidence ratio 1.89 [95% CI 1.64–2.33], $p<0.01$).

Cases occurred predominantly in males (209 [84%]) (Table 1). Butchering/slaughtering cattle as a source of infection was more common among male patients (143 [68%]); processing/handling meat was more common among female patients (28 [68%]). Case-patients' median age was 43 years [range 5–79]. The adjusted incidence risk ratios from the negative binomial model showed a stronger association of risk in males (4.95 [95% CI 2.91–8.42]) than in females (Table 2). Persons 50–64 years of age were at greater risk than were persons in all other age groups.

Author affiliations: University of Florida, Gainesville, Florida, USA (I. Kracalik, J.K. Blackburn); National Center for Disease Control and Public Health of Georgia, Tbilisi, Georgia (L. Malania, N. Tsertsvadze, J. Manvelyan, P. Imnadze, S. Tsanava); and Agrarian University of Georgia, Tbilisi (L. Bakanidze)

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[1]These authors contributed equally to this article.

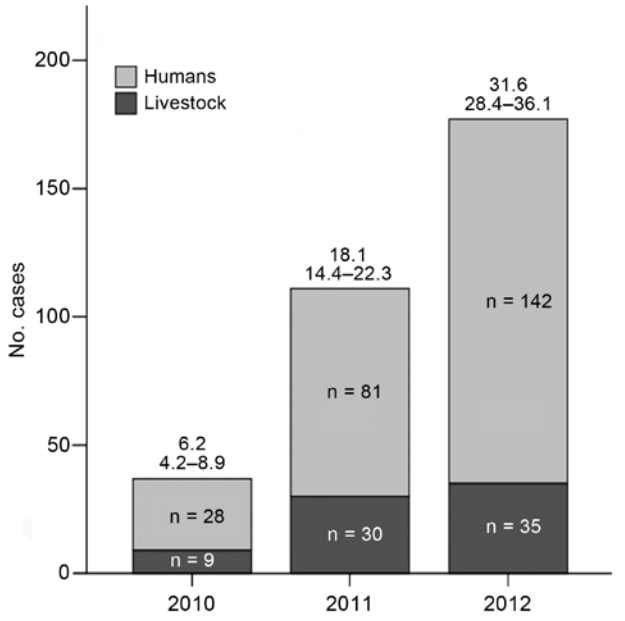

Figure 1. Total number of human cutaneous anthrax cases (light gray) and livestock cases (dark gray), Georgia, 2010–2012. Incidence rates (IRs) (95% CIs) of human cutaneous anthrax per million population are displayed above the bars.

Risk did not differ between case-patients who processed/handled meat and those who slaughtered/butchered cattle.

Empirical Bayes smoothed incidence per 10,000 population ranged from 0.11 (95% CI 0.03–0.37) to 213.8 (95% CI 72.8–552.6) (Figure 2, panel A). The kernel density analysis identified a higher risk per squared kilometer in the southeast and west (Figure 2, panel B). We identified 3 spatial clusters of high risk; the clusters comprised 58 communities with 197 cases (Figure 2, panel C). Cumulative incidence inside the clusters was 12.8/10,000 population compared with 0.96/10,000 population outside the clusters. The proportion ($\chi^2 = 0.65$, $p = 0.4$) of urban and rural communities (22 and 36, respectively) within clusters did not differ from the proportion outside clusters (urban: 14; rural: 24). Clusters 1 and 2 in the southeast bordered the densely populated urban area of the capital (Tbilisi) and were associated with grazing lands, whereas cluster 3 bordered the urban area of Poti in the west and was associated with a higher percentage of croplands.

Conclusions

Despite an apparent decline in HCA cases worldwide (*8*), incidence in Georgia has increased and is now comparable with that in Turkey, where the disease is hyperendemic (*1*,*9*,*10*). Our analysis identified clusters of communities that were of historical importance for HCA risk and new communities that represent areas of (re)emergence (*6*).

Our findings indicate that HCA cases were concentrated within specific areas associated with agriculture and in close proximity to urban centers (Figure 2), consistent with research linking urban livestock trading centers and markets to outbreaks and higher rates of HCA (*5*,*9*–*11*). Sick and dying animals are often slaughtered and brought to market quickly to mitigate economic losses, thereby exacerbating exposure risk while limiting livestock reporting (*10*,*11*). HCA is primarily associated with rural, agrarian areas (*2*,*3*,*12*), but we found no difference in the proportion of urban and rural communities in high-risk clusters. This association with urban zones suggests reliance on these areas for commerce, coupled with increased agricultural employment from 25% of the population in 1990 to ≈55% in 2010 (*5*,*13*). One hypothesis is that contaminated meat is brought into urban areas and sold at informal meat markets that because of fiscal constraints have little to no regulation.

Cases were predominantly linked to contact with infected livestock or meat, as observed elsewhere (*2*,*3*,*9*,*11*). Field work was a self-reported source of infection in 27 (10.7%) cases. This risk factor is not well documented and might reflect recall bias or unwillingness to admit to slaughtering infected animals. Our findings that males were at higher risk than females for HCA, as reported elsewhere (*2*,*14*) might reflect occupational exposures or gender roles, with males slaughtering/butchering livestock and

Table 1. Characteristics of human cutaneous anthrax patients, Georgia, 2010–2012

Characteristic	Male case-patients		Female case-patients	
	No., n = 209	Population*	No., n = 42	Population*
Age, y				
5–19	14	522,736	2	506,785
20–34	57	458,998	8	480,276
35–49	62	443,820	12	502,732
50–64	61	292,713	15	361,370
65–79	15	196,171	5	286,839
Self-reported infection source				
Slaughtering cattle	143		3	
Processing meat	39		28	
Field work/sowing and harvesting crops	20		7	
Unknown	7		3	

*Population estimates were obtained from the Georgian State Statistical Office (GeoStat, http://geostat.ge) and are based on median year population totals for the study period.

Table 2. Results of the negative binomial regression model examining risk factors for human cutaneous anthrax, Georgia

Patient characteristic	IRR*		95% CI†	p value
	Univariate	Adjusted		
Age, y				
5–19	0.12	0.11	0.05–0.26	<0.01
20–34	0.57	0.46	0.23–0.91	0.03
35–49	0.65	0.58	0.30–1.14	0.11
50–64	Referent	Referent		–
65–79	0.37	0.36	0.16–0.78	0.01
Sex				
F	Referent	Referent		–
M	5.75	4.95	2.91–8.42	<0.01
Self-reported infection source				
Slaughtering/butchering cattle	Referent	Referent		–
Processing/handling meat	0.45	0.75	0.40–1.39	0.36
Field work/sowing and harvesting crops	0.18	0.26	0.13–0.51	<0.01
Unknown	0.07	0.09	0.04–0.20	<0.01

*χ^2 goodness-of-fit test indicated the model fit the data (df = 31, χ^2 = 40.71, p = 0.11). IRR, incidence risk ratio.
†Wald 95% CIs.

females more involved in preparing meat (*14*). In contrast, no gender differences were reported in Turkey or Kazakhstan (*3*,*9*). Although age was not a risk factor in other regions (*2*,*3*), persons 50–64 years of age in this study were at higher risk for infection (Table 2), reflecting changing sociocultural practices related to an agrarian lifestyle.

Although the true cause of the increase in HCA cases is unknown, it is probably due to a combination of factors

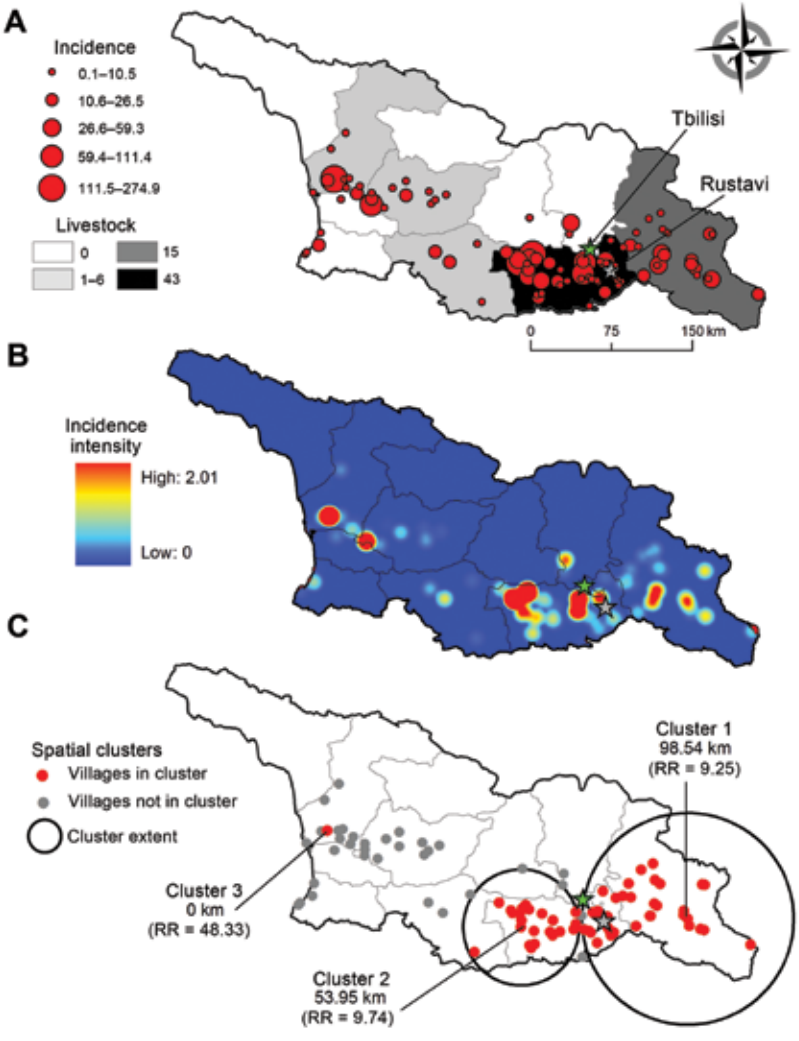

Figure 2. A) Empirical Bayes Smoothing cumulative incidence (per 10,000 population) of human cutaneous anthrax at the community level, Georgia, 2010–2012. Green star indicates the location of the capital, Tbilisi; gray star indicates the fourth most populous city, Rustavi. The total number of livestock cases during the study period is shown by region. B) Risk surface representing the estimated smoothed cumulative incidence per square kilometer. C) Spatial clustering of human cutaneous anthrax cases as determined by using SaTScan (*7*). RR, risk ratio.

related to decreased public health funding and agricultural reform resulting in a shift from collectivization to private ownership (*1*,*5*,*13*). These factors, coupled with limited veterinary control and the cessation of compulsory livestock vaccination, have played plausible roles in the increased incidence of HCA. Livestock cases were out of sync temporally and spatially with human cases, indicating anthropocentric reporting or high incidence from relatively few animals, although differences in the aggregation of livestock cases limit the inference of spatial synchrony. Controlling HCA requires controlling the disease in livestock (*1*,*2*,*9*) and highlights the need for a One Health approach. Our findings can be used to formulate public health interventions aimed at controlling anthrax in livestock and increasing awareness of the disease, particularly in urban areas. Given the limited resources available, future efforts should focus on high-risk areas for livestock surveillance and control, such as targeted vaccination campaigns (*15*).

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Mr Kracalik is a spatial epidemiologist at the Spatial Epidemiology and Ecology Research Laboratory, Department of Geography, University of Florida. His primary research interests include bacterial zoonoses and spatial analysis.

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Address for correspondence: Jason K. Blackburn, Spatial Epidemiology and Ecology Research Laboratory, Department of Geography, 3141 Turlington Hall, University of Florida, Gainesville, FL 32611, USA; email: jkblackburn@ufl.edu

Melioidosis Caused by *Burkholderia pseudomallei* in Drinking Water, Thailand, 2012

Direk Limmathurotsakul, Gumphol Wongsuvan, David Aanensen, Sujittra Ngamwilai, Natnaree Saiprom, Patpong Rongkard, Janjira Thaipadungpanit, Manas Kanoksil, Narisara Chantratita, Nicholas P.J. Day, and Sharon J. Peacock

We identified 10 patients in Thailand with culture-confirmed melioidosis who had *Burkholderia pseudomallei* isolated from their drinking water. The multilocus sequence type of *B. pseudomallei* from clinical specimens and water samples were identical for 2 patients. This finding suggests that drinking water is a preventable source of *B. pseudomallei* infection.

Burkholderia pseudomallei is a Tier 1 select agent and the cause of naturally acquired melioidosis in Southeast Asia, northern Australia, the Indian subcontinent, and areas of South America (*1*). The organism is present in soil and surface water, and most melioidosis cases are believed to result from bacterial inoculation or inhalation (*1*). Ingestion has been increasingly suspected to be an alternative route of infection. *B. pseudomallei* isolated from a community water supply in Australia was genetically identical to that causing disease in clusters (*2*,*3*), although no direct evidence was available to show that affected cases had consumed contaminated water. The pattern of infection after ingestion in an experimental model includes multiple organ dissemination and hepatosplenic abscesses, which are common features of human melioidosis and supportive evidence for ingestion as a route of human infection (*4*).

Our previous study in Ubon Ratchathani Province in northeastern Thailand investigated the activities of daily living associated with acquisition of melioidosis (*5*). Households of participants who resided ≤100 km of Sappasithiprasong Hospital in Ubon Ratchathani were visited, and water samples were collected from all sources of drinking water and from tap water (*5*). Culture of these samples for *B. pseudomallei* provided borderline statistical evidence to suggest that consuming water containing *B. pseudomallei* was associated with melioidosis (conditional odds ratio 2.2, 95% CI 0.8–5.8, p = 0.08) (*5*). We performed a study to further evaluate the role of ingestion as a route of infection, and we identified the genotypes of *B. pseudomallei* isolated from patients and the water supplies consumed by them.

The Study

During July 2010–December 2011, we collected and cultured 576 water samples from the households of 142 case-patients and 288 controls (*5*). In brief, 5 L of water was collected from each source of drinking water and tap water, regardless of consumption. If the water was filtered or boiled by the householder before consumption, samples were collected for culture. Locations from which water samples were collected were recorded by using the Epicollect Program (*6*). For each sample, 1 L was passed through two 0.45-μm filters (500 mL through each filter), and the remaining 4 L was passed through 2.5 g of sterile diatomaceous earth (Celite; World Minerals Corporation, San Jose, CA, USA).

Filters were cultured on Ashdown agar to obtain a quantitative bacterial count, and diatomaceous earth was cultured in selective broth containing (15 mL of threonine–basal salt plus colistin broth) to obtain a sensitive, qualitative method. Broth was incubated at 40°C in air for 48 h, after which 10 mL of the upper layer was streaked onto an Ashdown agar plate to achieve single colonies, incubated at 40°C in air, and examined every 24 h for 7 days. If enrichment broth cultures showed positive results but filters on Ashdown agar showed negative results, the quantitative count was defined as <1 CFU/L. Ten colonies of *B. pseudomallei* were randomly picked from the primary plate and saved for genotyping. If there were <10 colonies, all primary plate colonies were used, and the number was adjusted to 10 after subculture of the enrichment broth.

Genotyping was performed by using pulsed-field gel electrophoresis and multilocus sequence typing (MLST) as described (*7*,*8*). In brief, isolates from clinical and water samples from the same patient were subjected to electrophoresis on the same gel. Colonies with an identical banding pattern were classified as the same genotype, and colonies with ≥1 different patterns were further genotyped by using MLST (*8*). A map was drawn by using the R program (www.r-project.org/) and OpenStreetMap (www.openstreetmap.org) data.

Author affiliations: Mahidol University, Bangkok, Thailand (D. Limmathurotsakul, G. Wongsuvan, S. Ngamwilai, N. Saiprom, P. Rongkard, J. Thaipadungpanit, N. Chantratits, N.P.J. Day, S.J. Peacock); Imperial College London, London, UK (D. Aanensen); Sappasithiprasong Hospital, Ubon Ratchathani, Thailand (M. Kanoksil); Churchill Hospital, Oxford, UK (N.P.J. Day); and Addenbrooke's Hospital, Cambridge, UK (S.J. Peacock)

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Table 1. Culture of *Burkholderia pseudomallei* from household drinking water in Ulbon Ratchathani, Thailand, 2012*

Source of drinking water†	No. positive samples/no. tested (%)	Median quantitative count of *B. pseudomallei*, CFU/L (range)
Well	1/27 (4)	31
Bore hole	10/84 (12)	18 (<1–65)
Collected rain	0/160 (0)	NA
Tap		
Available but not consumed	26/178 (15)	<1 (<1–63)
Consumed	6/95 (6)	1 (<1–13)
Bottled	0/32 (0)	NA
Total	43/576 (7)	1 (<1–65)

*Households of participants who resided ≤100 km of Sappasithiprasong Hospital in Ubon Ratchathani were visited. NA, not applicable.
†Samples from water that was consumed were collected, together with tap water samples, which were collected regardless of ingestion history.

A total of 43 (7%) of 576 water samples were culture positive for *B. pseudomallei* (Table 1). The rate of positivity did not differ between the rainy season (June–November) and dry season (December–May) (8% vs. 6%; p = 0.32, by Fisher exact test) (Figure 1). Positive water samples were geographically distributed across the sampling area (Figure 2). The median quantitative count of *B. pseudomallei* in water was 1 CFU/L (interquartile range <1–13 CFU/L, range <1–65 CFU/L) (Table 1). Of the 43 culture-positive water samples, 21 (7%) of 288 were from control households and 22 (15%) of 142 were from case-patient households. Ten of these case-patients with melioidosis reported drinking from contaminated water sources in the 30 days before the onset of illness.

We compared genotypes of *B. pseudomallei* in clinical specimens and water samples from the 10 case-patients who consumed water that was subsequently shown to contain *B. pseudomallei*. A total of 91 colonies from 10 water samples and 1 colony isolated from blood culture (*7*) or sputum (*3*) from each case-patient was examined by using pulsed-field gel electrophoresis and MLST. The median number of different genotypes observed per water sample was 3 (range 1–6). Two case-patients were infected with a *B. pseudomallei* genotype that was also present in their drinking water (Table 2).

Conclusions

Our finding that drinking water, including public tap water, in northeastern Thailand contains viable *B. pseudomallei* is a public health concern. Public tap water in Thailand, to which melioidosis is highly endemic, needs to be safe and free from *B. pseudomallei* contamination. *B. pseudomallei* can survive in water for prolonged periods (*9*). The National Tap Water Quality Assurance Program in Thailand currently does not include *B. pseudomallei* detection (*10*) and this fact warrants review. Unlike observations in Hong Kong (*11*), all collected rainwater specimens were culture negative for *B. pseudomallei*. The reasons for this finding are not known, but it may be that the bacterial count was below the detection limit.

Clusters of melioidosis cases with the same genotype in Australia might be related to a temporary stoppage of the water purification process (*2*,*3*). Clustering has not been reported from Thailand, and might be explained by the high degree of genetic diversity of *B. pseudomallei* in water in this setting (*12*). The validity of a study that reported the detection of *B. pseudomallei* in 6 (7%) of 85 drinking water samples in Italy is questionable (*13*) because the bacterial isolates were not confirmed to be *B. pseudomallei* by using specific identification methods, and there have been no reported cases of indigenous melioidosis in Italy (*14*).

We propose that ingestion was the probable route of melioidosis acquisition in the 2 patients who each had matching *B. pseudomallei* genotypes in their clinical sample and drinking water. Although other routes of infection, such as inoculation injury, are possible for these 2 patients, genetic diversity of *B. pseudomallei* in even a small area in Thailand indicates that the likelihood of observing an identical genotype for an isolate in water and an isolate from a human that was acquired by soil inoculation is probably low (*12*). Infection with >1 strain of *B. pseudomallei* is rare (*15*), but we might not have linked clinical and water strains in some cases because we did not pick appropriate colonies for genotyping from a water sample that contained multiple genotypes. In summary, evidence from a case–control study (*5*) and our molecular study suggests that most *B. pseudomallei* infections are acquired by inoculation, but a proportion of cases are caused by ingestion, and such cases are potentially preventable.

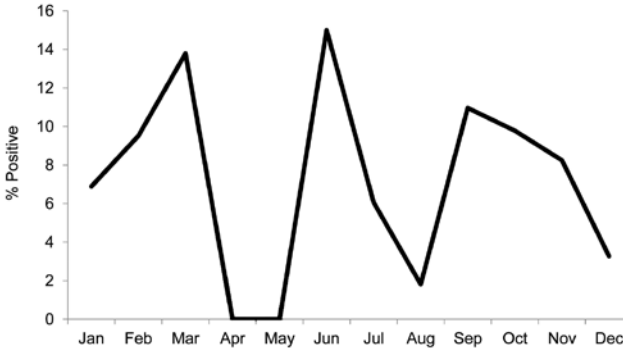

Figure 1. Percentage of water samples positive for *Burkholderia pseudomallei*, Thailand, 2012.

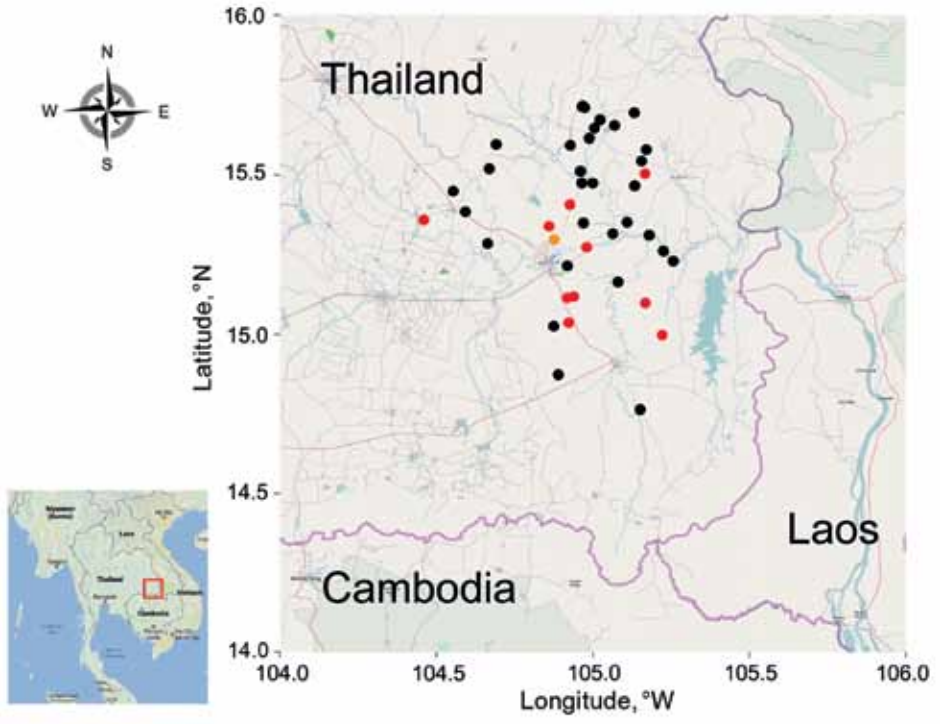

Figure 2. Ubon Ratchathani Province in northeastern Thailand and locations where water samples were tested for *Burkholderia pseudomallei*, 2012. Location of wells, bore holes, and tap water samples that were positive are indicated by orange, red, and black circles, respectively. The red square in the inset indicates the study area in Thailand.

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Table 2. Genotyping of *Burkholderia pseudomallei* isolated from clinical specimens and water samples for 2 patients, Thailand, 2012*

Patient no./age, y/sex	Sample type	No. colonies tested	PFGE pattern (no. colonies)	No. bands different from clinical sample	Sequence type determined by MLST	Clinical features and outcome
1/78/F	Blood	1	A (1)	NA	208	No known risk factors for melioidosis; patient had acute onset of severe pneumonia and septic shock and died 48 h after hospital admission. Blood, sputum, and urine specimens were positive for *B. pseudomallei*. Abdominal ultrasound scan was not performed. Patient had a history of consuming untreated well water.
	Well water	10	A (2)	0	208	
			B (2)	8	54	
			C (4)	11	58	
			D (2)	12	309	
2/88/M	Blood	1	E (1)	NA	48	Patient had diabetes, acute onset of pneumonia, and right hemiparesis. Blood and sputum specimens were positive for *B. pseudomallei*. Computer tomography scan of the brain showed a ruptured mycotic aneurysm, leading to acute hematoma in the left frontoparietotemporal lobe and multiple septic emboli. Abdominal ultrasonogram was not performed. Patient had a history of consuming untreated well water and tap water. Septic shock developed on day 19 and the patient died on day 24 after hospital admission.
	Tap water	10	F (7)	3	48	
			G (2)	6	47	
			H (1)	14	696	

*Genotyping was performed by using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) as described (*7*,*8*). NA, not applicable.

National Institute for Health Research Cambridge Biomedical Research Centre.

Dr Limmathurotsakul is a clinician scientist at the Mahidol–Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Bangkok, Thailand. His research interests are the epidemiology of melioidosis and *Burkholderia pseudomallei* in the environment.

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Address for correspondence: Direk Limmathurotsakul, Mahidol–Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Rd, Bangkok 10400, Thailand; email: direk@tropmedres.ac

Fatal Systemic Morbillivirus Infection in Bottlenose Dolphin, Canary Islands, Spain

Eva Sierra, Daniele Zucca, Manuel Arbelo, Natalia García-Álvarez, Marisa Andrada, Soraya Déniz, and Antonio Fernández

A systemic morbillivirus infection was diagnosed postmortem in a juvenile bottlenose dolphin stranded in the eastern North Atlantic Ocean in 2005. Sequence analysis of a conserved fragment of the morbillivirus phosphoprotein gene indicated that the virus is closely related to dolphin morbillivirus recently reported in striped dolphins in the Mediterranean Sea.

Since the first morbillivirus outbreak affecting bottlenose dolphins (*Tursiops truncatus*) along the mid-Atlantic Coast of the United States during the late 1980s (*1*), some mass die-off episodes affecting the global cetacean population have occurred (*2*–*5*). Three cetacean morbilliviruses have been identified: porpoise morbillivirus, isolated from harbor porpoises that died along the coast of Ireland (*6*); dolphin morbillivirus (DMV), first identified in striped dolphins from the Mediterranean Sea (*2*); and pilot whale morbillivirus, isolated from a long-finned pilot whale stranded in New Jersey, USA (*7*). Other members of the *Paramyxoviridae* family that have affected various marine mammal populations worldwide are phocine distemper virus and canine distemper virus (CDV) (*8*). Since early July 2013, a widespread die-off of >500 bottlenose dolphins occurred along the US mid-Atlantic Coast that probably resulted from morbillivirus infection (*9*).

We report a unique morbillivirus that caused documented systemic infection in a bottlenose dolphin from the eastern North Atlantic Ocean. Although this case occurred nearly 9 years ago, the nucleotide sequence of a fragment of the morbillivirus phosphoprotein (*P*) gene shows high homology with sequences recently reported for morbilliviruses from striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea. This information is of value because no other sequences of morbillivirus from bottlenose dolphins are available in GenBank, despite the seriousness of this fatal disease, which causes mass deaths of marine mammals worldwide.

Author affiliation: University of Las Palmas de Gran Canaria, Arucas, Canary Islands, Spain

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Case Report

On July 18, 2005, a juvenile female bottlenose dolphin was stranded alive in Arrieta, Lanzarote (Canary Islands, Spain). The animal was 250 cm long and in moderate body condition, according to anatomic parameters. The animal died shortly after stranding, and a complete standardized necropsy was performed within 6 hours after death. Required permission for the management of stranded cetaceans in the Canarian archipelago is issued by the environmental department of the Canary Islands' government.

Tissues of all the major organs and lesions were collected and stored in neutral buffered 10% formalin fixative solution for histologic and immunohistochemical analyses. Fixed tissue samples were trimmed and then routinely processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for examination by light microscopy. A monoclonal antibody against the nucleoprotein of CDV (MoAb CDV-NP, VMRD, Inc., Pullman, WA, USA) known to react with DMV was used as primary antiserum. Immunohistochemical analysis was performed on selected samples of brain, intestinal, lymphatic, pancreatic, pulmonary, renal, and splenic tissues, as described (*4*). Samples of lung, spleen, and brain were collected and held frozen at -80°C until processed for molecular virology.

On gross examination, the most remarkable findings were moderate to severe multiorgan parasitic infection, mainly cirripedes (*Xenobalanus* sp.) in the caudal fin; larval cestodes (*Phyllobothrium* sp. and *Monorygma grimaldi*) within the subcutaneous and peritoneum tissues; nematodes (*Anisakis* spp.), trematodes (*Pholeter* sp.), and taeniform cestodes in the stomachs and intestine, and trematodes (*Nasitrema* sp.) and nematodes (*Crassicauda* sp.) within both pterygoid sinuses; bilateral serous-suppurative and proliferative arthritis at the scapula-humeral junction; and generalized lymphadenomegalia. Within the brain; the leptomeninges were enlarged and fibrotic at the cortex area.

Histologically, the lesions were consistent with findings from the gross examination. Other histopathologic findings were multifocal nonsuppurative hepatitis, adrenalitis, and bronchointerstitial pneumonia. In lung, a multifocal suppurative bronchitis associated with larval and adult nematode parasites (morphologically identified as *Halocercus* spp. and *Stenurus* spp.) were observed. Alveolar septa were expanded by macrophages and lymphocytes admixed with scattered karyorrhectic debris. Alveoli were often lined by hyperplastic type II pneumocytes and filled with large numbers of multinucleated syncytial cells.

The immunohistochemical study demonstrated morbilliviral antigen in bronchiolar epithelium, type 2 pneumocytes, and multinucleate (syncytial) cells from lung (Figure 1).

The syncytia also were found within the lymph nodes, pancreas, spleen, kidney, and intestine, and they were characterized by a moderate amount of eosinophilic cytoplasm and 2 to >20 nuclei, often containing weak stained eosinophilic inclusion bodies. Lymphocytolysis was remarkable within the spleen and multiple lymph nodes and were characterized by loss of cellular detail and accumulation of karyorrhectic and eosinophilic cellular debris. Syncytia from lymph nodes, spleen, pancreas, and kidney also were immunostained against CDV antibody. Severe nonsuppurative meningitis (with >20 layers of lymphohistiocytic cells), perineuritis, and encephalomyelitis were found within the nervous system. Multiple microhemorrhages were a common associated lesion. The brain showed severe inflammatory changes, but only a few neurons were immunopositive, and they were limited to some lymphohistiocytic cells surrounding vessels, glial, and endothelial cells. The epithelial tropism of the virus was demonstrated immunohistochemically within the lung, intestine, kidney, and pancreatic duct epithelium.

Molecular detection of cetacean morbillivirus was performed by a 1-step reverse transcription PCR of a 426-bp conserved region of the *P* gene, as described (*10*). All the samples tested from this dolphin were reverse transcription PCR positive for morbillivirus. Sequences were the same in all positive samples from the animal.

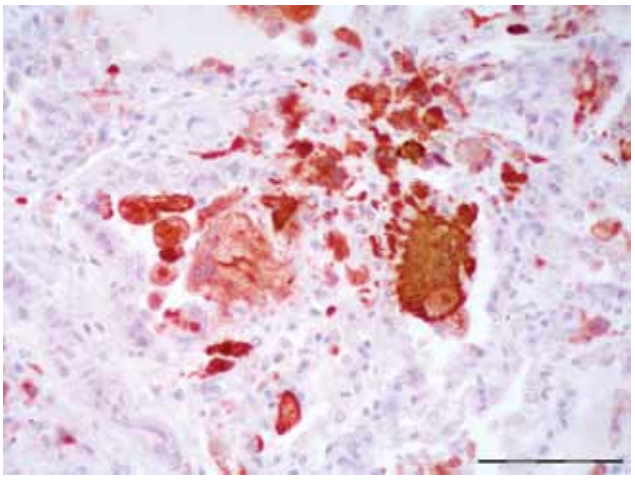

Figure 1. Lung from necropsy of a bottlenose dolphin (*Tursiops truncatus*) that had fatal systemic morbillivirus infection, Canary Islands, Spain, 2005. Positive intracytoplasmic and intranuclear immunoperoxidase staining of morbilliviral antigen (red) within hyperplastic type II pneumocytes, macrophages and multinucleated syncytial cells. Avidin-biotin-peroxidase with Harris hematoxylin counterstain. Scale bar indicates100 μm.

We conducted a BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) search to compare sequenced products with sequences described in GenBank for morbillivirus. The common sequence of 411-nt fragment of the *P* gene showed a 99% homology with those sequences obtained in 2007 and 2011 from striped dolphins stranded along the coastline of the Mediterranean Sea.

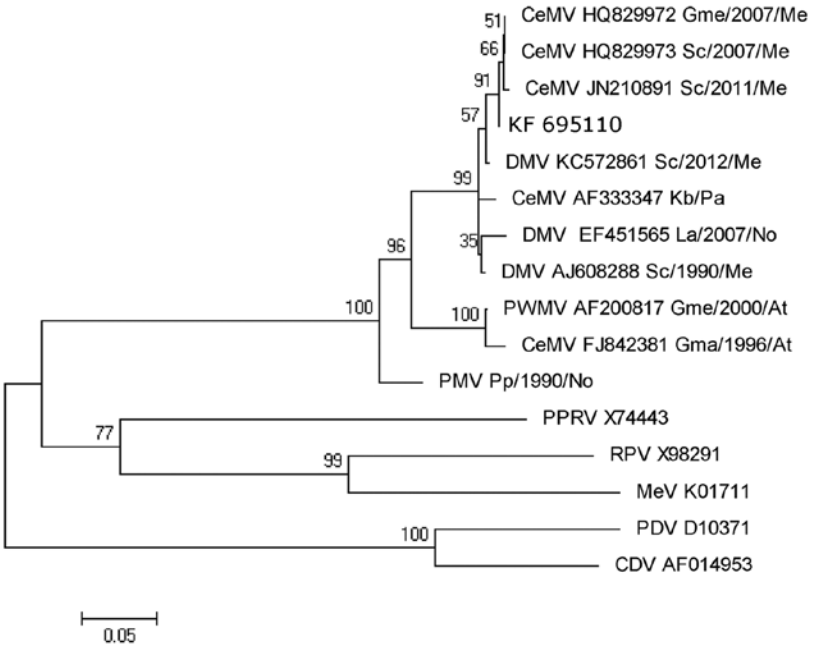

Figure 2. Phylogram of morbillivirus phosphoprotein gene sequences. MEGA 5.0 software (www.megasoftware.net) was used to construct the maximum-likelihood phylogenetic trees. A Tamura-Nei substitution model and a bootstrap resampling (1,000 replicates) were used to assess the reliability of the trees. Bootstrapping values are indicated as percentages next to bifurcations. The new isolate from this study has the GenBank accession no. KF695110. Sequence names include the virus name (CeMV, cetacean morbillivirus; DMV, dolphin morbillivirus; PWMV, pilot whale morbillivirus; PMV, porpoise morbillivirus; PPRV, peste-des-petits-ruminants virus; RPV, rinderpest virus; MeV, measles virus; PDV, phocine distemper virus; CDV, canine distemper virus; GeneBank accession numbers (when available); cetaceans species (Gme, *Globicephala melas*; Sc, *Stenella coeruleoalba*; Kb, *Kogia breviceps*; La, *Lagenorhynchus albirostris*; Gma, *Globicephala macrorhynchus*; Pp, *Phocoena phocoena*; Tt, *Tursiops truncatus);* and the year and the geographic area of the stranding (Me, Mediterranean Sea; Pa, Pacific Ocean; No, North Sea; At, Atlantic Ocean. Scale bar indicates nucleotide substitutions per site.

We used MEGA 5.0 software (www.megasoftware.net) to construct maximum-likelihood phylogenetic trees. A bootstrap resampling (1,000 replicates) was used to assess the reliability of the trees (Figure 2).

Conclusions

We describe a systemic morbillivirus infection in a bottlenose dolphin in the Canary Islands. A previous morbillivirus infection had been described in a bottlenose dolphin found dead along the Atlantic coast of Mauritania in 1998; the virus was exclusively detected by hybridization in the stomach tissue sample (*11*).

Other cases that illustrate the systemic and neurologic features of the disease have been described in bottlenose dolphins in the Mediterranean Sea (*12–14*), and the first reported case of a fatal morbillivirus infection in cetaceans within the Southern Hemisphere (southwestern Pacific Ocean) has been described (*15*). No sequences from these studies were provided to GenBank, although the phylogenetic analyses showed high homology with other DMV. The *P* gene fragment of the virus obtained from the current study is molecularly almost identical to that reported in striped dolphins in the Mediterranean Sea during the last 5 years. This fact supports the hypothesis that transmission occurs between species, as it was reported in the 2006–2007 Mediterranean epizootic between pilot whales (*Globicephala melas* and *G. macrorhynchus*) and striped dolphins (*3*), and demonstrates that dolphin populations of the Mediterranean Sea and the Atlantic Ocean are in contact through the Gibraltar Straits. This particular point is critical to better understand the epidemiology and transmission of morbilliviruses between cetaceans and could be essential to clarifying the infection source of the die-off of bottlenose dolphins alson the East Coast of the United States (*9*).

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Dr Sierra is a researcher at the Institute for Animal Health at the University of Las Palmas de Gran Canaria. Her areas of expertise include veterinary pathology (including whale and dolphin pathology) and pathogenesis of animal infectious diseases, and her research interests include muscular pathologies on whales and dolphins.

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Address for correspondence: Eva Sierra, Institute for Animal Health, Veterinary School, University of Las Palmas de Gran Canaria, Canary Islands, Spain; email: esierra@becarios.ulpgc.es

Co-circulation of West Nile Virus Variants, Arizona, USA, 2010

Jessica A. Plante,[1] Kristen L. Burkhalter,[1] Brian R. Mann, Marvin S. Godsey, Jr., John-Paul Mutebi, and David W. C. Beasley

Molecular analysis of West Nile virus (WNV) isolates obtained during a 2010 outbreak in Maricopa County, Arizona, USA, demonstrated co-circulation of 3 distinct genetic variants, including strains with novel envelope protein mutations. These results highlight the continuing evolution of WNV in North America and the current complexity of WNV dispersal and transmission.

West Nile virus (WNV) emerged in the Americas in 1999 after an outbreak of neuroinvasive disease in humans, birds, and horses in New York, New York. The virus spread rapidly across North America and was detected in Arizona in 2003. In 2004, Arizona experienced a large outbreak (214 neuroinvasive cases and 16 deaths, second only to California in that year), followed by ≈50–60 neuroinvasive cases per year during 2005–2008.

An outbreak in 2010 resulted in 107 neuroinvasive cases and 15 deaths, the largest number of cases for a state that year. WNV activity in Maricopa County, which includes the city of Phoenix and surrounding municipalities, where numerous human cases were reported in the town of Gilbert, was investigated by a team from the Centers for Disease Control and Prevention (Fort Collins, CO, USA) working with local and state public health officials. Epidemiologic and entomologic findings from those investigations have been reported (*1*,*2*) We describe the molecular and phenotypic characterization of WNV isolates obtained from that outbreak.

The Study

As part of the Centers for Disease Control and Prevention investigation, Vero cell culture isolates of WNV were obtained from pools of *Culex quinquefasciatus* (Say) mosquitoes collected during August 1–9, 2010, at multiple sites in the study areas (Figure 1). Nucleotide sequences (GenBank accession nos. KF704145–KF704159) for the envelope (E) protein–coding regions were determined for 15 strains and subjected to phylogenetic analysis. The AZ10 E gene sequences were distributed in 3 robust monophyletic clusters (designated A, B, C; posterior probabilities 0.99) as determined by using applied relaxed clock Bayesian coalescent analysis (Figure 2, panel A).

Isolates from all 3 clusters were detected from the Gilbert study sites. All AZ10 isolates encoded the E-159 Val→Ala mutation that is characteristic of genotypes described since 2002 (*4*). The 6 strains in cluster A, which were only obtained from pools collected in Gilbert, also encoded a conservative Leu→Ile mutation at E-312, a surface exposed residue in the putative receptor binding domain III (EIII) known to be a variable site in multiple WNV genetic lineages, including strains of lineage 2 currently circulating in Europe (*5*–*9*). The 2 strains in cluster B, collected in Glendale and Gilbert, Arizona, encoded a conservative Ser→Thr mutation at E-275.

To further characterize the phylogenetic relationships of these AZ10 isolates, 1 strain from each cluster was selected for full-length genomic sequencing and comparison of the encoded open reading frames to 486 additional genomic sequences from North America available in GenBank. This analysis also supported the concurrent circulation of 3 distinct variants in Gilbert and the surrounding areas of Maricopa County during the 2010 outbreak (Figure 2, panel B). Strain AZ10-581 (cluster A) grouped with the recently described SW/WN03 genotype (*10*), and was most closely related to a South Dakota 2005 strain and 2 other strains that each encoded the E-L312I mutation. Strain AZ10-91 (cluster B) grouped with 2004–2005 Arizona and New Mexico isolates also belonging to a clade of the SW/WN03 genotype. Other SW/WN03 genotype viruses did not encode the E-275 mutation in AZ10-91 and AZ10-372. Strain AZ10-892 grouped with other recently described Arizona 2010/2011 isolates (*4*) and a New York 2004 strain belonging to the dominant NA/WN02 genotype, confirming persistence or reintroduction of that genotype in the southwestern United States (*4*). Nucleotide divergence from NY99 ranged from 0.58% to 0.66% for the AZ10 strains and divergence between the 3 clusters was up to ≈1.2% (Table 1).

The presence of the E-312 coding mutation was of particular interest. Most sequences for lineage 1 WNV strains encode Leu at E-312, whereas lineage 2 strains encode Val or Ala. E-312 lies in an exposed loop of EIII, where it may contribute to the antigenic and/or putative receptor binding activities of the domain (*6*). To assess the effects of the Leu→Ile mutation and tolerance for alternative amino acid

Author affiliations: University of Texas Medical Branch, Galveston, Texas, USA (J.A. Plante, B.R. Mann, D.W.C. Beasley); and Centers for Disease Control and Prevention, Fort Collins, Colorado, USA. (K.L. Burkhalter, M.S. Godsey, Jr., J.P. Mutebi)

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[1]These authors contributed equally to this article.

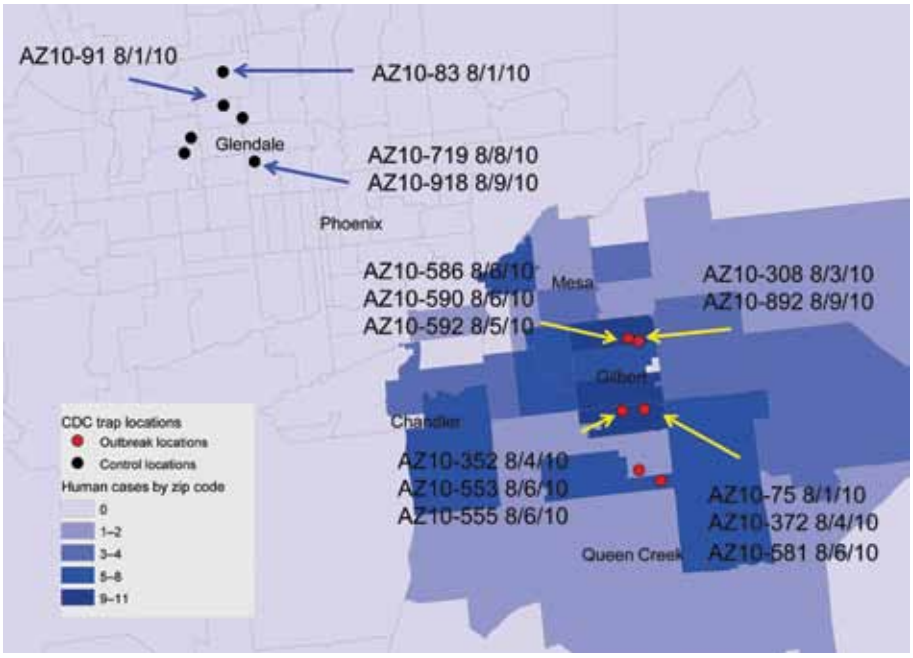

Figure 1. Distribution of mosquito sampling sites in Maricopa County, Arizona, USA, during the 2010 West Nile virus (WNV) outbreak investigation and collection dates/locations of pools yielding indicated WNV isolates used for molecular and/or phenotypic analysis. Gray lines indicate individual zip code boundaries. CDC, Centers for Disease Control and Prevention.

substitutions at this site, we engineered 5 E-312 mutants by using an NY99 infectious clone (NY99ic) encoding alternative amino acids that are each only a single nucleotide substitution away from the wild-type Leu codon (CUU) (Table 2). (Although Phe also requires only a single nucleotide change from the Leu codon, it occurs naturally in some lineage 1 and 2 WNV strains and was not included in this analysis.) Mutagenesis, in vitro ligation, and transcription of genome equivalent RNA and virus recovery were performed as described (*11*). All mutant viruses were readily recovered from transfected Vero cells and grew to peak titers comparable to the parental NY99ic virus, and the introduced mutations were stable through 3 additional Vero cell passages.

Virulence of AZ10-75 and AZ10-581 (cluster A), AZ10-91 (cluster B), AZ10–892 (cluster C), and the recovered NY99ic E-312 mutants was compared with wild-type NY99ic after intraperitoneal inoculation of 3- to 4-week-old female Swiss Webster mice (Table 2) as described (*11*). The AZ10 strains and all E-312 mutants had 50% lethal doses (LD_{50}s) and average survival times comparable with that of NY99ic, with the exception of the L312P mutant, which was markedly attenuated (630 PFU/LD_{50} vs. 0.3 PFU/LD_{50}, and prolonged survival time). Antigenic characteristics of viruses encoding L312I mutations were also compared by assessing their neutralization by monoclonal antibodies 7H2 and 5H10 and a polyclonal rabbit antiserum against the EIII region as described (*5*). AZ10-75, AZ10-581 and all E-312 variants were effectively neutralized by the monoclonal antibodies and antiserum (Table 2).

Conclusions

Detection of a Leu→Ile mutation at residue 312 in EIII and its apparent persistence since first detection in the 2005 South Dakota isolate were major findings given the variable nature of this residue in other WNV lineages and the presumed importance of EIII in antigenicity and receptor binding activity of E protein. Although other parameters that could contribute to the selection of E-312 variants in nature remain to be explored, analysis of engineered E-312 mutants suggested that most nonsynonymous single nucleotide mutations at this site, including the Leu→Ile substitution in some AZ10 isolates, have no major effect on virulence of NY99-derived WNV in mice and were not associated with major changes in antigenicity.

The 2010 epidemic of WNV disease in the Maricopa County area was associated with co-circulation of 3 distinct WNV variants. The high mouse virulence of all strains tested suggests that signature nucleotide and amino acid changes associated with the different genotypes involved (*4,10*) were probably not linked to major changes in virulence for mammalian hosts, and that all 3 variants might have contributed to human disease in the 2010 outbreak. Some nonstructural protein mutations have been shown to influence virulence in avian hosts (*12*), and that phenotype remains to be determined for these strains or other recently identified WNV variants.

Detection of multiple sequence variants has been associated with outbreaks in the United States in as early as 2002 (*13*), but co-circulation of variants in relatively narrow spatial and temporal contexts, such as that observed in

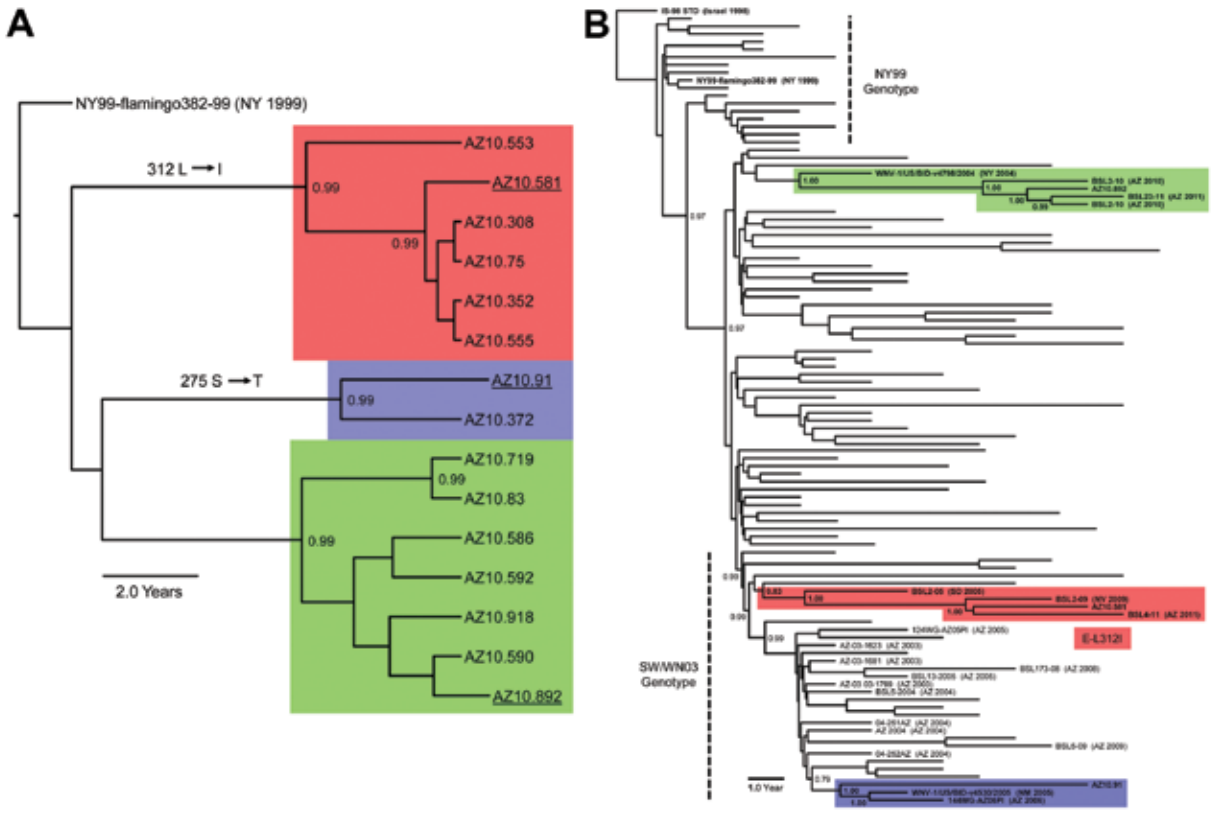

Figure 2. A) Bayesian phylogenetic tree of envelopes genes of all described Arizona, USA, 2010 isolates of West Nile virus (WNV) (n = 15). Isolates grouped in 3 distinct monophyletic clusters designated A (red), B (blue), and C (green). B) Bayesian phylogenetic tree of full-length encoded open reading frame for 3 Arizona, USA, 2010 isolates: AZ10.581 (red), AZ10.892 (green), AZ10.91 (blue), and 100 representative North American WNV isolates. All applied relaxed clock Bayesian methods used the generalized time reversible + invariant sites + Γ_4 substitution model with a lognormal molecular clock and triplicate 50 million state runs produced in BEAST v1.6.2 (*3*). Inferred phylogenetic trees were edited in FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Consistent phylogenetic topologies with additional neighbor-joining and maximum-likelihood methods further validated these inferred relationships. Posterior probabilities ≥0.90 are indicated for highlighted nodes. Scale bars indicate divergence time in years.

Maricopa County, has been a feature of recent investigations, including WNV transmission in El Paso, Texas, and Ciudad Juarez, Mexico, during 2010 (*14*) and during the 2012 outbreak in Dallas, Texas (*15*). These findings highlight the current complexity and dynamic nature of WNV transmission in the United States and suggest that co-circulation of multiple variants, with continued introduction or reintroduction of variants into disease-endemic areas, will be a major feature of future outbreaks.

Acknowledgments

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Table 1. Nucleotide divergence for open reading frame sequences between representative West Nile virus strains and other closely related strains from North America, Arizona, USA, 2010*

Strain	Nucleotide divergence (%) from			
	NY99	Cluster A	Cluster B	Cluster C
AZ10–581	0.58	0.25–0.52	0.66–0.85	0.61–1.10
AZ10–91	0.66	0.74–1.04	0.49–0.52	0.69–1.16
AZ10–892	0.65	0.84–1.18	0.92–1.04	0.27–0.62

*Strains used for analysis in each cluster are those color coded with representative AZ10 strains and shown in Figure 2, panel B. For cluster A: BSL2–2005 (SD 2005; GenBank accession no. DQ666452), BSL2–09 (NV 2009, JF957175), BSL4–11 (AZ 2011, JQ700438); cluster B: v4530 (NM 2005, HM756677), 144WG-AZ06PI (AZ 2006, GQ507482); cluster C: v4798 (NY 2004, HM756671), BSL3–10 (AZ 2010, JF957186), BSL2–10 (AZ 2010 JF957185), BSL23–11 (AZ 2011, JQ700440).

Table 2. Mouse virulence and antigenic characteristics of selected isolates of West Nile virus and NY99ic-derived E-312 variants, Arizona, USA, 2010*

Strain/variant	Mouse neuroinvasiveness		Neutralization indices ± SD		
	ip LD_{50}, PFU	AST ± SD (days)	7H2	5H10	α-EIII
NY99ic	0.3	8.7 ± 1.8	1.5 ± 0.2	1.5 ± 0.2	2.4 ± 0.3
AZ10–75	0.5	8.7 ± 2.2	1.3 ± 0.5	1.3 ± 0.3	2.1 ± 0.3
AZ10–581	0.8	9.8 ± 2.4	1.2 ± 0.2	1.3 ± 0.1	2.5 ± 0.2
AZ10–91	0.5	7.8 ± 0.9	ND	ND	ND
AZ10–892	0.3	8.6 ± 2.0	ND	ND	ND
NY99–312F	1.3	9.8 ± 1.9	1.5 ± 0.4	1.5 ± 0.4	2.5 ± 0.4
NY99–312H	0.8	8.5 ± 1.9	2.0 ± 0.1	1.7 ± 0.3	2.9 ± 0.0
NY99–312I	0.3	8.5 ± 1.4	1.8 ± 0.4	1.4 ± 0.3	2.5 ± 0.4
NY99–312P	630	**13.0 ± 0.0**	2.2 ± 0.1	2.0 ± 0.1	2.6 ± 0.2
NY99–312R	2.0	8.5 ± 2.2	1.8 ± 0.2	1.7 ± 0.1	2.7 ± 0.0

*Average survival time (AST) for each strain/variant was determined on the basis of animals in all dose groups that did not survive. Value significantly different ($p<0.05$ by Student *t*-test) from NY99ic is indicated in **boldface**. ip, intraperitoneal; LD_{50}, 50% lethal dose; ND, not determined.

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Ms Plante is a doctoral student at the University of Texas Medical Branch, Galveston, TX. Her research interests include molecular biology of flaviviruses.

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Address for correspondence: David W. C. Beasley, Department of Microbiology and Immunology, University of Texas Medical Branch, 301 University Blvd, Galveston, TX 77555-0609, USA; email: d.beasley@utmb.edu

Replicative Capacity of MERS Coronavirus in Livestock Cell Lines

Isabella Eckerle, Victor M. Corman, Marcel A. Müller, Matthias Lenk, Rainer G. Ulrich, and Christian Drosten

Replicative capacity of Middle East respiratory syndrome coronavirus (MERS-CoV) was assessed in cell lines derived from livestock and peridomestic small mammals on the Arabian Peninsula. Only cell lines originating from goats and camels showed efficient replication of MERS-CoV. These results provide direction in the search for the intermediate host of MERS-CoV.

Coronaviruses (CoV) in the genera *Alphacoronavirus* and *Betacoronavirus* (order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*) infect a broad range of mammals, including humans (*1*). The human CoVs (HCoVs) HCoV-HKU1, HCoV-229E, HCoV-NL63, and HCoV-OC43 typically cause mild to moderate respiratory tract infection; however, the disease course can be more severe in a minority of patients. In 2002–2003, an epidemic of severe lower respiratory tract infection with a case-fatality rate of ≈10% was caused by severe acute respiratory syndrome (SARS)–CoV (*2*). In 2012, another CoV associated with severe respiratory disease emerged on the Arabian Peninsula and was termed Middle East respiratory syndrome (MERS)–CoV (*3*).

Both SARS-CoV and MERS-CoV are zoonotic viruses, and their presumed origin is in bats. SARS-related CoVs were identified in *Rhinolophus* spp. bats in China and Europe (*4*,*5*), and MERS-related CoVs were found in *Pipistrellus* bats in Europe and in *Neoromicia* bats in South Africa (*6*,*7*). As with SARS-CoV, it is expected that MERS-CoV might be transmitted to humans by an intermediate animal host, and neutralizing antibodies against MERS-CoV have been found in Arabian camels originating from Oman, Spain, and Egypt (*8*,*9*).

We investigated replication of MERS-CoV in cell lines of the most abundant mammalian livestock species and representative peridomestic small mammals on the Arabian Peninsula. To estimate MERS-CoV permissiveness of cell cultures derived from these animals, we compared MERS-CoV replication and infectious virus production with that in bat- and primate-derived cells known to be permissive for MERS-CoV. The MERS-CoV receptor dipeptidyl peptidase 4 (DPP-4) is expressed in epithelial cells of the lung and kidney, and patients with MERS-CoV consistently show severe involvement of both organs; thus, we focused on lung and kidney cells in potential animal hosts (*10*,*11*).

The Study

Using enhanced respiratory personal protection equipment in a Biosafety Level 3 facility, we cultivated, in parallel, cell lines from goats, sheep, cattle, camelids (dromedary and alpaca), rodents, insectivores, bats, and human and nonhuman primates (Table). Cells were checked to ensure the absence of mycoplasma contamination and genotyped for their species of origin by sequencing of the mitochondrial cytochrome c subunit oxidase I gene (*12*). All cells expressed DPP-4, as determined by immunofluorescence staining (Figure 1) and Western blot analysis (data not shown). Because several of the ungulate cell lines had not previously been used for viral infection experiments, we determined permissiveness of all cell lines for Rift Valley fever virus (RVFV) clone 13, a virus mutant known to be attenuated yet broadly infectious for ungulate cell lines (*13*). Triplicate infections with multiplicities of infection (MOIs) of 0.5 infectious units/cell resulted in highly consistent levels between cells (maximal variation 3.2-fold) (Figure 2, panel A). In addition, to demonstrate the ability of the cells to support CoV replication, we infected all cell lines in triplicate (MOIs of 0.5) with bovine CoV strain Nebraska. The strain replicated to high levels in all cell lines; replication varied by ≤52.9-fold, which constitutes small relative variations in light of the overall levels of replication (Figure 2, panel B). We conducted MERS-CoV infections under the same conditions and with MOIs of 0.5.

In addition to livestock cell lines, we used rodent, insectivore, bat, and primate cell lines in the experiments (Table). Bat and primate cells known to be permissive for MERS-CoV served as controls. MERS-CoV–inoculated cells were incubated for 1 h and then washed twice before supernatant was harvested (0 h after incubation) (Figure 2, panel C). We quantified virus replication by using the *upE* assay (*14*), a MERS-CoV real-time reverse transcription PCR that screens upstream of the *E* gene. Replication was seen in lung and kidney cell lines derived from goats (*Capra hircus*) and in umbilical cord and kidney cells from camelids (*Camelus dromedarius* and *Llama pacos*) (Figure 2, panel C). Efficient replication (≥9.3 $\log_{10}$ virus RNA

Author affiliations: University of Bonn Medical Centre, Bonn, Germany (I. Eckerle, V.M. Corman, M.A. Müller, C. Drosten); and Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany (M. Lenk, R.G. Ulrich)

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Table. Characteristics of mammalian cell lines used in a study designed to narrow the search for the intermediate mammalian host of Middle East respiratory syndrome coronavirus

Designation	Species of origin	Mammalian order	Organ of origin	Cell type
ZN-R	Goat (*Capra hircus*)*	*Artiodactyla*	Kidney	Primary
ZLu-R	Goat (*C. hircus*)*	*Artiodactyla*	Lung	Primary
LGK-1-R	Alpaca (*Llama pacos*)*	*Artiodactyla*	Kidney	Primary
TT-R.B	Arabian camel (*Camelus dromedarius*)*†	*Artiodactyla*	Umbilical cord	Immortalized
PO	Sheep (*Ovis aries*)*	*Artiodactyla*	Kidney	Immortalized
KN-R	Cattle (*Bos taurus*)*	*Artiodactyla*	Kidney	Primary
KLu-R	Cattle (*B. taurus*)*	*Artiodactyla*	Lung	Primary
MyglaAEC.B	Bank vole (*Myodes glareolus*)*	*Rodentia*	Trachea	Immortalized
Crocsu-Lu	Lesser white-toothed shrew (*Crocidura suaveolens*)*	*Soricomorpha*	Lung	Immortalized
PipNi	Common pipistrelle (*Pipistrellus pipistrellus*)*	*Chiroptera*	Kidney	Immortalized
A549	Human (*Homo sapiens*)	*Primates*	Lung	Immortalized
Vero E6	African green monkey (*Chlorocebus aethiops*)	*Primates*	Kidney	Immortalized

*Species of origin was confirmed by sequencing of the cytochrome c subunit oxidase I gene.
†Parents and the calf phenotypically resembled *C. bactrianus*. The most likely explanation is cross-breeding between the 2 species with mitochondria stemming from a *C. dromedarius* mother in one of the former generations.

genome equivalents/mL of cell culture supernatant) was seen in the goat kidney cells; this replication level was similar to that in Vero E6 cells (interferon-deficient primate kidney cells). Goat lung cells, alpaca kidney cells, and dromedary umbilical cord cells also showed strong replication, but virus did not replicate in sheep, cattle, rodent, or insectivore cells. In addition, expression of the receptor, DPP-4, was confirmed in all cells, including the nonpermissive sheep, cattle, rodent, and insectivore cells.

After following virus growth in all permissive cells for another 20 h, we harvested supernatants and, to confirm the production of infectious virus particles, we titrated the supernatants by using a plaque assay in Vero cells. MERS-CoV replication was seen in all permissive cells except TT-R.B (Figure 2, panel D), and all permissive cells showed cytopathic effects. The highest production of virus particles was in goat lung and kidney cells (1.0×10^7 and 2.7×10^6 PFU/mL, respectively). This level of replication was comparable to that in human lung cells (A549) and Vero E6 (Figure 2, panel D).

Conclusions

Transmission of MERS-CoV between humans is still limited, and the identification of an intermediate animal host could enable the development of public health measures to prevent future spread of the virus among humans. Although MERS-CoV neutralizing antibodies have been detected in camels from Oman, Spain, and Egypt, the virus has not previously been detected in camels (*8,9*). An informed focusing of investigations on a select group of species, such as camels, could benefit epidemiologic investigations. To identify potential intermediate host species of MERS-CoV, we used in vitro testing to determine virus permissiveness in select cell culture models. In general, cell lines cannot depict the full pathogenicity of in vivo infection because infection is influenced by epithelium-specific differentiation of target cells and the presence of immune cells. However, for viruses such as CoVs, whose tropism is believed to be determined mainly by the availability of an appropriate entry receptor (*10*), epithelial cell cultures could indeed constitute valid surrogates of virus

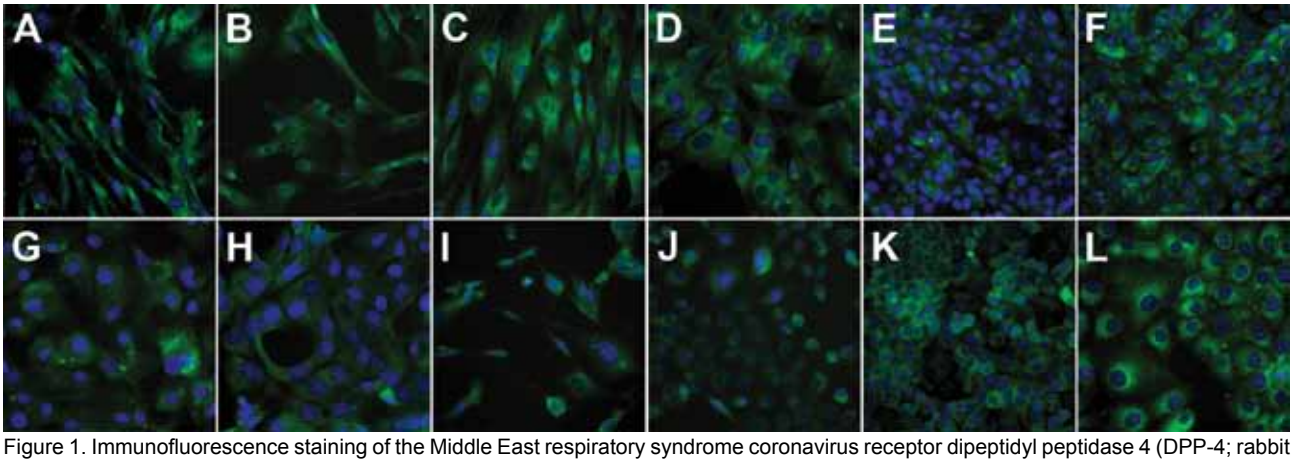

Figure 1. Immunofluorescence staining of the Middle East respiratory syndrome coronavirus receptor dipeptidyl peptidase 4 (DPP-4; rabbit anti-CD26/DPP-4 polyclonal antibody; Bioss Inc., Woburn, MA, USA) in cell lines used to guide the search for the intermediate host of the virus. Counterstaining of nuclei was done by using 4′,6-diamidino-2-phenylindole. Cell lines: A) ZN-R, B) ZLu-R, C) LGK-1-R, D) TT-R.B, E) PO, F) KN-R, G) KLu-R, H) MyglaAEC.B, I) Crocsu-Lu, J) PipNi, K) A549, and L) Vero E6. Magnification 400-fold.

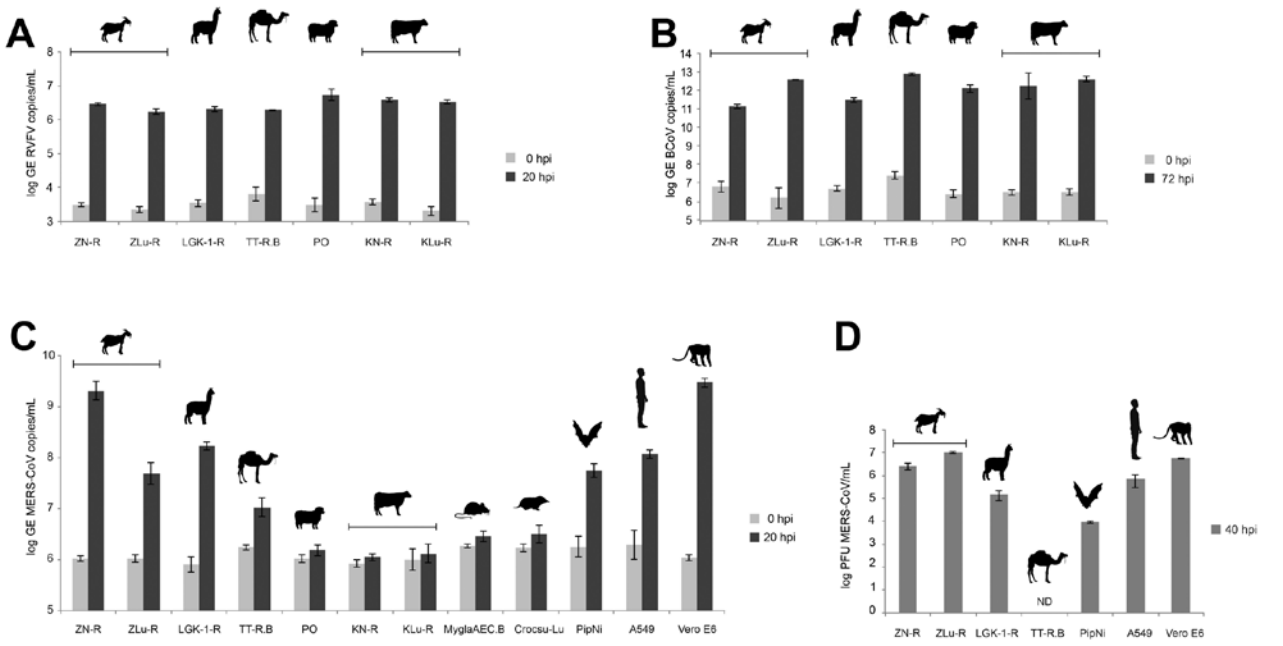

Figure 2. Analysis of the replication of Rift Valley fever virus (RVFV) clone 13 (A), bovine coronavirus (BCoV) (B), and Middle East respiratory syndrome coronavirus (MERS-CoV) (C) and of the production of infectious MERS-CoV particles (D) in cell lines derived from livestock and peridomestic small mammals on the Arabian Peninsula. Cell lines of human, bat, and primate origin were used as controls. Replication levels for each virus used are given as log of the genome equivalents (GEs) (A–C) or as plaque-forming units (PFUs). Vertical bars indicate ranges; horizontal bars indicate cell line origins. Using panel C as a reference, symbols represent (left to right) goat, alpaca, Arabian camel, sheep, cattle, bank vole, shrew, bat, human, and African green monkey. ND, not detected; hpi, hours postincubation.

permissiveness in vivo. With these limitations in mind, our results are in concordance with the findings of MERS-CoV neutralizing antibodies in camels and with information regarding patient contact with animals in reports of 2 human cases of MERS-CoV infection (*11*,*15*). One of the patients owned a farm on which camels and goats were kept. Before onset of his own illness, the patient reported illness in several goats on his farm. The patient did not have direct contact with animals, but he reported having eaten goat meat and having had contact with one of the animal caretakers, who suffered from respiratory disease (*15*). The second patient reported direct contact with a diseased camel shortly before onset of his symptoms (*11*).

In our study, production of infectious virus particles was seen in goat lung and kidney cells and in camelid kidney cells. Excretion patterns indicative of kidney infection should be investigated once further clues to the identity of the MERS-CoV animal reservoir become available. Our preliminary findings suggest that ungulates, such as goats and camels, are a possible intermediate host of MERS-CoV; thus, exposure to urine and feces from these animals might constitute a source of human infection. Moreover, food products derived from these animals (e.g., meat and milk) should be tested for their potential to transmit MERS-CoV. The results of our study suggest that investigations into the MERS-CoV animal reservoir and intermediate host should focus on caprid (e.g., goats) and camelid hosts, and we identified several new cell lines for use in virus isolation studies.

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Dr Eckerle is a virologist at the Institute of Virology in Bonn, Germany. Her primary research interest is characterization of novel and emerging zoonotic viruses.

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Address for correspondence: Christian Drosten, Institute of Virology, University of Bonn Medical Centre, 53105 Bonn, Germany; email: drosten@virology-bonn.de

Investigation of Inhalation Anthrax Case, United States

Jayne Griffith, David Blaney, Sean Shadomy, Mark Lehman, Nicki Pesik, Samantha Tostenson, Lisa Delaney, Rebekah Tiller, Aaron DeVries, Thomas Gomez, Maureen Sullivan, Carina Blackmore, Danielle Stanek, Ruth Lynfield, and the Anthrax Investigation Team[1]

Inhalation anthrax occurred in a man who vacationed in 4 US states where anthrax is enzootic. Despite an extensive multi-agency investigation, the specific source was not detected, and no additional related human or animal cases were found. Although rare, inhalation anthrax can occur naturally in the United States.

Anthrax is a naturally occurring disease, affecting herbivores that ingest bacterial spores when consuming contaminated vegetation or soil (*1*). *Bacillus anthracis* spores are highly resistant to weather extremes and can remain viable in soil and contaminated animal products, such as bones or hides, for many years (*2*,*3*). Heavy rains or flooding can bring spores to the surface or concentrate organic material and spores in low-lying areas. As surface water evaporates, spores may attach to growing vegetation or become concentrated in soil around roots (*3*,*4*).

Humans can become infected from exposure to infected animals or contaminated animal products (including meat, hides, and hair) or to contaminated dust associated with these products (*1*). Historically, inhalation anthrax was considered an occupational hazard for those working in wool and goat hair mills and tanneries (*5*). Recently, inhalation anthrax cases have resulted from exposure to African-style drums made of animal hides and from bioterrorist attacks (*6*,*7*). State or local health departments, the Centers for Disease Control and Prevention (CDC), and the Federal Bureau of Investigation undertake epidemiologic and criminal investigations whenever a clinical isolate is confirmed as *B. anthracis*.

Author affiliations: Minnesota Department of Health, St. Paul, Minnesota, USA (J. Griffith, S. Tostenson, A. DeVries, M. Sullivan, R. Lynfield); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (D. Blaney, S. Shadomy, M. Lehman, N. Pesik, L. Delaney, R. Tiller); US Department of Agriculture, Atlanta (T. Gomez); and Florida Department of Health, Tallahassee, Florida, USA (C. Blackmore, D. Stanek)

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The Study

On August 5, 2011, a community hospital laboratory contacted the Minnesota Department of Health to submit for identification a *Bacillus* sp. blood isolate obtained from a patient hospitalized with pneumonia. The identification of *B. anthracis* was confirmed by nucleic acid amplification and gamma phage. CDC typed the isolate as GT59 using multiple-locus variable-number tandem repeat analysis specific for 8 loci (*8*). CDC also sequenced the strain and performed whole-genome single nucleotide polymorphism analysis. The isolate was related to strains with genotypes generally associated with imported animal products and most closely related to a strain obtained from a 1965 investigation of a case of cutaneous anthrax in a worker at a New Jersey gelatin factory, which used bone imported from India (*9*,*10*).

The patient, a 61-year-old Florida resident, had begun a 3-week trip with his wife on July 11, 2011. They drove through North Dakota, Montana, Wyoming, and South Dakota, where animal anthrax is sporadic or enzootic (Figure). While traveling, they walked in national parks, collected loose rocks, and purchased elk antlers. On July 29, they drove through herds of bison and burros, frequently stopping while animals surrounded their vehicle. The man was hospitalized on August 2, following onset of illness while he was en route to Minnesota. His condition was treated with antimicrobial drugs, according to published recommendations, supplemented with anthrax immune globulin, and he fully recovered (*11*). He and his wife were interviewed to identify potential sources of exposure. The Federal Bureau of Investigation found no concern for bioterrorism.

The couple had no contact with dead animals, but reported dusty conditions while driving through the herds. The patient had not traveled abroad during the past year or been exposed to tanneries, wool or goat hair mills, bone meal, African drums, or illicit drugs. He crafted metal and stone jewelry and knives with elk-antler handles in a home workshop. One month before illness onset, he had constructed fishing flies using hair from a healthy elk he hunted in Kentucky 8 months previously.

Because his wife may have experienced the same exposure, postexposure prophylaxis was provided, including a 60-day course of oral antimicrobial drugs and a 3-dose series of Anthrax Vaccine Adsorbed (Emergent Biosolutions, Lansing, MI, USA), under an investigational new drug protocol. Symptoms of anthrax did not develop; however, she was unable to provide blood for serologic testing before starting postexposure prophylaxis.

[1]Members of the Anthrax Investigation Team are listed at the end of this article.

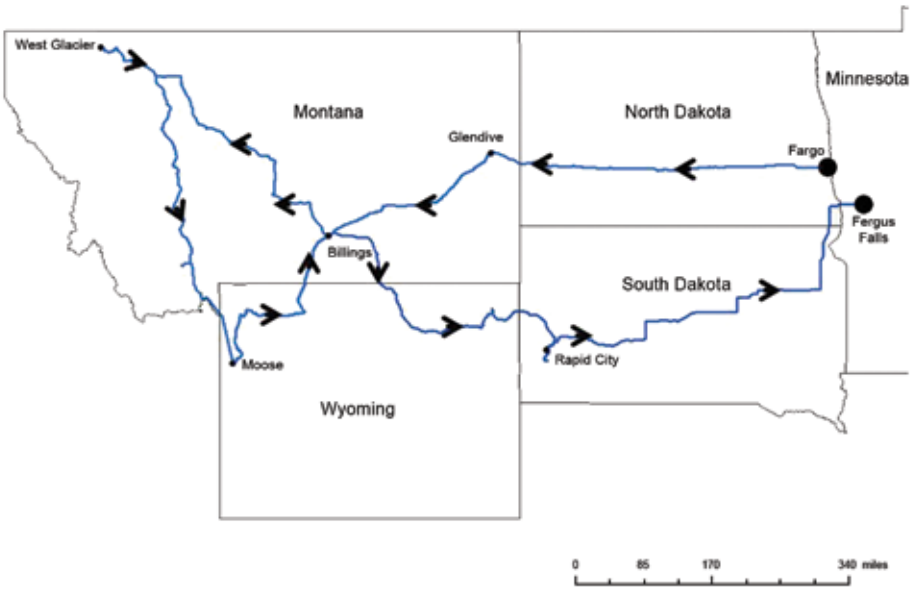

Figure. Route traveled by anthrax case-patient in the United States, July 11, 2011 to August 2, 2011.

Environmental sampling of the entire trip route was not feasible because the travel route involved several thousand miles over 3 weeks, specific suspected exposure locations were lacking, and recovering *B. anthracis* from environmental samples (*12*), particularly soil (*13*), gives variable results and is inefficient. To identify an exposure source and focus the environmental investigation, investigators obtained targeted samples from the vehicle and personal items where spores were likely to be found. From the patient's vehicle and contents, 47 environmental samples were collected, and 18 environmental samples were obtained from his home workshop and garage (Table), following procedures of the National Institute for Occupational Safety and Health and the Environmental Protection Agency (*14*). All samples were processed and cultured by using CDC's Laboratory Response Network culture and PCR methods at the Minnesota Department of Health and the Florida Department of Health. *B. anthracis* was not detected in any of the 65 samples.

Multiple agencies, including CDC, state public and animal health departments of Minnesota, North Dakota, Montana, Wyoming, South Dakota, and Florida, and other federal and state partners, conducted enhanced retrospective surveillance to identify other possible human or animal anthrax cases during June 1–August 31, 2011. Infection preventionists, medical examiners, and coroners were asked if they were aware of cases of unexplained death or fulminant illness potentially caused by anthrax. The US Department of Agriculture Veterinary Services and Wildlife Services/Animal and Plant Health Inspection Service, the Southeastern Cooperative Wildlife Disease Study, the US Geological Survey National Wildlife Health Center, the National Park Service, and state wildlife and veterinary agencies were asked to report unexplained animal die-offs and animal deaths consistent with anthrax. In addition, ≈450 Laboratory Response Network and 7 National Animal Health Laboratory Network laboratories in the participating states, the National Veterinary Services Laboratories, and other veterinary and wildlife laboratories were asked to review records for non-hemolytic *Bacillus* spp. isolates identified during the surveillance period.

No associated anthrax cases or isolates were found. A single bovine case was reported in Aurora County, South Dakota, in August 2011; however, the isolate was determined to be of an unrelated genotype (GT3). No other anthrax-associated die-offs in wildlife or domestic animals were identified, and no other suspected or confirmed human cases of anthrax were identified or reported during this period.

Conclusions

We did not find a specific exposure associated with anthrax infection for this case-patient and no other human or related animal cases. The clinical isolate genotype and sequence closely matched several previous environmental sample isolates from North America, however, no epidemiologic links were identified. The case-patient was exposed to airborne dust while traveling through areas where anthrax was enzootic; however, testing of vehicle air filters in which the dust was concentrated was negative for *B. anthracis*. Nonetheless, the patient may have been exposed through contact with an unidentified contaminated item. This investigation was limited by the poor sensitivity expected for soil sampling and lack of data to guide additional focused environmental sampling, precluding widespread random sample collection and testing along the route traveled.

Table 1. Samples tested for presence of *Bacillus anthracis* during investigation of inhalation anthrax case, United States, 2011*

Sample type	Description (no. specimens)	PCR result	Culture result
Vacuum sock†	Vehicle floorboards (3)	ND	ND
Vacuum sock	Vehicle seats (2)	ND	ND
Macrofoam swab‡	Vehicle instrument panel (2)	ND	ND
Macrofoam swab	Vehicle arm rests (2)	ND	ND
Macrofoam swab	Vehicle dashboard (2)	ND	ND
Macrofoam swab	Vehicle steering wheel	ND	ND
	Vehicle exterior		
Macrofoam swab	Vehicle windshield	ND	ND
Macrofoam swab	Vehicle driver's window seam	ND	ND
Macrofoam swab	Vehicle hub caps (4)	ND	ND
Macrofoam swab	Vehicle intake grill	ND	ND
Bulk	Scraping of debris on Vehicle wheel hubs (5)	ND	ND
Bulk	Vehicle cabin filter (2)	ND	ND
Vacuum sock	Vehicle cabin filter	ND	ND
Vacuum sock	Vehicle engine filter	ND	ND
	Personal items		
Macrofoam swab	Boots (2)	ND	ND
Bulk	Boots (2) (dirt removed from soles)	ND	ND
Macrofoam swab	Antlers (4)	ND	ND
Macrofoam swab	Fishing rod/lures (2)	ND	ND
Bulk	Rinsate of fishing lures	ND	ND
Bulk	Rinsate of rocks (6)	ND	ND
Macrofoam swab	Rock sifter	ND	ND
	Residence		
Macrofoam swab	Garage electrical box		ND
Macrofoam swab	Garage workbench surface		ND
Macrofoam swab	Electrical outlets (5)		ND
Macrofoam swab	Home air conditioner unit		ND
Bulk	Air intake filter		ND
Macrofoam swab	Rocks		ND
Macrofoam swab	Fishing gear		ND
Macrofoam swab	Mounted animal heads (2)		ND
Macrofoam swab	Computer modem		ND
Macrofoam swab	Surfaces (2)		ND
Bulk	Vacuum bag contents (2)		ND

*ND, not detected; blank spaces indicate test was not performed.
†www.cdc.gov/niosh/topics/emres/surface-sampling-bacillus-anthracis.html.
‡www.ncbi.nlm.nih.gov/pmc/articles/PMC2730285.

It is unusual for inhalation anthrax case-patients to have no identified exposure source (*5*,*9*,*15*). Inhalation of spore-contaminated soil has been suggested as a possible source of infection for bison in anthrax outbreaks in Canada (*16*). A heavy equipment operator acquired inhalation anthrax during a bison outbreak in Canada, where he dragged carcasses to burial sites and was exposed to airborne dust during operations (*2*).

Chronic pulmonary disease or immunosuppression may increase a person's susceptibility to inhalation anthrax (*9*,*15*). This case-patient had a decades-long history of chemical pneumonitis, and although he reported no respiratory difficulties, perhaps his risk was increased. He also had a history of mild diabetes; diabetes has been observed in other anthrax patients (*9*).

This report highlights the challenges of investigating cases of anthrax when no specific suspected source exists. Anthrax, either naturally-occurring or bioterrorism-related, is a major public health concern, and timely recognition is critical. Clinicians and public health professionals should be cognizant that naturally acquired anthrax can occur in the United States and take appropriate steps to rapidly diagnose and investigate such cases.

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Members of the Anthrax Investigation Team with affiliations: Civil Support Team, Minnesota National Guard, St. Paul, Minnesota, USA (Robb Mattila); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (Cari Beesley, William Bower, J. Bradford Bowzard, Gregory Burr, Leslie A. Dauphin, Mindy Glass Elrod, Chad Dowell, Jay Gee, Marta Guerra, Alex Hoffmaster, Chung Marston, Stephan Monroe, Meredith Morrow, Carol Rubin, David Sue, Rita Traxler, Tracee Treadwell, Katherine Hendricks Walters); Florida Department of Health, Bureau of Laboratories, Tampa, Florida, USA (P. Amuso); Florida Department of Health, Disease Control and Health Protection, St. Petersburg, Florida, USA (Pat Ryder, Ann Schmitz); Hennepin County Medical Center, Minneapolis, Minnesota, USA (Mark Sprenkle); Lake Region Healthcare, Fergus Falls, Minnesota, USA (Dawn Henkes); Minnesota Department of Health, St. Paul (Courtney Demontigny, Heather Fowler,

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Ms. Griffith is the bioterrorism epidemiologist at the Minnesota Department of Health, Minneapolis. She is responsible for bioterrorism disease surveillance and biodetection program responses in the state.

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Address for correspondence: Jayne Griffith, Minnesota Department of Health, 625 Robert St North, PO Box 64975, St. Paul, MN 55164, USA; email: jayne.griffith@state.mn.us

Genetic Characterization of Coronaviruses from Domestic Ferrets, Japan

Yutaka Terada, Shohei Minami, Keita Noguchi, Hassan Y.A.H. Mahmoud, Hiroshi Shimoda, Masami Mochizuki, Yumi Une, and Ken Maeda

We detected ferret coronaviruses in 44 (55.7%) of 79 pet ferrets tested in Japan and classified the viruses into 2 genotypes on the basis of genotype-specific PCR. Our results show that 2 ferret coronaviruses that cause feline infectious peritonitis–like disease and epizootic catarrhal enteritis are enzootic among ferrets in Japan.

An epizootic catarrhal enteritis (ECE) was first recognized in domestic ferrets (*Mustelo putorius furo*) in the United States in 2000 (*1*). The causative agent of ECE was demonstrated to be a novel ferret coronavirus (FRCoV) belonging to the genus *Alphacoronavirus* (*1*,*2*). Ferrets with ECE showed general clinical signs of lethargy, anorexia, and vomiting and had foul-smelling, green mucous–laden diarrhea. A systemic infection of ferrets closely resembling feline infectious peritonitis (FIP) was subsequently reported among ferrets in the United States and Europe. The causative agent was also shown to be an *Alphacoronavirus*, which was named ferret systemic coronavirus (FRSCV) (*3*,*4*); this virus was found to be genetically distinct from those associated with ECE and from 2 viruses assigned to different genotypes (*5*). Other cases of ECE and ferret infectious peritonitis have since been described in the United States and in Europe (*2*–*4*,*6*,*7*). One case of pathology-confirmed FIP-like disease has been described among domestic ferrets in Japan (*8*). The goal of this study was to determine the prevalence of coronavirus among domestic ferrets seen by veterinarians in various parts of Japan.

The Study

Fecal samples were collected during August 2012–July 2013 from 79 ferrets from 10 animal hospitals scattered across 5 prefectures in Japan. Most of the ferrets were brought to veterinarians for clinical signs such as diarrhea, abdominal masses, and hypergammaglobulinemia; some had signs unrelated to coronavirus infection or were asymptomatic (Table 1). The diarrhea tended to be mild, unlike with ECE, and was found in coronavirus-negative and -positive animals.

RNA was extracted from fecal samples by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), and reverse transcription PCR (RT-PCR) was performed by using the QIAGEN OneStep RT-PCR Kit (QIAGEN) using coronavirus consensus primers IN-6 and IN-7, which amplify the open reading frame (ORF) 1b region, encoding RNA-dependent RNA polymerase (RdRp). This primer pair can amplify nucleic acids from many coronaviruses in the subfamily *Coronavirinae* (*9*). Of 79 samples, 33 (41.8%) were positive for coronaviruses by RT-PCR (Table 2). Nucleotide sequences were determined for the amplified fragments and used to construct a phylogenetic tree (Figure 1). The coronaviruses detected in this study belonged to the genus *Alphacoronavirus* but formed a separate species from those of other species. The identities with feline coronavirus, transmissible gastroenteritis virus, porcine respiratory coronavirus, and mink coronavirus were 73.5%–75.9%, 73.5%–76.1%, 73.8%–76.1%, and 80.2%–84.0%, respectively.

On the basis of additional sequence data, a new primer pair was designed: forward FRCoV RdRp-F1 (5′-GTT GGT TGC TGC ACA CAT AG-3′) and reverse FRCoV RdRp-R1 (5′-GGA GAA GTG CTT ACG CAA ATA-3′). Results for RT-PCR using this new primer set showed that 44 (55.7%) of 79 samples were positive for coronavirus, which was a higher number than that obtained by using the published coronavirus consensus primers (55.7% vs. 41.8%) (Table 2). Two samples that had positive results by consensus primers had negative results by the new primers: sample 22 had many mutations in the primer binding site (Figure 1), whereas sample 40 had few mutations.

On the basis of the partial sequences of the spike gene, Wise et al. (*5*) reported that the known ferret coronaviruses could be divided into 2 genotypes: genotype 1, which included the agent of FIP-like disease, and genotype 2, which included the causative agent of ECE. To differentiate between these genotypes in the positive samples from our testing, RT-PCR was carried out by using 2 pairs of genotype-specific primers: forward primer 5′-CTG GTG TTT GTG CAA CAT CTA C-3′ and reverse primer 5′-TCT ATT TGC ACA AAA TCA GAC A-3′ for genotype 1, and forward primer 5′-GGC ATT TGT TTT GAT AAC GTT G-3′ and reverse primer 5′-CTA TTA ATT CGC ACG AAA TCT GC-3′ for genotype 2 (*5*). Among these ferrets, 30 (38.0%) were infected with genotype 1 and 17 (21.5%)

Author affiliations: Yamaguchi University, Yamaguchi, Japan (Y. Terada, S. Minami, K. Noguchi, H.Y.A.H. Mahmoud, H. Shimoda, K. Maeda); Kagoshima University, Kagoshima, Japan (M. Mochizuki); and Azabu University, Kanagawa, Japan (Y. Une)

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Table 1. Detection of FRCoV from ferrets with clinical signs, Japan

Sample type	No. (%) samples			
	Diarrhea, n = 34	Hypergammaglobulinemia, n = 6	Abdominal mass, n = 14	Nonrelated signs/ asymptomatic, n = 33
All FRCoV-positive samples†	25 (73.5)	5 (83.3)	7 (50.0)	17 (51.5)
Genoype I samples‡	17 (50.0)	2 (33.3)	4 (28.6)	10 (30.3)
Genoype II samples§	7 (20.6)	1 (16.7)	4 (28.6)	7 (21.2)

*FRCoV, ferret coronavirus; RT-PCR, reverse transcription PCR.
†RT-PCR was carried out by using FRCoV-specific primers.
‡RT-PCR was carried out by using type 1 FRCoV-specific primers (*5*).
§RT-PCR was carried out by using type 2 FRCoV-specific primers (*5*).

with genotype 2; 8 (10.1%) ferrets were infected with both genotypes of coronaviruses (Figure 2). Samples 27 and 28 were from ferrets that lived in the same house and harbored the same ferret coronavirus but that were born on different farms, indicating that horizontal transmission had occurred. The nucleotide sequences of the amplified genes confirmed that these coronaviruses also fell into genotypes 1 and 2 (Figure 2).

Our results indicate that both genotypes of coronavirus have been spreading within the ferret population in Japan for some time, and some ferrets have been coincidentally infected with both genotypes. Of note, most ferrets that were positive for genotype 1 ferret coronavirus in this study did not show FIP-like disease (Table 1), indicating that infection with genotype 1 ferret coronavirus does not always cause FIP-like disease. Genotype 1 ferret coronavirus has also been detected from asymptomatic ferrets in the Netherlands (*11*).

To further investigate virus transmission routes, oral swab specimens were collected from 14 of the 79 ferrets and examined by RT-PCR using primers FRCoV RdRp-F1 and FRCoV RdRp-R1. Five (35.7%) specimens were positive (data not shown), providing a route leading to infection of susceptible animals. Coronaviruses are known to cause both respiratory and intestinal diseases in various animal species; therefore, ferret coronaviruses should be investigated in respiratory disease.

Conclusions

We established a sensitive RT-PCR method using a new primer pair to detect coronavirus sequences and demonstrated that ferret coronaviruses are widespread among ferrets in Japan. We determined the partial nucleotide sequences of the spike gene of 23 strains and found they were clearly divided into 2 genotypes, 1 and 2 (Figure 2). The reported ferret coronaviruses associated with FIP-like disease, designated as genotype 1 by Wise et al. (*5*), all fell within genotype 1 phylogenetically, whereas all published ECE-causing strains fell within genotype 2. This finding leads to a possible conclusion that FIP-like disease–causing strains (i.e., FRSCVs) are variants of what has been designated genotype 1 ferret coronaviruses. Because we found no relationship between the 2 genotypes of ferret coronavirus and the type of disease (Table 1), we cannot determine whether FIP-like and ECE-like ferret coronaviruses circulate independently as distinct entities or evolve, like feline coronaviruses, from more ubiquitous and less pathogenic enzootic strains. Nonetheless, the addition of these 23 new isolates to the phylogenetic tree of ferret coronaviruses tends to support the latter conclusion. Without extensive animal passage studies, virus isolation, and coronavirus-free ferrets, this theory may be difficult to confirm. However, additional evidence tends to link virulent pathotypes of ferret coronaviruses to specific mutational events. Nucleotide sequences of the 3c-like protein genes of FRSCV, MSU-1 (DDBJ/EMBL-Bank/GenBank accession no. GU338456), MSU-S (GU459059), and WADL (GU459058), showed that 2, MSU-1 and WADL, possessed a truncated 3c-like protein gene (*5*), similar to that described for FIP viruses of cats (*12–14*). FIP-causing viruses of cats also contain a second mutation in the spike gene (*15*), which was not investigated in our study. The existence of 2 major genotypes of Japanese ferret coronaviruses is also reminiscent of the serotype I and II feline coronaviruses. Without ferret coronaviruses that can be grown in cell culture, however, such serologic differentiation will be difficult.

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We express our appreciation to the many veterinarians who collected and donated clinical samples.

Table 2. Comparison of results for detection of FRCoV in ferret fecal samples by RT-PCR using coronavirus consensus and FRCoV-specific primers, Japan

Coronavirus consensus primers	FRCoV-specific primers		Total no. (%)
	No. positive samples	No. negative samples	
No. positive samples	31	2	33 (41.8)
No. negative samples	13	33	46 (58.2)
Total no. (%)	44 (55.7)	35 (44.3)	79

*FRCov, ferret coronavirus; RT-PCR, reverse transcription PCR.

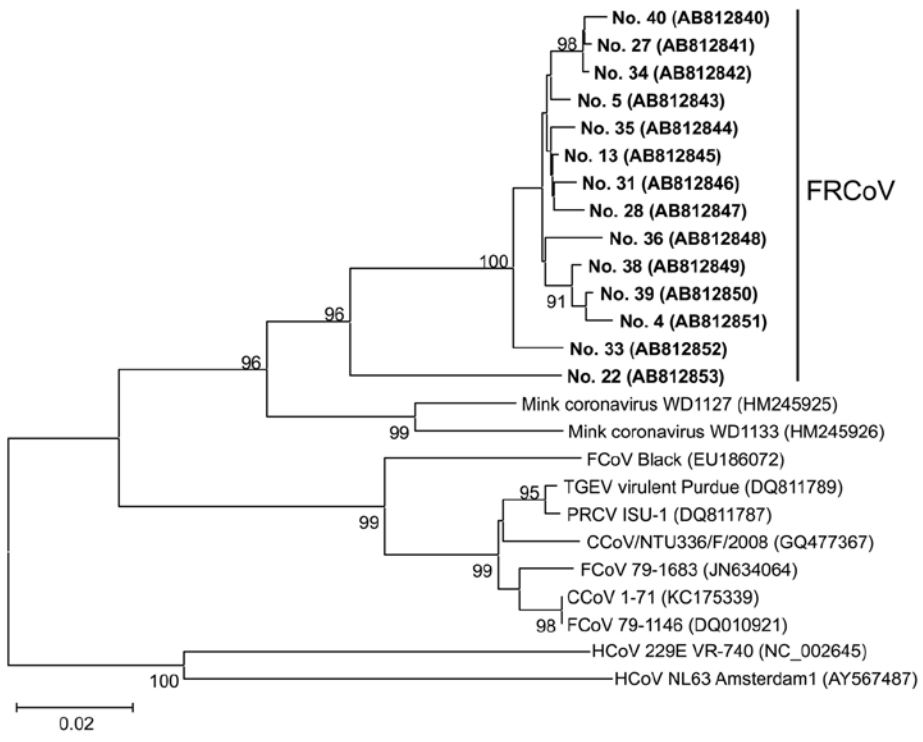

Figure 1. Phylogenetic tree constructed on the basis of the nucleotide sequences of the partial RNA-dependent RNA polymerase–encoding regions of ferret coronaviruses (FRCoVs) isolated in Japan (shown in boldface; sample IDs are indicated) compared with other coronaviruses (CoVs). The tree was constructed by the neighbor-joining method in MEGA5.0 software (*10*); bootstrap values of >90 are shown. DDBJ/EMBL-Bank/GenBank accession numbers for the nucleotide sequences are shown in parentheses. Human CoVs (HCoVs) 229E and NL63, which belong to the *Alphacoronavirus* genus, were used as the outgroup. CCoV, canine coronavirus; FCoV, feline coronavirus; TGEV, transmissible gastroenteritis virus; PRCoV, porcine respiratory coronavirus. Scale bar indicates nucleotide substitutions per site.

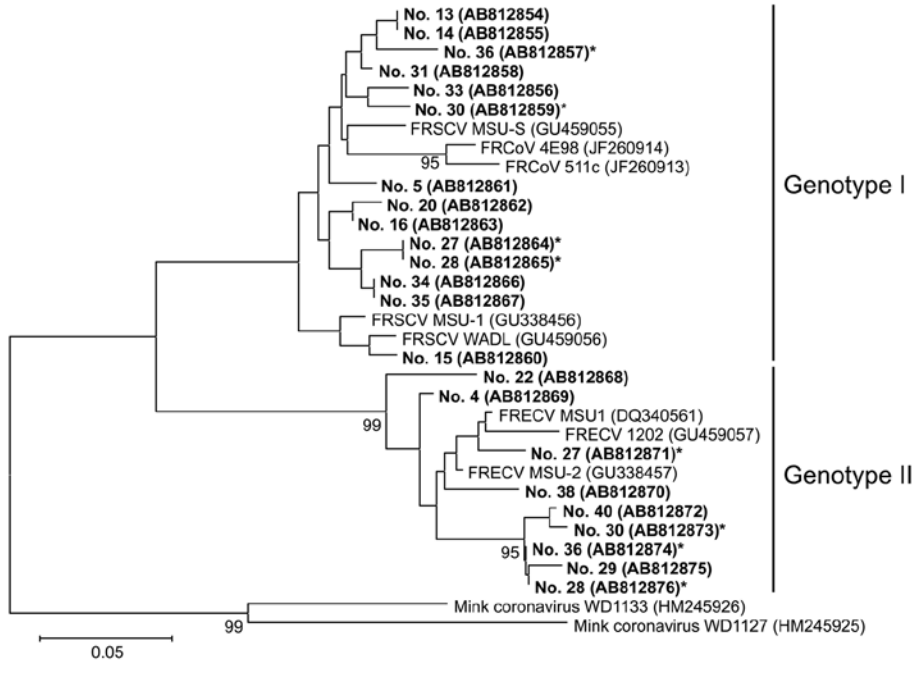

Figure 2. Phylogenetic tree based on the nucleotide sequences of partial S genes of ferret coronaviruses (FRCoVs) isolated in Japan (shown in boldface; sample IDs are indicated) compared with other coronaviruses (CoVs). The tree was constructed by the neighbor-joining method in MEGA5.0 software (*10*); bootstrap values of >90 are shown. Asterisks indicate samples from ferrets infected with FRCoVs of both genotypes1 and 2. DDBJ/EMBL-Bank/GenBank accession numbers for the nucleotide sequences are shown in parentheses. FRSCV, ferret systemic coronavirus; FRECV, ferret enteric coronavirus. Scale bar indicates nucleotide substitutions per site.

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Mr Terada is a PhD student in the Joint Faculty of Veterinary Medicine at Yamaguchi University, Yamaguchi, Japan. His research interest is evolution of coronaviruses.

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Address for correspondence: Ken Maeda, Laboratory of Veterinary Microbiology, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan; email: kmaeda@yamaguchi-u.ac.jp

Crimean-Congo Hemorrhagic Fever Virus, Greece

Anna Papa, Persefoni Sidira, Victor Larichev, Ludmila Gavrilova, Ksenia Kuzmina, Mehrdad Mousavi-Jazi, Ali Mirazimi, Ute Ströher, and Stuart Nichol

Seroprevalence of Crimean-Congo hemorrhagic fever virus (CCHFV) is high in some regions of Greece, but only 1 case of disease has been reported. We used 4 methods to test 118 serum samples that were positive for CCHFV IgG by commercial ELISA and confirmed the positive results. A nonpathogenic or low-pathogenicity strain may be circulating.

Crimean-Congo hemorrhagic fever (CCHF) virus (CCHFV; genus *Nairovirus*, family *Bunyaviridae*) causes severe disease in humans and circulates in many areas of Africa, Asia, and Europe. The disease is characterized by a sudden onset of high fever, chills, severe headache, dizziness, and back and abdominal pains; additional signs and symptoms include nausea, vomiting, diarrhea, and neuropsychiatric and cardiovascular changes. In severe cases, hemorrhagic manifestations are present, ranging from petechiae to large areas of ecchymosis and bleeding from different sites of the body. Case-fatality rates range from 10% to 50% (*1*). Humans become infected with CCHFV through tick bites, mainly from *Hyalomma* spp. ticks, or by direct contact with blood or tissues from viremic livestock or infected humans. The geographic range of CCHFV is the most extensive among tick-borne viruses related to human health, coinciding with the distribution of *Hyalomma* spp. ticks (*1*).

Greece is a country in the south of the Balkan Peninsula. Although sporadic cases or outbreaks of CCHF are often observed in other Balkan countries, including Bulgaria, Albania, and Kosovo (*2*–*5*), only 1, fatal, CCHF case has been reported in Greece (*6*). The causative strain, Rhodopi-2008, clusters with other pathogenic Balkan CCHFV strains; however, it differs by >20% at the nucleotide level from the CCHFV strain AP92, which was isolated from *Rhipicephalus bursa* ticks collected in 1975 from goats in Vergina village in northern Greece (*7*). Human disease has not been associated with the AP92 strain, and only a few mild cases associated with an AP92-like strain have been reported in Turkey (*8*).

Recent studies have shown that the seroprevalence of CCHFV antibodies (IgG) in the general population of Greece is ≈4%, but large differences are seen between regions (range 0%–27%) (*9*–*11*). All of these studies used commercial ELISA kits to detect CCHFV IgG. Our aim was to retest these IgG-positive samples by using additional serologic methods to evaluate the accuracy of the results of the earlier studies.

The Study

We investigated 118 serum samples that had shown positive test results for CCHFV IgG during 3 recent seroprevalence studies in Greece (*9*–*11*). The ages of the seropositive persons ranged from 26 to 90 years (median 73 years). Oral consent had been given by all participants, and the seroprevalence studies were approved by the Ethics Committee of the Medical School of Aristotle University of Thessaloniki, Greece. For control, 20 CCHFV IgG–negative samples were tested. All positive samples had been tested by using a commercial ELISA (Vektor-Best, Novosibirsk, Russia) and stored at –70°C.

We tested all the samples by 3 methods: 1) an in-house, sandwich-capture ELISA described by the Ivanovsky Institute of Virology (Moscow, Russia), which uses inactivated sucrose-acetone–extracted whole-cell antigen of CCHFV strain UZ10145 prepared from the brain of infected newborn white mice (*12*); 2) an in-house sandwich/indirect ELISA developed by the Centers for Disease Control and Prevention (Atlanta, GA, USA), which uses cells infected with CCHFV strain IbAr 10200 as the antigen (*13*); and 3) a commercial immunofluorescence assay (IFA; CCHFV Mosaic 2, Euroimmun Medizinische Labordiagnostika AG, Lübeck, Germany), which uses cells transfected with CCHFV glycoprotein and nucleoprotein (CCHFV strain IbAr 10200) and control-transfected cells. Serum dilution of 1:100 was used in both ELISAs. IFA was performed according to the manufacturer's instructions by using 1:100 serum dilution; when a negative result was obtained, the test was repeated at lower dilutions (1:40 and 1:16). In addition to these methods, we tested 48 of the 118 CCHFV IgG-positive samples by microneutralization assay using the CCHFV strain IbAr 10200; the samples selected for this test method were from all geographic regions of Greece (*14*). This procedure was performed in a high-containment Biosafety Level 4 laboratory at the Karolinska Institute (Stockholm, Sweden).

Author affiliations: Aristotle University of Thessaloniki School of Medicine, Thessaloniki, Greece (A. Papa, P. Sidira); D.I. Ivanovsky Institute of Virology, Moscow, Russian Federation (V. Larichev, L. Gavrilova, K. Kuzmina); Swedish Institute for Communicable Disease Control, Stockholm, Sweden (M. Mousavi-Jazi, A. Mirazimi); National Veterinary Institute, Uppsala, Sweden (A. Mirazimi); Linköpings University Institute of Clinical and Experimental Medicine, Linköping, Sweden (A. Mirazimi); and Centers for Disease Control and Prevention, Atlanta, Georgia, USA (U. Ströher, S. Nichol)

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Table. Results of ELISA and IFA testing of 118 serum samples initially positive for CCHFV by ELISA, Greece*

Result	No. (%) samples			
	Initial ELISA†	CDC ELISA‡	Ivanovsky Institute ELISA§	IFA¶
High-positive	76 (64.4)	61 (51.7)	73 (61.8)	78 (66.1)
Positive	29 (24.6)	43 (36.4)	30 (25.4)	26 (22.0)
Low-positive	13 (11.0)	13 (11.0)	13 (11.0)	13 (11.0)
Negative	NA	1 (0.8)	2 (1.7)	1 (0.8)

*IFA, immunofluorescence assay; CCHFV, Crimean-Congo hemorrhagic fever virus; CDC, US Centers for Disease Control and Prevention; NA, not applicable.
†Samples were initially collected and tested for IgG by ELISA (Vektor-Best, Novosibirsk, Russia) as part of 3 previous seroprevalence studies (*9*–*11*).
‡CDC, Atlanta, Georgia, USA (*13*).
§Ivanovsky Institute of Virology, Moscow, Russia (*12*).
¶CCHFV Mosaic 2; Euroimmun Medizinische Labordiagnostika AG, Lübeck, Germany.

ELISA results were noted as high-positive, positive, or low-positive according to the optical density in relation to the respective cutoff value. IFA grading was done on the basis of serum dilution: 1:16, low-positive; 1:40, positive; and ≥1:100, high-positive.

Both ELISAs and the IFA found CCHFV IgG in 116 (98.3%) of 118 samples (Table). One sample was negative by all methods, including the neutralization test, and 1 sample had positive results on 1 ELISA and showed a low-positive result by IFA (this sample was not tested by neutralization test). The microneutralization assay detected CCHFV in 47/48 samples tested; titers ranged from 10 to 200 (mean 105). All IgG-negative samples had negative results by both ELISAs and by IFA.

Conclusions

Our results confirm previous findings that the CCHFV seroprevalence in Greece is high, especially in areas where livestock husbandry is a major occupation. All the methods used to test these samples produced similar results, which suggests that these methods are reliable laboratory tools for CCHFV seroprevalence studies. The commercial ELISA previously used had been used in various seroprevalence studies, including a study performed in response to a CCHF outbreak in western Afghanistan (*15*). That study reported (as unpublished data) that this kit had been previously compared with an in-house ELISA from the US Army Medical Research Institute for Infectious Diseases (Fort Detrick, MD, USA), and results of the commercial and in-house ELISAs were comparable for all samples tested.

An advantage of the in-house ELISAs, which also run each sample with a negative antigen (background), is that they efficiently reduce the number of false-positive results. The commercial IFA includes control-transfected cells for the same reason. Furthermore, this IFA enables the detection of antibodies against CCHFV nucleoprotein or glycoprotein antigens. The viral antigens in this specific IFA and in the applied in-house ELISAs are expressed in eukaryotic cells, which suggests that the glycosylation and 3D structure of the viral proteins were authentic in the assays we used.

Neutralization testing for CCHFV is a relatively difficult technique because the procedure must be performed in a Biosafety Level 4 environment and because nairoviruses produce weaker neutralizing antibody responses than do members of other *Bunyaviridae* family genera (*1*). In our study, CCHFV-neutralizing antibodies were detected in all but 1 of the samples tested.

Given that only 1 CCHF case has been reported in Greece, the high seroprevalence in specific regions might be related to a nonpathogenic or low-pathogenicity strain, such as the CCHFV strain AP92. The confirmation of the high CCHFV seroprevalence in specific areas of Greece needs further investigation to detect undiagnosed CCHF cases or elucidate the factors playing a role in subclinical infections.

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Dr Papa is a professor of microbiology at the Aristotle University of Thessaloniki and head of the National Centre for Arboviruses and Hemorrhagic Fever Viruses in Greece. Her research interests include diagnostics and molecular epidemiology of arthropod- and rodent-borne viruses.

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Address for correspondence: Anna Papa, Department of Microbiology, School of Medicine, Aristotelian University of Thessaloniki, Thessaloniki 54124, Greece; email: annap@med.auth.gr

Trace-Forward Investigation of Mice in Response to Lymphocytic Choriomeningitis Virus Outbreak

Laura Edison, Barbara Knust, Bret Petersen, Julie Gabel, Craig Manning, Cherie Drenzek, Ute Ströher, Pierre E. Rollin, Douglas Thoroughman, Stuart T. Nichol, and the Multistate LCMV Outbreak Working Group[1]

During follow-up of a 2012 US outbreak of lymphocytic choriomeningitis virus (LCMV), we conducted a trace-forward investigation. LCMV-infected feeder mice originating from a US rodent breeding facility had been distributed to >500 locations in 21 states. All mice from the facility were euthanized, and no additional persons tested positive for LCMV infection.

Lymphocytic choriomeningitis virus (LCMV), a rodent-borne arenavirus, causes inapparent infection in mice but can cause febrile illness, aseptic meningitis, encephalitis, and severe birth defects in humans (www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/lcmv.htm) (*1*). LCMV also can cause disseminated disease with substantial mortality among infected organ transplant recipients (*2*). The reservoir is the common house mouse, *Mus musculus*, but other rodents can become infected and transmit infection to humans. LCMV is endemic among house mice throughout the world, with antibody seroprevalence of 5%–13% in the United States (*3*). LCMV is easily maintained after being introduced into a captive mouse population because mice can persistently shed the virus. LCMV can be transmitted to humans through direct or aerosol contact with urine, feces, or saliva of infected rodents; through transplantation of infected organs; and from mother to fetus (*1*). Sporadic cases occur from exposure to peridomestic house mice, and outbreaks from exposure to infected rodents, particularly hamsters, kept as pets or used for laboratory experimentation have been reported (*2*,*4*–*7*). No outbreaks have been linked to contact with frozen mice.

During summer 2012, state and local agencies and the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) investigated an outbreak of LCMV in the United States. A total of 31 (32%) of 97 tested employees of 3 rodent breeding facilities were infected because of the likely introduction of LCMV into the captive breeding population by wild mice. LCMV aseptic meningitis was diagnosed in 4 employees, and diagnostic testing of the breeding population identified LCMV infection among mice but not rats; no hamsters were bred at the facility (facility A in [*8*,*9*]). All mice originating from this captive breeding population were considered potentially infected and had been distributed to rodent purchasing facilities in multiple states by an Indiana rodent distributor, facility B. We describe the trace-forward investigation of live mice distributed by facility B and the public health measures taken to prevent additional human LCMV infections.

The Study

During July and August 2012, investigators from CDC's Viral Special Pathogens Branch reviewed shipping records from facility B and subsequent distributors and notified health departments in states that had received potentially infected mice during January 1–May 7, 2012; frozen mice were considered a low public health risk and were not traced. Health departments were provided with a list of facilities that had purchased these mice, educational resources about LCMV, and an algorithm to determine whether potentially infected mice remained at these purchasing facilities resulting from the presence, comingling, or breeding of these mice, which would maintain LCMV among the mouse population (Figure 1). As a result of varying state statutes concerning regulation and licensing of pet stores and animal breeders or distributors, the government agencies that had jurisdiction to perform these investigations included local and state departments of public health, environmental health, food safety, and agriculture.

State investigators interviewed purchasing facility managers by telephone, mail, email, or in person to determine whether potentially infected mice remained on the premises and to encourage euthanization of these mice. Interviews also assessed whether pregnant, ill, or immunocompromised employees might have been exposed to LCMV by directly handling potentially infected mice or bedding or equipment used for the mice. Because of risk for severe disease, facility managers were asked to offer serologic testing to these employees for LCMV IgM and IgG, which was performed by CDC by using ELISA as described (*10*). No additional case-finding activities were

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (L. Edison, B. Knust, B. Petersen, C. Manning, U. Ströher, P.E. Rollin, D. Thoroughman, S.T. Nichol); Georgia Department of Public Health, Atlanta (L. Edison, J. Gabel, C. Drenzek); and Kentucky Department for Public Health, Lexington, Kentucky, USA (D. Thoroughman)

DOI: http://dx.doi.org/10.3201/eid2002.130861

[1]Additional members of the Multistate LCMV Outbreak Working Group who contributed data are listed at the end of this article.

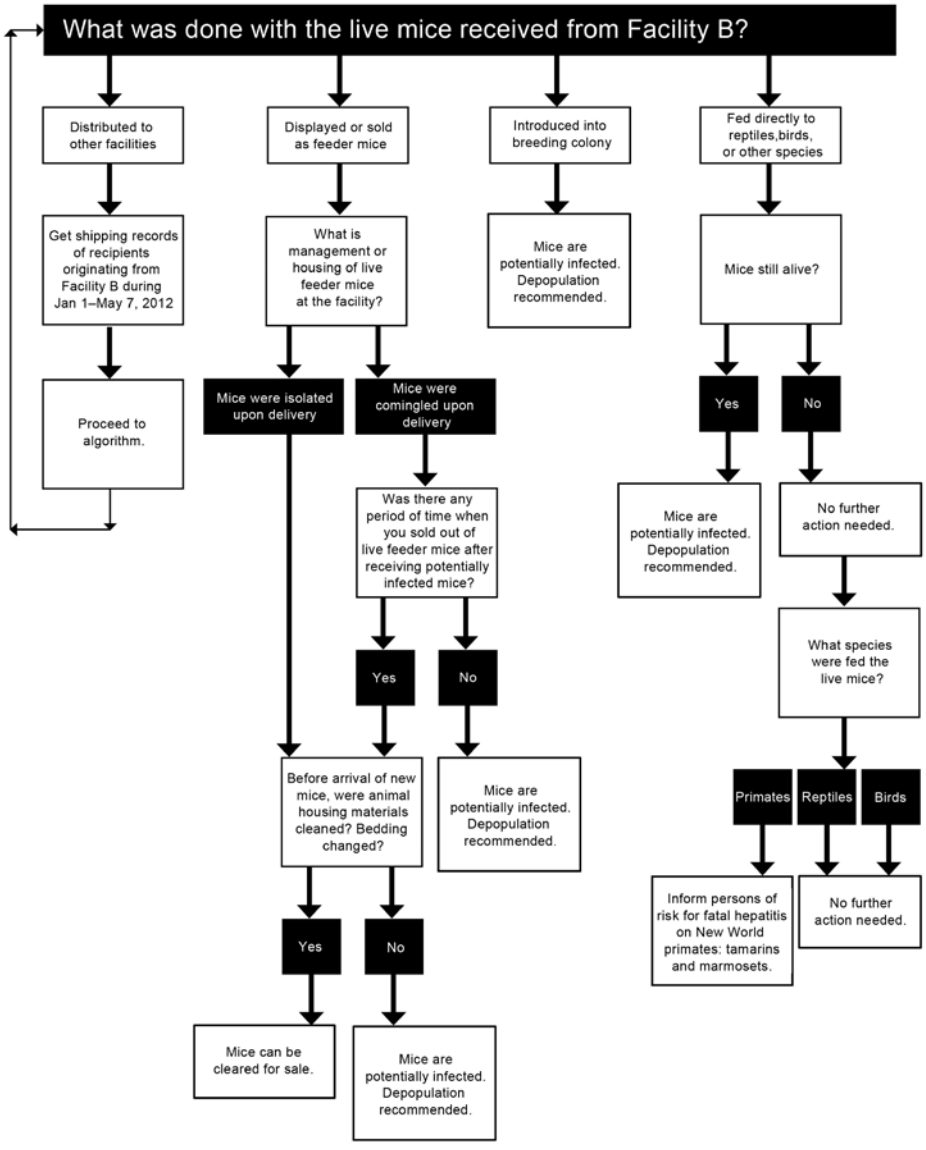

Figure 1. Algorithm used to determine whether mice were potentially infected with lymphocytic choriomeningitis virus (LCMV) during a multistate investigation, United States, 2012. This algorithm was used to determine whether 1) potentially infected mice remained at the facilities being assessed, 2) mice from the original shipment remained, 3) offspring from these mice remained, or 4) shipments of mice had been comingled or had shared equipment with mice from the original shipment. LCMV is easily maintained in a mouse colony, and a clear break among the population (i.e., a time when no remaining mice are maintained and equipment is disinfected) is necessary to ensure that no ongoing infection continues.

conducted. Because of resource limitations, diagnostic testing of live mice at purchasing facilities was not conducted.

Reviews of shipping records indicated that ≈304,000 live mice distributed by facility B were shipped to 561 purchasing facilities: 543 pet stores, 11 breeders or distributors, and 7 zoos or aquariums in 21 states, potentially exposing thousands of employees and pet store mouse purchasers to LCMV. Facility B had shipped mice to 4 subsequent distributors; the largest was located in Georgia, and it had shipped >183,000 mice to 420 purchasing facilities in 16 states (Figure 2). Interviews of facility managers at purchasing facilities revealed that 48% still had potentially infected mice; >10,000 mice were subsequently euthanized. The most common reason for still having potentially infected mice was comingling of rodent shipments, followed by breeding or still having mice from the original shipments.

Serologic testing was performed on blood samples from 34 pet store or zoo employees from 6 states who self-identified as pregnant or ill, were potentially exposed to LCMV, and agreed to serologic testing. Fourteen were pregnant; 1 had aseptic meningitis; and 23 reported nonspecific symptoms including fever, headache, body aches, cough, and vomiting. All persons tested were negative for antibodies against LCMV.

Conclusions

These captive feeder mice had a wide and complex distribution chain, potentially exposing thousands of persons to LCMV. No additional human cases were identified after distribution of these mice; none of the pet store or zoo employees tested had serologic evidence of infection. Although no additional human cases were identified,

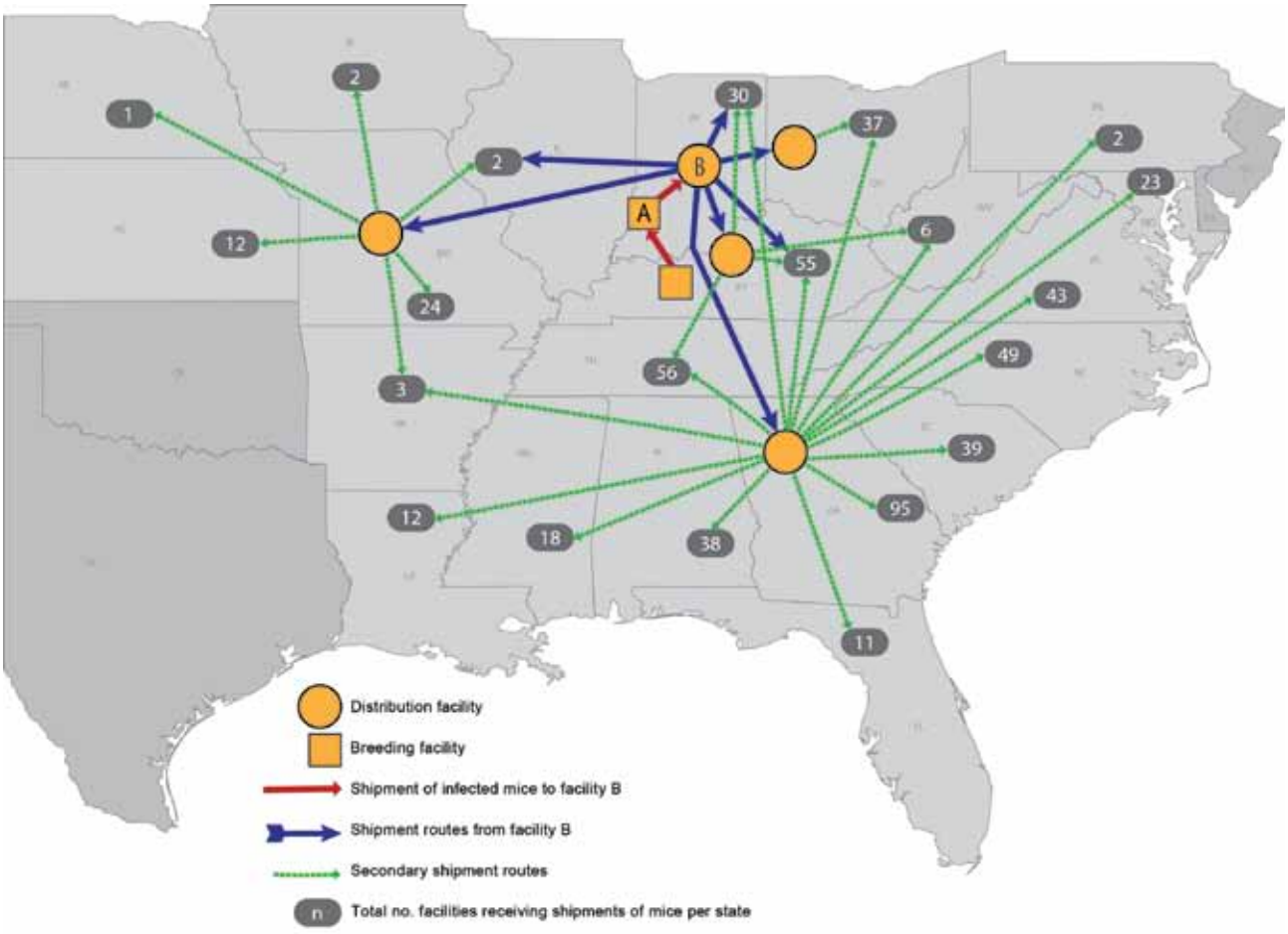

Figure 2. The distribution of mice potentially infected with lymphocytic choriomeningitis virus originating from facility A to ≈500 pet stores and other animal facilities in 21 states, United States, 2012.

euthanasia of all potentially infected rodents was recommended to mitigate potential risk.

Wild mice that access captive breeding populations are often the source of infection of captive rodent populations (*8,11*). After being introduced, LCMV transmission is easily maintained among mouse colonies and is difficult to recognize because mice do not appear ill. Because persistently infected mice pass infection to their offspring, the number of infected mice in a breeding colony can quickly multiply. Mice can be persistently infected without having serologic evidence of infection (*12*); thus, LCMV can be missed by serologic screening alone. Therefore, preventing introduction of the virus into breeding colonies, depopulation of infected rodents, and correct use of personal protective equipment are the most efficient ways to mitigate human exposure. We recommend preventive measures at each point in the distribution process, both domestically and abroad (Table) (*13–15*). More research is needed to develop methods for detecting LCMV in rodents at distributors and pet stores.

Our investigation had several limitations. Employees tested were a fraction of those who had had contact with potentially infected mice. Also, pet store mouse purchasers and purchasing facility employees were difficult to contact, and no pet store customers were tested. Thus, the true number of infected persons is unknown.

Rodent breeders and distributors can fall through a regulatory gap in the United States. Frozen feeder mice are considered pet food and can be regulated by the Food and Drug Administration (FDA), but neither FDA nor the US Department of Agriculture has the authority to regulate live mice and rats because they are not regulated under the Animal Welfare Act (7 CFR 2132, May 13, 2002, www.aphis.usda.gov/animal_welfare/downloads/awa/awa.pdf) and the Food, Drug and Cosmetics Act (21 CFR 500, April 1, 2012, www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=500&showFR=1). Therefore, regulatory authority falls to the states, which have an array of regulations governing the handling, breeding, and distribution of rodents, including the licensing of pet breeders

Table. Recommendations for the prevention and control of lymphocytic choriomeningitis virus among rodents and persons who handle them

Preventing lymphocytic choriomeningitis virus (LCMV) from being introduced into a rodent breeding colony
• All rodents introduced into the breeding colony facility should come from a facility with a biosecurity and monitoring program for LCMV in place.
• Contact between wild mice and breeding colony animals should be prevented through exclusion and by trapping of escaped and feral mice; rodent traps should be placed at the perimeter of the facility, in rodent rooms, and in areas where feed is stored.
• Any feral rodents or colony rodents that escape should be removed and euthanized.
• Testing for LCMV should be included in the routine health monitoring for the colony.
Preventing the spread of LCMV within a rodent breeding colony
• Movement of animals between rooms should be restricted; replacement breeding animals remain within the room they were born, and animals only leave rooms to be removed from the facility.
• Equipment used in handling used cages or bedding should not be shared between rooms or used to handle clean cages or bedding, and such equipment should be disinfected regularly.
• Employees should wear waterproof washable or disposable footwear that can be cleaned between rooms, and they should wear designated coveralls or laboratory coats for each building or room. Footwear should be disinfected before exit and entry into each room.
• After LCMV is discovered, all rodents in the affected rooms or colony should be euthanized.
Preventing infection or the spread of infection at the rodent distributor or pet store
• Rodents should be purchased only from suppliers with biosecurity and monitoring programs.
• Comingling of rodents from different shipments and between rodent species should be prevented.
• Contact between wild mice and captive rodents should be prevented through exclusion and by trapping of escaped rodents and wild mice; rodent traps should be placed at the perimeter of the facility, in rodent rooms, and in areas where feed is stored.
• Equipment used in handling used cages or bedding should not be shared between shipments of rodents or used to handle clean cages or bedding, and such equipment should be disinfected regularly.
Preventing infection among persons who handle rodents
• Employees who handle rodents should be educated about the risk for LCMV, and educational material should be distributed at the point of purchase in pet stores.
• Gloves should be used during handling of live or frozen rodents, used bedding, and dirty cages; hands should be promptly washed when gloves are removed.
• Pregnant and immunocompromised persons should be advised not to directly handle rodents or clean cages.
• Employees should not eat, drink, or smoke in rodent rooms.
Handling rodents with known LCMV infection
• A respirator with a filter of ≥N95 rating, filtering face piece, elastomeric half or full mask, or powered air-purifying respirator should be worn; respiratory capacity testing and fit testing are necessary for all persons wearing such gear.
• Gloves, waterproof and washable footwear, and coveralls should be worn; disinfectant should be used to clean the external surfaces of the protective gear, and workers should wash their hands after removing gloves.

and distributors (*15*; Thomas Edling, pers. comm.), as was evident in this trace-forward investigation. Because of the lack of consistent regulation, we recommend that state and federal partners and rodent industry advisory groups work with breeders, distributors, and pet stores to increase awareness of LCMV infection and implement recommended best practices (Table) to prevent introduction of LCMV into captive rodent populations, prevent subsequent dissemination of potentially infected rodents, and reduce the potential for human exposure and disease among employees and consumers of pet stores and rodent breeding facilities.

Additional members of the Multistate LCMV Outbreak Working Group who contributed data: Jennifer House (Indiana State Department of Health); Gary Balsamo (Louisiana Department of Health and Hospitals); Erin Delaune (Louisiana Office of Public Health); Susan Weinstein (Arkansas Department of Health); Bryan Buss, Manjiri Joshi, and Andre Braith (Nebraska Department of Health and Human Services); Katherine Feldman (Maryland Department of Health and Mental Hygiene); Elizabeth Santini (Pennsylvania Department of Agriculture); Virginia Dato and James Rankin (Pennsylvania Department of Health); Stephanie Cox and Kate Habicht (South Carolina Department of Health and Environmental Control); Ellen Salehi, Mary DiOrio, Kim Quinn, and Kathleen Smith (Ohio Department of Health); Sara Robeson, Kathy Fowler, and John Poe (Kentucky Department for Public Health); Clay Horton and Jessica Austin (Green River District Health Department, Kentucky); and Heather Henderson and Rand Carpenter (Tennessee Department of Health).

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Dr Edison is an Epidemic Intelligence Service officer assigned to the Georgia Department of Public Health, Acute Disease Epidemiology Section, CDC, Atlanta, Georgia. Her research interests include zoonotic diseases, health care–associated infections, and outbreak investigations.

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Address for correspondence: Laura Edison, Georgia Department of Public Health, Acute Disease Epidemiology Section, 2 Peachtree St NW Suite 14-232, Atlanta, GA 30303, USA; email: kgq2@cdc.gov

Rift Valley Fever Outbreak, Southern Mauritania, 2012

Abdourahmane Sow,[1] Ousmane Faye,[1] Yamar Ba, Hampathé Ba, Diawo Diallo, Oumar Faye, Cheikh Loucoubar, Mohamed Boushab, Yahya Barry, Mawlouth Diallo, and Amadou Alpha Sall

After a period of heavy rainfall, an outbreak of Rift Valley fever occurred in southern Mauritania during September–November 2012. A total of 41 human cases were confirmed, including 13 deaths, and 12 Rift Valley fever virus strains were isolated. Moudjeria and Temchecket Departments were the most affected areas.

Rift Valley fever virus (RVFV; genus *Phlebovirus*, family *Bunyaviridae*) periodically causes outbreaks in humans and livestock (*1*), mostly in sub-Saharan Africa (*2*,*3*). Rift Valley fever (RVF) in humans is characterized by a mild, acute, febrile illness with spontaneous recovery, although 1%–2% of cases may evolve to more severe disease, such as acute hepatitis, encephalitis, retinitis, or a hemorrhagic syndrome (*4*,*5*). In Mauritania, RVF outbreaks have been reported repeatedly; the first occurred in 1987 after the building of the Diama dam, which had ecologic and environmental effects that favored a large-scale outbreak that resulted in 200 human deaths (*6*). Since then, RVF epizootics/epidemics have been reported in Mauritania in 1993, 1998, 2003, and 2010 (*7*–*10*).

After 2-fold increase of rainfall in Mauritania during 2012 (annual total 269 mm compared with 137 mm in 2011, as measured at Temchekett station), abortions among pregnant domestic ruminant livestock were reported in the Tagant, Brakna, Trarza, Assaba, and Hodh-El-Gharbi regions of southern Mauritania, starting in September 2012 (Figure 1). By November 2012, many human patients with hemorrhagic fever, 13 of whom died, had been reported in the same regions. Preliminary biological investigation of suspected RVF cases confirmed 23 human cases; this finding led to a multidisciplinary field investigation in these regions during November 18–29, 2012. Here we report the results of this investigation and laboratory findings from this outbreak.

The Study

During the human outbreak investigation, the following case definitions were used: 1) a suspected case was illness in any patient living in Tagant, Brakna, Trarza, Assaba, and Hodh-El-Gharbi regions during September 1–November 29, 2012, that included fever and influenza-like syndrome, whether associated with bleeding or neurologic symptoms or not; 2) a probable case was a case in a patient with a suspected case who died before being tested for RVF infection markers; 3) a confirmed case was a case in a patient with a suspected case whose serum sample was positive for RVF IgM and/or who had positive results by RVF molecular assay. Contacts were defined as family members and neighbors of patients with confirmed and probable cases who showed risk for exposure to RVFV.

For the entomologic investigation, arthropods were collected by using CDC light and animal-baited traps and aspirators, sorted by species and sex (for mosquitoes) or polyspecific pools (for the other arthropods) in the field on chill table, and stored in liquid nitrogen until testing for presence of virus. Human samples were tested for IgM and IgG and by reverse transcription PCR for RVFV (*11*). RVFV isolation from mosquitoes and human samples was attempted by inoculation in AP61 cell lines, followed by indirect immunofluorescence assay, as described (*12*). Partial sequencing of the G2 and nonstructural coding regions of the small segement of RVFV detected or isolated was also performed. Data were analyzed by using R software (www.r-project.org), χ^2 test was used to compare difference between 2 proportions, and significance was set at $p<0.05$. Logistic regression was used to search for association between confirmed status and sociodemographic risk factors. Model accuracy was tested by using the Hosmer Lemeshow test; if $p<0.05$, the model was rejected, but otherwise, the model was considered adequate.

A total of 288 persons had serum samples tested: 23 had confirmed cases, 47 had suspected cases, and 218 were contacts. Median age of those sampled was 24 (range 2–86) years. Forty-one (14%) persons had evidence of recent RVFV infection (20 positive for IgM, 15 by PCR, 6 both), and 24 (8%) had evidence of past infection (positive for IgG). Twelve RVFV strains were isolated from PCR-positive human samples, and phylogenetic analysis was performed on the medium and small genome segments for

Author affiliations: Unité des arbovirus et des Fièvres Hémorragiques Virales de l'Institut Pasteur de Dakar, Dakar, Senegal (A. Sow, Ousmane Faye, Oumar Faye, C. Loucoubar, A.A. Sall); Unité d'Entomologie Médicale de l'Institut Pasteur de Dakar, Dakar (Y. Ba, D. Diallo, M. Diallo); Institut National de Recherche et de la Santé Publique de Nouakchott, Nouakchott, Mauritania (H. Ba); Ministère de la Santé de la République Islamique de Mauritanie, Mauritania (M. Boushab); and Centre National d'Etude et de Recherches Vétérinaires, Nouackchott (Y. Barry)

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[1]These authors contributed equally to this article and are co–first authors.

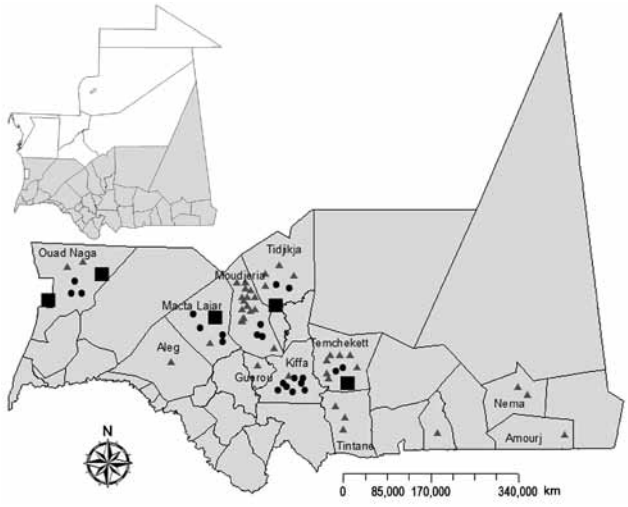

Figure 1. Geographic distribution of confirmed and probable cases of Rift Valley fever among humans and animals, southern Mauritania (gray shading), 2012. Triangles, confirmed human cases; dots, probable human cases; squares, confirmed animal cases.

6 of these isolates. These strains clustered in the West Africa lineage and formed a group with RVF strains that were isolated in northern Mauritania during the 2010 outbreak (*10*) (Figure 2).

The overall attack rate for this outbreak was 0.039% (Table 1), with 41 confirmed cases, including 13 deaths, and 22 probable cases. The outbreak may have started as early as July 2012, when 2 probable cases were reported at Ouad-Naga, but the first confirmed case was detected on September 9 (week 38) at the same location, after which confirmed and probable cases increased progressively from week 38 to week 41 (first week of October), when the peak of the epidemic was reached. Case reports then decreased progressively; the last confirmed and probable cases were reported during weeks 43 and 44, respectively (last 2 weeks of October). Letfatar and Marvek villages in Moudjeria and Temchekett Departments were the most affected areas during this outbreak. The attack rate was 26% (11 confirmed cases, including 2 deaths, and 2 probable cases) in Letfatar and 20% (5 confirmed cases and 1 probable case) in Marvek. In addition, 75% (18/24) of the patients who had positive results for RVF IgG (most [85.9%] adults) were found in Tagant region; IgG prevalence was 54% and 21% in Moudjeria and Tidjigja, respectively.

Persons 15–44 years of age were most affected during this RVF outbreak, representing 70.7% (29/41) of confirmed cases and 68.2% (15/22) of probable cases (Table 1). Housewives and shepherds had significantly higher attack rates than did persons in other occupational

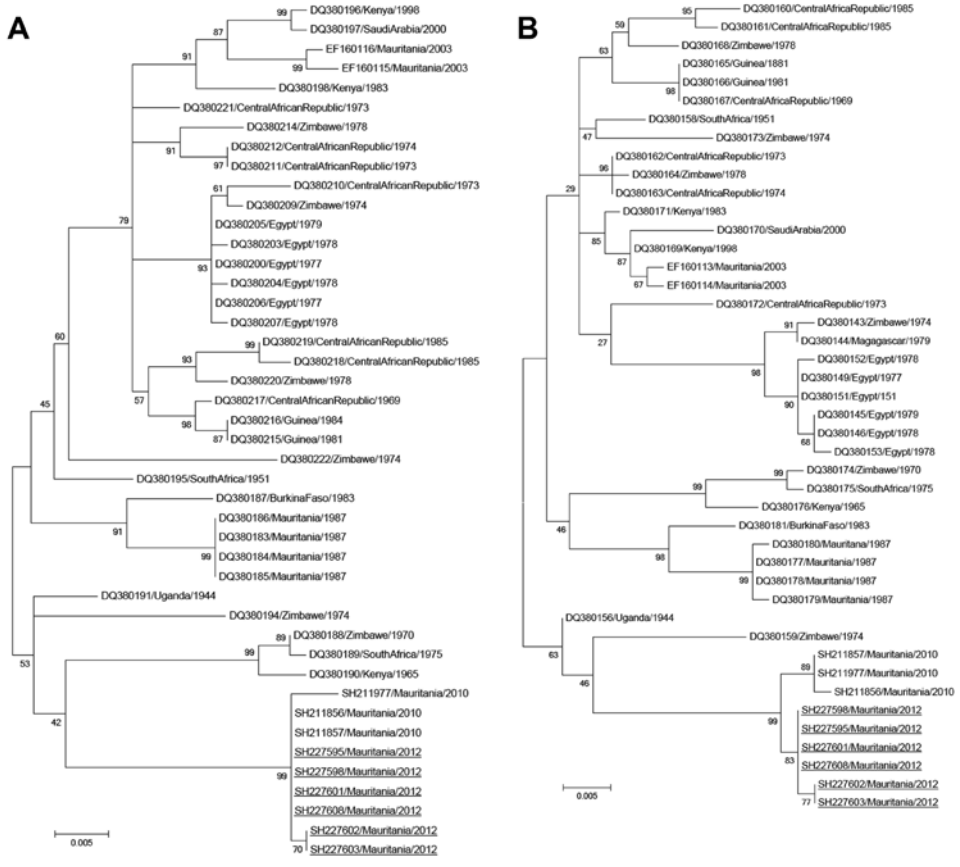

Figure 2. Phylogenetic trees for the medium (M) segments (680 nt) (A) and small (S) segments (531 nt) (B) of 6 Rift Valley fever virus isolates from southern Mauritania, 2012 (underlined), showing relationships among strains isolated from different localities and countries. The strains from 2012 grouped with strains isolated in northern Mauritania in 2010, which suggests re-emergence from an enzootic focus. GenBank accession numbers are KF648851–KF648856 for the M segments and KF648857–KF648862 for the S segments of the isolates identified in this study. GenBank accession numbers, countries, and year of isolation are given for the other strains. Scale bars indicate nucleotide substitutions per site.

Table 1. Attack rates and lethality of human Rift Valley fever cases, by region, sex, and age, southern Mauritania, November 2012

Category	No. confirmed cases (no. deaths)	No. probable cases	Attack rate, cases/ 1,000 population	Death rate, %	Total population
Region					
Moudjeria	16 (4)	3	0.39	25.0	48,751
Tiguidja	4 (3)	2	0.128	75.0	46,944
Tamcheket	7 (0)	2	0.22	0	40,887
Tintane	3 (1)	0	0.035	33.0	84,649
Kiffa	1 (0)	8	0.088	0	102,057
Macta lajar	2 (1)	4	0.095	50.0	62,857
Ouad Naga	2 (1)	3	0.143	50.0	34,898
Guerou	1 (1)	0	0.024	100.0	41,844
Aleg	1 (0)	0	0.011	0	88,077
Djigueni	1 (0)	0	0.017	0	58,619
Amourj	1 (1)	0	0.001	100.0	936,619
Nema	2 (1)	0	0.024	50.0	84,242
Sex					
M	18 (7)	10	0.036	38.9	782,614
F	23 (6)	12	0.041	26.1	847,830
Age group, y					
0–14	5 (0)	2	0.011	0	658,699
15–44	29 (12)	15	0.071	41.4	622,831
45–64	3 (0)	3	0.021	0	291,849
≥65	4 (1)	2	0.105	25.0	57,065
Total	41 (13)	22	0.039	31.7	1,630,444

categories (36.6%; p = 0.09), probably because of exposure to potentially infected mosquitoes during agricultural work or direct contact with viremic livestock, infected tissues, and aborted animals.

Analysis of signs and symptoms among patients with confirmed RFV showed that 73.2% (30/41) had mild signs and symptoms (fever, headache, arthralgia, and myalgia), whereas 26.8% (11/41) developed hemorrhagic symptoms, mainly epistaxis (6/11 [54.5%]) and hematemesis (2/11 [18.2%]). Patients with probable cases showed more severe signs of the disease, including gingival bleeding (43%), epistaxis (36%), gastrointestinal bleeding (21%), neurologic signs (14%), and petechiae (14%). Differential diagnosis also enabled the identification of 1 case of Crimean-Congo hemorrhagic fever in Moudjeria; 12 additional persons showed signs of past infection.

A total of 292 arthropods, including 152 mosquitoes belonging to 13 species, were collected during November at 12 sites (1 trap-night/site) (Table 2). *Aedes vexans*, *Culex poicilipes, Culex antennatus*, and *Mansonia uniformis* comprised 52.6% of the mosquitoes collected; these 4 species are known to be RVFV vectors in the subregion. No RVFV strains were isolated from mosquitoes, but the investigations were carried out after most breeding sites had dried up and after the start of a vector control campaign by the Mauritanian authorities, which limited the number of specimens collected.

Conclusions

An RVF outbreak in southern Mauritania during late 2012 consisted of 41 confirmed cases, including 13 deaths, and 22 probable cases. The extent of this outbreak is probably underestimated because some RVF cases may be asymptomatic or misdiagnosed as malaria (*13*); underdiagnosis could also explain why the case-fatality rate (31.7%) for this outbreak was higher than that for previous outbreaks in Mauritania in 1998 (16%) (*7*) and 2003 (6.67%) (*8*). Phylogenetic analysis suggests that this outbreak resulted from re-emergence from an enzootic focus; RVF isolates from 2012 are closely related to isolates obtained in 2010 in northern Mauritania (Figure 2), which suggests endemicity of RVF in Mauritania that can be maintained by vertical transmission (*14*).

To facilitate identification of RVFV among animals and humans in Mauritania, an active surveillance system should be implemented or reinforced in these areas, and RVF should be primarily suspected for nonmalarial acute febrile illness (*15*). In addition, the identification of 1 acute and 12 past Crimean-Congo hemorrhagic fever infections during the investigation indicates the co-circulation of the 2 viruses, as previously shown (*8*), and calls for systematic differential diagnostics or a syndromic approach to hemorrhagic fever surveillance to avoid a large-scale epidemic.

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Table 2. Arthropods collected during investigation of Rift Valley fever outbreak, by region, southern Mauritania, 2012

Species	Region				Total
	Moudjeria	Ouad Naga	Tamcheket	Tidjikja	
Mosquitoes					
Aedes sudanensis	–	–	–	1	1
Ae. vexans	–	–	–	45	45
Anopheles funestus	–	–	–	1	1
An. pharoensis	7	–	–	–	7
An. rhodesiensis	–	–	8	–	8
An. rhodesiensis male	–	–	21	–	21
An. rufipes	15	–	–	–	15
An. ziemanni	5	–	–	–	5
Culex antennatus	6	–	–	13	19
Cx. decens	–	–	–	1	1
Cx. decens male	–	–	–	1	1
Cx. neavei	2	–	–	–	2
Cx. poicilipes	15	–	–	–	15
Cx. quinquefasciatus	–	3	–	1	4
Cx. quinquefasciatus male		6	–	–	6
Mansonia uniformis	1	–	–	–	1
Total no. mosquitoes	51	9	29	63	152
Other arthropods					
Culicoides spp.	6	–	1	12	19
Phlebotomus spp.	108	–	11	2	121
Total no. arthropods	165	9	41	77	292

*–, none found.

Dr Sow is a medical doctor with a master of public health degree who works in the arbovirus and viral hemorrhagic fever unit at Institut Pasteur, Dakar, Senegal. His research interests include outbreak investigations, epidemiology, and modeling arbovirus emergences.

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Address for correspondence: Amadou Alpha Sall, Unité des Arbovirus et virus de fièvres hémorragiques, Institut Pasteur de Dakar, BP 220 Dakar, Senegal; email: asall@pasteur.sn

Reemergence of Rift Valley Fever, Mauritania, 2010

Ousmane Faye, Hampathé Ba, Yamar Ba, Caio C.M. Freire, Oumar Faye, Oumar Ndiaye, Isselmou O. Elgady, Paolo M.A. Zanotto, Mawlouth Diallo, and Amadou A. Sall

A Rift Valley fever (RVF) outbreak in humans and animals occurred in Mauritania in 2010. Thirty cases of RVF in humans and 3 deaths were identified. RVFV isolates were recovered from humans, camels, sheep, goats, and *Culex antennatus* mosquitoes. Phylogenetic analysis of isolates indicated a virus origin from western Africa.

Rift Valley fever (RVF) is an acute febrile viral disease that affects domestic ruminants and humans. The disease in animals is characterized by abortions among pregnant females and high mortality rates for offspring (*1*). The causative pathogen, RVF virus (RVFV), is a member of the family *Bunyaviridae*, genus *Phlebovirus*, and its genome consists of 3 RNA segments: large (L), medium (M), and small (S) (*2*). In human infections, symptoms are generally mild but may evolve to severe symptoms, such as hemorrhaging, meningoencephalitis, and retinopathy, with fatal outcomes (*3*). RVF is endemic to sub-Saharan Africa, Egypt, Saudi Arabia, and Yemen (*4*). In western Africa, a major RVF outbreak occurred in Mauritania and Senegal in 1987 and resulted in 220 human deaths (*5*). This outbreak was followed by epizootics/epidemics in 1998 and 2003 in different provinces in Mauritania (Hodh El Gharbi, Assaba, Brakna, Trarza, and Gorgol Provinces) (*6*,*7*).

In October 2010, the health services of Adrar Province in Mauritania were informed of the deaths of 2 girls with hemorrhagic syndromes and 2 persons with acute fever, arthralgia, and headaches in Amogjar Province. Blood samples collected from a sick patient were sent to Institut Pasteur in Dakar, Senegal where RVFV IgM and RVFV genome were detected. During October 28–November 11, 2010, suspected RVF cases were identified in several cities Adar Province (Atar, Aoujeft, Chinguetti), Inchiri Province (Akjoujt), and Hodh el Gharbi Province (Tintane, Kobeni) (Figure 1), and high mortality rates among camels, sheep, and goats were reported in Adrar and Inchiri Provinces (*8*). Clinical samples from humans and animals with suspected RVF were positive for this virus.

These cases led to identification of 2 additional confirmed cases of acute RVF (positive for IgM against RVFV or RVFV RNA) in Adrar and Inchiei Provinces. Multidisciplinary investigations involving epidemiologists, virologists, entomologists, and veterinary teams in areas where the cases occurred were conducted in December 2010 as part of other investigations (*8*), describe the extent of the outbreak, and investigate possible RVF reemergence factors in Mauritania. We report results of laboratory and field investigations among humans, animals, and mosquitoes during the 2010 outbreak.

The Study

During October–December 2010, surveillance and investigation teams collected 80 clinical samples from 36 humans with suspected RVF and 44 of their contacts in Atar, Aoujeft, Akjoujt, Chingetti, Kobeni, and Tintane Moughatas (Figure 1). A human with a suspected case of RVF was defined as any patient with fever associated with or without hemorrhage, jaundice, or neurologic symptoms during October–December. A human contact was defined as a healthy person living within the immediate environment (i.e., same household or neighborhood) of a human or animal with a confirmed case of RVF. Serum samples from suspected case-patients were tested by using real time reverse transcription PCR, ELISA (for detection of IgM), and virus isolation.

Samples were obtained from animals living near suspected or confirmed case-patients, and a questionnaire about the species, sex, age and reproductive status of female animals was completed by their owners. Sampling of mosquitoes was conducted in December 2010 by using animal-baited traps placed in and around houses in which suspected and/or confirmed case-patients were identified. RVFV isolation and identification was performed on mosquitoes and human serum samples as described (*9*). Coding regions for RVFV nonstructural protein and glycoprotein 2 were amplified by using reverse transcription PCR and described sets of primers (*10*,*11*) and then purified after electrophoresis on agarose gels, and sequenced for phylogenetic analyses.

IgM against RVFV or viral RNA genome was detected in 30 (37.5%) of 80 patients, and 6 RVFV isolates were recovered (Table 1). The dates of symptom onset for confirmed cases of RVF in humans indicated that the outbreak probably started at the end of October, peaked in November, and decreased the second week of December. Among 30 patients (8 contacts and 22 suspected case-patients) with acute RVFV infections, the male: female ratio

Author affiliations: Institut Pasteur, Dakar, Senegal (O. Faye, Y. Ba, O. Faye, M. Diallo, A.A. Sall); Institut National de Recherche en Santé Publique, Nouakchott, Mauritania (H. Ba, I. O. Elgady); and University of São Paulo, São Paulo, Brazil (C.C.M. Freire, P.M.A. Zanotto)

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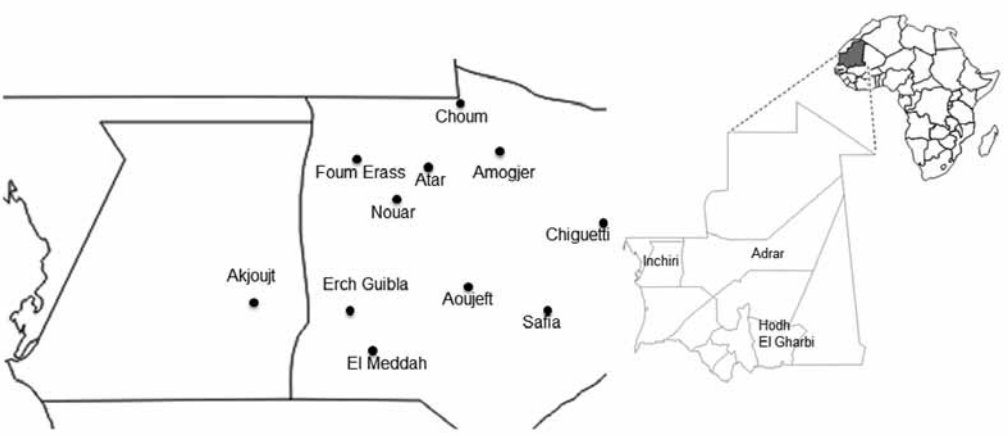

Figure 1. Location of study sites tested for patients with confirmed Rift Valley fever, Mauritania, 2010.

was 5:1 (25:5), and the median age was 35 years (range 17–70 years); 3 deaths were reported. Except for 1 man (a teacher), all infected men were herdsmen and all infected women were housewives.

For the animal investigation, serum samples were obtained and processed from 83 small ruminants (70 goats and 13 sheep) with a median age 4 years (range 1–10 years) from all localities tested (Table 2, Appendix, wwwnc.cdc.gov/EID/article/20/2/13-0996-T2.htm). IgM against RVFV was detected in 23 (27.7%) of 83 animals. In addition, samples from 5 sick camels were collected in neighborhoods of persons with suspected and confirmed cases of RVF, and RVFV RNA was detected in 3 of these samples. Information for populations living in Adrar indicated that the first probable case of the disease among animals was in a sick camel in Aoujeft during the last week of October (*8*).

A total of 2,741 mosquitoes belonging to 5 genera and 11 species were collected for entomologic investigation. *Culex antennatus* was the most abundant mosquito species (83%), followed by *Anopheles gambiae (*9%) and *An. rufipes* (2%) mosquitoes (Table 3). Three RVFV strains were isolated from *Cx. antennatus* mosquitoes collected in Safia (Adrar Province).

Phylogenetic analysis based on RVFV partial small and medium RNA sequences showed a low level of variation between isolates from humans, animals, and mosquitoes. Isolates belonged to the West Africa lineage and were closely related to strains from Diawara (1998) and Hodh El Garbi (1998) in Mauritania and from Kenya (1965), Zimbabwe (1974), and Zambia (1975) (Figure 2).

Conclusions

After a 4-fold increase in rainfall in Mauritania from 2009 through 2010 (59 mm vs. 196 mm; Aoujeft Weather Station). RVF clinical cases were confirmed in Adrar and Inchiri Provinces. Although RVF was reported in some provinces of Mauritania in 1987, 1998, and 2003 (*5–7*), cases in humans from Adrar and Inchiri Provinces have not been previously reported. However, in 2010 a total of 30 laboratory-confirmed human case-patients were recorded in these 2 provinces; 3 of the case-patients died. Both areas had a drought in recent years or their average rainfall was not >60 mm before 2010. The study showed animal abortion rates of 26.13% (23/88) and 30.68% (27/88) in 2 groups of small ruminants living near patients with recent RVFV infections. Three camels were infected with RVFV, and the cases were probably linked to human infections. The first human death from RVF was reported on November 11 in the village of Tawaz, in which a high mortality rates were observed in camel populations (*11*).

Serologic evidence of RVF in camels from northern Sudan and southern Egypt in 1977 (*12*) led to camels being suspected as key contributors in RVFV transmission (*13*). The involvement of camels in human infections could explain the high male:female ratio (25:5) for infections in humans because men are more involved in animal

Table 1. Seroepidemiologic and virologic results for humans, Rift Valley Fever outbreak, Mauritania, 2010*

City	No. tested	No. positive for IgM against RVFV	No. positive for RVFV by RT-PCR	No. with isolated virus
Atar	54	12	10	3
Aoujeft	12	1	7	3
Akjoujt	10	0	4	0
Chinguetti	2	0	1	0
Kobeni	1	0	0	0
Tintane	1	0	0	0
Total	80	0	22	6

*RVFV, Rift Valley fever virus; RT-PCR, reverse transcription PCR.

Table 3. Virologic results for mosquitoes tested during a Rift Valley fever outbreak, Mauritania, 2010*

Mosquito species	No. tested	No. mosquito pools	No. mosquitoes positive for RVFV by RT-PCR	No. with isolated virus
Aedes vexans	13	3	0	0
Anopheles gambiae	259	10	0	0
Anopheles pharoensis	51	7	0	0
Anopheles rufipes	47	7	0	0
Anopheles pretoriensis	3	1	0	0
Anopheles gambiae	36	2	0	0
Anopheles pharoensis	2,276	46	4	4
Anopheles rufipes	16	4	0	0
Anopheles pretoriensis	30	5	0	0
Total	2,736	85	4	3

*RVFV, Rift Valley fever virus; RT-PCR, reverse transcription PCR.

husbandry. In 2006, a similar sex difference for infection was observed in Kenya, where men had a prevalence rate of IgG against RVFV that was 3 times higher than that in women (*14*).

Entomologic investigations in Mauritania showed that only *Cx. antennatus* mosquitoes were infected with RVFV. Similar finding were reported for previous RVF outbreaks in Mauritania. Furthermore, *Cx. antennatus* mosquitoes were found to be infected with RVFV in Nigeria in 1967 and in Kenya during 1981–1984, and vector competence was demonstrated in laboratory studies (*15*). Phylogenetic analysis suggests reemergence of the local RVFV focus and emphasizes the need to strengthen RVF surveillance and other control measure in Mauritania after heavy rainfall.

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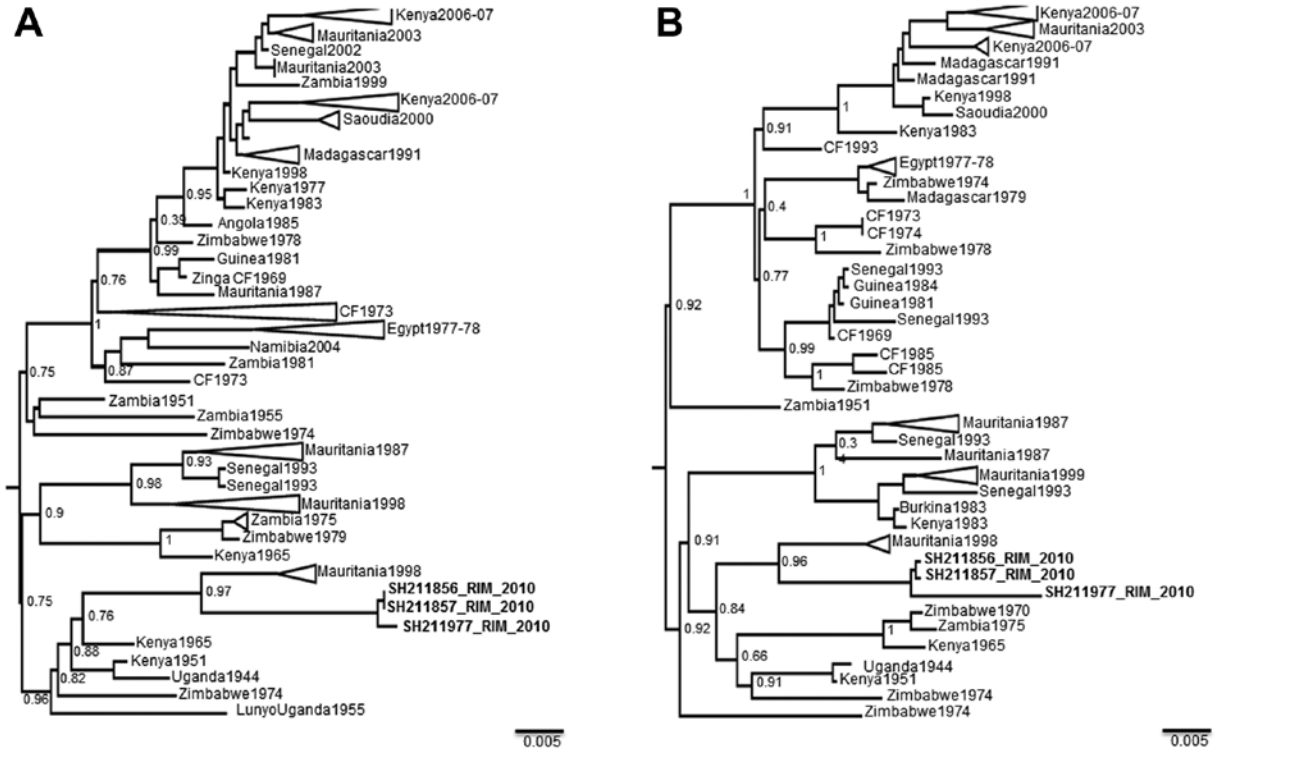

Figure 2. Maximum-likelihood trees for the RNA A) small and B) medium segments of Rift Valley fever virus. Trees show relationships among strains isolated from different localities and countries. Samples from Mauritania 2010 are indicated in **boldface** and designated SH211856_RIM_2010, SH211857_RIM_2010, and SH211977_RIM_2010. GenBank accession nos. are KF717588, KF717589, and KF717590 for the medium segment and KF717591, KF717592, and KF717593 for the small segment. Values along the branches indicate bootstrap values. Scale bars indicate nucleotide substitutions per site.

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Dr Ousmane Faye is a virologist and acarologist at the Arbovirus and Viral Hemorrhagic Fever Unit, Institut Pasteur, Dakar, Senegal. His primary research interests include the virology and vectorial transmission of arboviruses and viral hemorrhagic fevers.

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Address for correspondence: Amadou A. Sall, Unité des Arbovirus et Virus de Fièvre Hémorragique, Institut Pasteur, 36 Ave Pasteur, Boîte Postale 220, Dakar, Senegal; email: asall@pasteur.sn

Burkholderia pseudomallei Isolates in 2 Pet Iguanas, California, USA

Ashley M. Zehnder,[1] Michelle G. Hawkins, Marilyn A. Koski, Barry Lifland, Barbara A. Byrne, Alexandra A. Swanson, Michael P. Rood, Jay E. Gee, Mindy Glass Elrod, Cari A. Beesley, David D. Blaney, Jean Ventura, Alex R. Hoffmaster, and Emily S. Beeler

Burkholderia pseudomallei, the causative agent of melioidosis, was isolated from abscesses of 2 pet green iguanas in California, USA. The international trade in iguanas may contribute to importation of this pathogen into countries where it is not endemic and put persons exposed to these animals at risk for infection.

Burkholderia pseudomallei, a gram-negative bacterium, is the causative agent of melioidosis. Melioidosis is endemic in countries in Southeast Asia and in northern Australia, and has been sporadically reported from Central and South America (*1*). In the United States, most case-patients have traveled to disease-endemic areas (*2*).

B. pseudomallei infection occurs through direct cutaneous inoculation with soil or water containing *B. pseudomallei* and through ingestion or inhalation of aerosolized bacteria. In humans, the incubation period is typically 1–21 days, but some patients demonstrate clinical signs years after exposure (*1*). Acute melioidosis can manifest as a severe pneumonia and septicemia, with death rates >40% in countries where access to medical care is limited. In chronic melioidosis, abscesses occur in various organs, including the lungs, liver, spleen, and cutaneous sites (*1*,*3*). In animals, abscesses and acute illness are common (*4*).

Author affiliations: School of Veterinary Medicine, University of California, Davis, Davis, California, USA (A.M. Zehnder, M.G. Hawkins, M.A. Koski, B.A. Byrne); Stanford University School of Medicine, Stanford, California, USA (B. Lifland); Los Angeles County Department of Public Health, Los Angeles, California, USA (A.A. Swanson, M.P. Rood, E.S. Beeler); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (J.E. Gee, M.G. Elrod, C.A. Beesley, D.D. Blaney, A.R. Hoffmaster); and Sacramento County Department of Public Health, Sacramento, California, USA (J. Ventura)

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B. pseudomallei is classified by US federal agencies as a tier 1 select agent. Tier 1 agents are believed to pose the greatest threat for deliberate misuse and potential harm to public health. Multiple regulations restrict access to these agents and reduce the risk of their release from secure settings (*5*). Infection is generally diagnosed by culture. Commercially available bacterial identification systems may provide initial identification; however, *B. pseudomallei* may be misidentified by some systems (*6*). Other identification tests are available, including PCR and antigen detection; these are not commonly used outside disease-endemic regions (*3*,*7*,*8*).

Case Reports

A 2.5-year-old female green iguana (*Iguana iguana*) was referred to the William R. Pritchard Veterinary Medical Teaching Hospital, University of California, Davis, in 2007 for evaluation of a coelomic mass and leukocytosis ($40.0–42.0 \times 10^3$ cells/mL [reference $3–10 \times 10^3$ cells/mL]). Radiographs and ultrasound results confirmed multiple hepatic masses. Initial aspiration and culture of 1 mass yielded 3 colonies of a *Bacillus* spp. Empirical antimicrobial drug therapy with ceftazidime (20 mg/kg intramuscularly every 48 h for 30 d) was initiated. The iguana was brought for treatment of additional coelomic masses 6 months later. Aspiration of the masses and culture of the sample yielded small colonies of suspected *B. pseudomallei*; blood samples collected for culture were negative for the organism. Results of in-house biochemical testing and PCR were consistent with *B. pseudomallei* or *B. mallei*. The culture was submitted to Stanford University School of Medicine, Department of Comparative Medicine. The Biolog Dangerous Pathogens Database ID system (http://info.biolog.com/VetCampaign_vet_alt.html) identified *B. pseudomallei*. Laboratory Response Network PCR protocols and biochemical testing performed at the Santa Clara Public Health Laboratory (Santa Clara, CA, USA) confirmed *B. pseudomallei*.

The isolate was then sent to the Centers for Disease Control and Prevention (CDC) for further characterization by multilocus sequence typing (MLST), a method of molecular subtyping that compares sequences from 7 housekeeping genes (*9*). This isolate's sequence type (ST) isolate is ST518 (http://bpseudomallei.mlst.net/). No environmental testing was performed. Treatment included trimethoprim/sulfamethoxazole (20 mg/kg by mouth every 24 h) and doxycycline (10 mg/kg by mouth every 24 h), according to published treatment regimens and in-house susceptibility testing (*7*,*10*–*12*). Against recommendations, therapy was discontinued by the owner after 3 months. Re-evaluation

[1]Current affiliation: Stanford University School of Medicine, Stanford, California, USA.

of the patient 13 months after diagnosis revealed persistent leukocytosis. Euthanasia of the iguana was recommended, but the owner declined. The iguana died at home ≈2.5 years after initial diagnosis; a necropsy was not permitted.

In December 2012, a 1.6-year-old female green iguana was brought to a veterinarian in Los Angeles County, California, ≈400 miles south of the first case. An abscess, ≈40 mm in diameter, appeared on the iguana's left shoulder 2 days after it fell from a height of 1 meter. The abscess was surgically removed and submitted to a commercial veterinary laboratory, which cultured β-hemolytic *Streptococcus* spp. and large numbers of unidentified gram-negative rods. Marbofloxacin (7.5 mg/kg by mouth, every other day, for 40 days) was prescribed. The laboratory forwarded the unidentified isolate to the Sacramento Public Health Laboratory, which identified it as *B. pseudomallei* by using Laboratory Response Network PCR protocols and biochemical testing. The isolate was forwarded to CDC where MLST was performed. The isolate was ST518, which matched the isolate from the first infected iguana and another isolate recovered from a tourist from Arizona who had been infected in Costa Rica in 2010 (http://bpseudomallei.mlst.net/).

Staff from Los Angeles County Department of Public Health visited the iguana owner's home 3 weeks after the iguana completed marbofloxacin treatment. The iguana had a firm swelling on the left shoulder that was ≈25-mm in diameter, with a central flat, red crust that was 5 mm in diameter (Figure). The owner had not yet disinfected the animal's housing. Sterile rayon swabs in liquid Amies medium were used to collect swab specimens from the iguana: 1 from the bottom of the feet, 1 from inside the cloacal opening (vent), and 2 from the red crust on the shoulder swelling. Four specimens were also collected from the iguana's housing: 50 mL of water from its aluminum water bowl, a fecal sample in the water, a cloth containing feces from its tank, and a biofilm sample, recovered with a sterile polyurethane foam swab, from the inside of the emptied water bowl. The 8 samples underwent culture and type III secretion system real-time PCR for *B. pseudomallei* (*13*). PCR results from the water sample, the water bowl biofilm, and a shoulder swab specimen were positive. *B. pseudomallei* was cultured from the second shoulder swab specimen. CDC confirmed the isolate as *B. pseudomallei*. The owner euthanized the iguana, and tissue samples were collected in a Biosafety Level 3 cabinet at Los Angeles County Department of Public Health. The iguana's shoulder swelling had grown to ≈30 mm in diameter, and multiple 5-mm yellow masses were found in the iguana's liver, lungs, and spleen.

Both owners stated that the iguanas had been purchased at young ages from local pet stores, had been kept indoors, and had not left California. The pathogen's zoonotic potential was discussed with the owners and veterinarians in both cases. The owners were advised to consult with their physicians about potential exposure and were advised to use personal protective equipment (e.g., gloves, masks, and dedicated clothing) when handling the pets, to wash their hands afterward, and to disinfect the pets' environment with 0.5%–1% sodium hypochlorite. No pet-related *B. pseudomallei* infections in humans have been reported.

Conclusions

This report identifies *B. pseudomallei* isolates in 2 pet green iguanas and the environment of one of the infected pets. Their clinical disease developed when they were adults and was refractory to antimicrobial drug treatment. The second iguana suffered mild trauma shortly before its

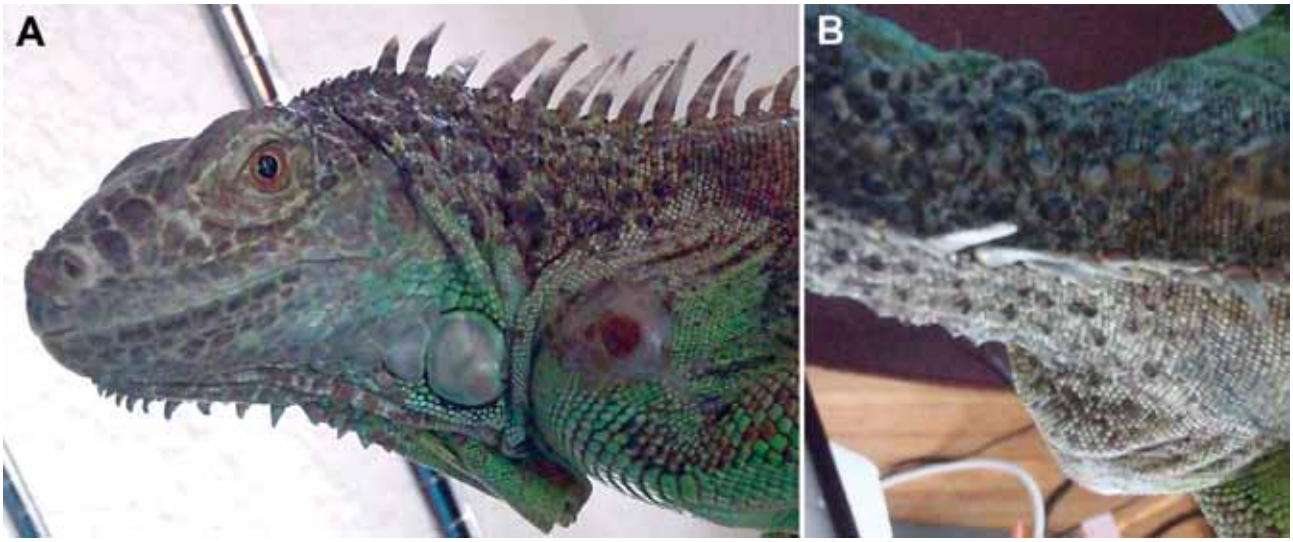

Figure. Abscess on shoulder of iguana infected with *Burkholderia pseudomallei*, California, USA, 2013. A) Lateral view. B) Dorsal view. Photo was taken at the time of sampling surface of animal and its environment.

abscess appeared. Human case reports suggest that trauma may trigger the onset of disease (*14*).

Most green iguanas entering the pet trade in the United States originate from Central America (*15*). Both iguanas in this report and a person exposed in Costa Rica were infected with isolates with an identical sequence type by MLST. These results suggest that the iguanas were infected in Central America and experienced prolonged incubation periods (>1.5 years).

Positive PCR results from the water bowl suggest environmental contamination. The open abscess on the second iguana tested positive by both PCR and culture and was the likely source. However, negative PCR and culture results from the iguana's vent, feet, and feces in the water bowl suggest contamination may not have been widespread.

Reptile owners, sellers, veterinarians, and staff in veterinary laboratories should be aware that green iguanas with abscesses may be infected with *B. pseudomallei* and that they should use appropriate personal protective equipment when handling them. Laboratories accepting samples from animals possibly infected with *B. pseudomallei* should anticipate risks associated with culturing this bacteria and take steps to protect workers.

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Dr Zehnder is a postdoctoral fellow in the Epithelial Biology Department at Stanford University School of Medicine. Her research interests focus on cancer biology and comparative medicine.

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Address for correspondence: Jay E. Gee, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop G34, Atlanta, GA 30333, USA; email: xzg4@cdc.gov

Congenital Rubella Syndrome in Child of Woman without Known Risk Factors, New Jersey, USA

Samantha I. Pitts, Gregory S. Wallace, Barbara Montana, Elizabeth F. Handschur, Debrah Meislich, Alethia C. Sampson, Suzanne Canuso, Jennifer Horner, Albert E. Barskey, Emily S. Abernathy, and Joseph P. Icenogle

We report a case of congenital rubella syndrome in a child born to a vaccinated New Jersey woman who had not traveled internationally. Although rubella and congenital rubella syndrome have been eliminated from the United States, clinicians should remain vigilant and immediately notify public health authorities when either is suspected.

Once a main cause of congenital abnormalities (*1*), congenital rubella syndrome (CRS) is now rare in the United States. However, rubella remains a common illness in countries without robust vaccination programs. Since rubella was declared eliminated in the United States in 2004 (*2*), 6 cases of CRS have been reported to the Centers for Disease Control and Prevention (CDC); 5 were likely imported cases (*3*). We describe the sixth case.

Case Report

In 2008, a full-term boy was born with microcephaly and a petechial rash. The infant's US-born mother reported no rash illness, travel history, or known contact with ill persons during the first 4 months of pregnancy. She lived with her 2 other children and the case-patient's father, who was born in Brazil and had not traveled internationally during the mother's pregnancy.

In 2002, the case-patient's mother had an equivocal rubella virus IgG titer of 5 (nonimmune <5) and in 2003 received measles, mumps, and rubella (MMR) vaccine. Vaccination status of the father was unknown. Maternal rubella virus IgG titer at 4 months' gestation was >400 IU/mL (immune >9).

The infant was delivered by urgent cesarean section because of cardiac decelerations during labor. Apgar scores at 1 and 5 minutes were 9 (of 10 total). At birth, the child weighed 2.7 kg (10th percentile), and he was 48.25 cm (15th percentile) in length and had a head circumference of 31.75 cm (<3rd percentile). He had a petechial rash on the face, back, and upper extremities and a systolic heart murmur. No jaundice or hepatosplenomegaly was noted.

Initial tests showed a leukocyte count of 13.8×10^9/L (reference 9–30) with atypical lymphocytes; a hemoglobin level of 202 g/L (reference 135–195); abnormal erythrocyte morphology, including macrocytosis, polychromasia, and poikilocytosis; and a platelet count of $98{,}000 \times 10^9$/L (reference 140–440). Computed tomographic scan of the head revealed 2 small calcifications in the left corona radiata. Results of neonatal hearing screening were normal. Urine samples were cultured for cytomegalovirus (CMV), and serum samples were tested for toxoplasma and parvovirus IgM; all results were negative. The infant was discharged from the hospital on day 4 with a suspected congenital infection.

At 16 days of age, the child was seen by an infectious disease physician. Examination revealed hepatosplenomegaly, and the systolic heart murmur was detected across the precordium, with radiation to the back. CMV quantitative PCR and testing for lymphocytic choriomeningitis IgM and IgG were requested, but not obtained. Congenital rubella was considered, but serologic tests were not ordered. A subsequent echocardiogram demonstrated supravalvular and peripheral pulmonic stenosis, a small patent ductus arteriosis, and a patent foramen ovale.

The child was delayed in attaining developmental milestones. At 6.5 months of age, he was referred to a geneticist, who requested multiple tests, including tests for rubella, CMV, and lymphocytic choriomeningitis. Rubella virus serum IgM was 7.2 (positive >1.0) and IgG was 59 IU/mL (immune >9). The local health department received notification of the test results and immediately initiated an investigation in collaboration with the New Jersey Department of Health. During multiple interviews, the child's family denied any exposure to international travelers or persons with a rash illness during the potential exposure period. No source contact or secondary transmission was identified. New Jersey Department of Health and the infectious disease physician requested that the child not attend day care and avoid exposure to unvaccinated infants until after his

Author affiliations: Johns Hopkins University School of Medicine, Baltimore, Maryland, USA (S.I. Pitts); New Jersey Department of Health, Trenton, New Jersey, USA (S.I. Pitts, B. Montana, E.F. Handschur, S. Canuso); Council of State and Territorial Epidemiologists, Atlanta, Georgia, USA (S.I. Pitts); Centers for Disease Control and Prevention, Atlanta (G.S. Wallace, A.E. Barskey, E.S. Abernathy, J.P. Icenogle); Cooper Medical School of Rowan University, Camden, New Jersey, USA (D. Meislich); and Burlington County Health Department, Westampton, New Jersey, USA (A.C. Sampson, J. Horner)

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first birthday, when he would be presumed to be free of infection. MMR vaccination at 12 months of age was withheld until completion of additional diagnostic testing.

When the infant was 7.5 months of age, rubella virus RNA was detected from a nasal wash sample by using reverse transcription PCR (RT-PCR); results of a nasal wash culture and serum RT-PCR were negative. CDC culture and RT-PCR results for nasal samples collected when the infant was 10 and 12 months of age were also negative. However, ELISAs performed by CDC on a serum sample collected when the infant was 12 months of age confirmed the presence of rubella virus IgM antibodies (Diamedix Rubella IgM Capture EIA; IVAX Corp., Miami, FL, USA) and IgG antibodies (Wampole Rubella IgG ELISA II; Alere, Waltham, MA, USA); results were 2.43 and 5.9, respectively (positive ≥1.1).

Conclusions

The clinical features and laboratory results for this case are most consistent with CRS, although initial testing was delayed until the infant was 6.5 months of age. Clinical findings for the case-patient (microcephaly, developmental delay, congenital heart disease) were compatible with CRS and met 3 of the Council of State and Territorial Epidemiologists' laboratory criteria for a confirmed case of CRS (*4*) (Table).

CRS occurs rarely in children of women with a history of vaccination or documented immunity by rubella virus titer. When CRS does occur, it is frequently a result of asymptomatic rubella reinfection in the mother (*5,6*). In this case-patient, CRS was likely the result of 2 rare events: his mother's lack of immunity after vaccination and her exposure to rubella in a highly immune population.

Table. Criteria of the Council of State and Territorial Epidemiologists (2009) met by patient with confirmed case of congenital rubella*

Criteria	Criteria met by case-patient
Clinical criteria	
Congenital heart disease†	Yes
Microcephaly	Yes
Developmental delay	Yes
Hepatosplenomegaly	Yes
Purpura	No
Cataracts/congenital glaucoma	No
Hearing impairment	No
Pigmentary retinopathy	No
Jaundice	No
Meningoencephalitis	No
Radiolucent bone disease	No
Laboratory criteria	
Demonstration of rubella IgM	Yes
Persistent rubella antibody in an infant	Yes
Positive rubella RT-PCR	Yes
Isolation of rubella virus	No

*RT-PCR, reverse transcription PCR. Source: (*4*).
†Usually patent ductus arteriosus or peripheral pulmonary artery stenosis.

Delays in diagnostic testing and reporting hindered the public health contact investigation of this case. Because the investigation began ≈1 year after the maternal exposure period, the persons involved had limited recall. Earlier diagnosis and reporting could have improved our contact investigation and provided for earlier isolation of the case-patient.

We were unable to identify a maternal rubella exposure. Given rubella's elimination from the United States, we speculate that the case-patient's mother may have had contact with an international traveler from a region with circulating rubella. In the years following documentation of rubella elimination in the United States, surveillance has identified a small number of circumstances in which foreign visitors without confirmed rubella transmitted the virus to US residents.

Several features of the laboratory testing bear further examination. The persistence of rubella IgM at 12 months has previously been reported in CRS cases (*7*). The child's rubella IgG level did not decline as would be expected as a result of the loss of maternal antibody; this lack of decline is consistent with rubella infection. In addition, the case-patient's serologic results were most consistent with CRS, given his clinical features, his low risk for postnatal rubella infection, and the fact that he was not vaccinated. When the infant was 7.5 months of age, RT-PCR was positive for 1 of 2 specimens. However, when the infant was 10 and 12 months of age, RT-PCR was negative, as were culture results when the infant was 7.5 and 10 months of age. These findings are consistent with the diagnosis of CRS because for ≈50% of children with CRS, culture results are negative by 3 months of age (*8*).

We found no evidence of secondary rubella transmission. A model has suggested that to interrupt rubella transmission, ≥87.5% of the population must be immune (*9*). On the basis of the 2010 National Immunization Survey, 86.1% (95% CI 80.4%–91.8%) of New Jersey children 19–35 months of age had received ≥1 doses of MMR vaccine, and 92.6% (95% CI 89.1%–96.1%) of adolescents 13–17 years of age had received ≥2 doses (*10*,*11*). Continued rubella elimination relies upon the maintenance of high levels of immunity in the population; thus, public health professionals should continue to strive to achieve high levels of rubella immunization in their communities.

Despite its elimination from the United States, rubella infection and CRS continue to occur rarely: 6 cases of CRS have been reported to CDC since 2004. Clinicians should remain vigilant to the possibility of rubella and CRS and immediately notify public health authorities when either is suspected.

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Dr Pitts is a Research and Clinical Fellow at Johns Hopkins University, Baltimore, MD. She is interested in infectious disease epidemiology and the use of surveillance data to inform public health decision-making.

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Address for correspondence: Samantha I. Pitts, Division of General Internal Medicine, Johns Hopkins University School of Medicine, 2024 E Monument St, Suite 2-602, Baltimore, MD 21287; email: spitts4@jhmi.edu

Lethal Factor and Anti-Protective Antigen IgG Levels Associated with Inhalation Anthrax, Minnesota, USA

Mark D. Sprenkle, Jayne Griffith, William Marinelli, Anne E. Boyer, Conrad P. Quinn, Nicki T. Pesik, Alex Hoffmaster, Joseph Keenan, Billie A. Juni, and David D. Blaney

Bacillus anthracis was identified in a 61-year-old man hospitalized in Minnesota, USA. Cooperation between the hospital and the state health agency enhanced prompt identification of the pathogen. Treatment comprising antimicrobial drugs, anthrax immune globulin, and pleural drainage led to full recovery; however, the role of passive immunization in anthrax treatment requires further evaluation.

Inhalation anthrax is a life-threatening disease caused by inhalation of *Bacillus anthracis* spores. In August 2011, the Minnesota Department of Health was contacted regarding a patient with pneumonia who had blood cultures positive for nonhemolytic gram-positive rod–shaped bacilli. We report the third naturally acquired case of inhalation anthrax reported in the United States since 1976 (*1*,*2*).

Case Report

A 61-year-old man with a history of impaired glucose tolerance, hypertension, and chemical pneumonitis without chronic lung disease sought care at a community hospital in northwestern Minnesota after experiencing 2 days of fever, productive cough, and exertional dyspnea. During recent travel through parks in the western United States, he had been exposed to animal antlers and hides, wild bison, and donkeys.

On hospital admission, the patient's temperature was 37.3°C, blood pressure 148/72, pulse 100, respiratory rate 20, and room air oxygen saturation 89% (median 95%, range 65%–100%). The right lung base had diminished breath sounds and apical crackles. Laboratory tests (Table 1) and chest imaging (Figure 1) were performed. Blood samples for culture were obtained, and ceftriaxone (2 g) and azithromycin (500 mg) (Table 2) were administered intravenously (IV) for presumed community-acquired pneumonia (CAP).

On hospital day 2, the patient had increasing tachycardia and higher oxygen requirements. A computed tomographic scan of the chest revealed multiple abnormalities (Figure 1). A *Bacillus* species was isolated from blood cultures and sent to the Minnesota Department of Health Public Health Laboratory (MDHPHL) for identification, and IV ciprofloxacin was initiated. On hospital day 3, the patient's condition continued to decline (Table 1). MDHPHL identified *B. anthracis* in the blood cultures, and meropenem and vancomycin were added to the treatment regimen. The isolate was sent to the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA).

On hospital day 4, the patient was transferred to a referral center with progressive respiratory failure requiring endotracheal intubation. He remained hemodynamically stable without need for vasopressor therapy. IV ciprofloxacin was continued, and IV rifampin and clindamycin were administered (Table 2). A chest tube was placed in the right pleural space, and 550 mL of serosanguineous fluid was drained during the initial 24 hours. Pleural fluid analysis showed a leukocyte count of 3,389 cells/mL (neutrophils 38%, lymphocytes 56%, monocytes 6%), a lactate dehydrogenase level of 352, and negative Gram stain results. On day 5, thoracentesis of the left pleural space drained 250 mL of serosanguineous fluid. Anthrax immune globulin (AIG) was requested from CDC on day 4 and administered on day 5 without adverse reaction.

The patient's disease course was complicated by nonoliguric renal failure; serum creatinine peaked at 1.5 mg/dL. On day 8, rifampin was discontinued; meropenem, which had been discontinued on day 5, was resumed for prophylaxis against nosocomial infection and improved central nervous system coverage of *B. anthracis* infection. After stabilization, the patient was maintained on volume control ventilation: tidal volume 500 mL, positive end–expiratory pressure 10 cm H_2O, and fraction of inspired oxygen 0.50. After day 6, renal dysfunction, hyponatremia, and thrombocytopenia gradually resolved. The patient was extubated on day 11, and the chest tube was removed on day 13; left-sided pleural effusion did not recur. He completed a 10-day course of clindamycin and a 14-day course of meropenem. Upon discharge on day 26, he was prescribed oral ciprofloxacin to complete 60 days of therapy.

MDHPHL identified the blood culture isolate as *B. anthracis* (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/2/13-0245-Techapp1.pdf). CDC performed

Author affiliations: Hennepin County Medical Center, Minneapolis, Minnesota, USA (M.D. Sprenkle, W. Marinelli); Minnesota Department of Health, St. Paul, Minnesota, USA (J. Griffith, B.A. Juni); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (A.E. Boyer, C.P. Quinn, N.T. Pesik, A. Hoffmaster, D.D. Blaney); and University of Minnesota, Minneapolis (J. Keenan)

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Table 1. Laboratory test results for patient being assessed and treated for inhalation anthrax, Minnesota, USA, 2011*

Test	Patient value, day of hospitalization†						Reference value‡
	1	3	4	5	7	9	
Hematologic							
White blood cell count, × 10^3/mm^3	7.6	13.9	27.4	12.0	10.7	15.6	4.0–10.0
Neutrophils, %	73.4	82.3	81.0	70.9	75.0	75.0	34.0–70.0
Lymphocytes, %	13.2	9.3	8.0	19.8	15.3	17.0	20.0–40.0
Monocytes, %	12.4	8.0	9.0	7.2	5.4	3.0	4.0–10.0
Hemoglobin, g/dL	17.0	17.0	16.6	14.5	12.8	12.7	13.1–17.5
Platelet count, × 10^3/mm^3	132	118	125	117	211	333	150–400
Serum chemistry							
Sodium, mEq/dL	139	129	121	125	128	132	135–148
Potassium, mEq/dL	3.9	4.2	3.8	3.7	3.4	4.3	3.5–5.3
Chloride, mEq/dL	95	89	84	86	92	98	100–108
Bicarbonate, mEq/dL	36	34	31	33	30	31	22–30
Serum urea nitrogen, mg/dL	17	16	18	29	40	27	8–22
Creatinine, mg/dL	1.0	0.9	0.8	1.2	1.3	0.9	0.7–1.4
Glucose, mg/dL	248	229	158	198	186	189	70–100
Calcium, mg/dL	8.7	8.6	8.6	7.7	7.3	7.3	8.5–10.5
Lactate, mmol/L	ND	ND	2.2	ND	ND	ND	0.7–2.1
Albumin, g/dL	3.6	3.0	ND	2.4	2.1	2.1	3.4–5.0
Alkaline phosphatase, U/L	58	60	ND	63	76	80	38–126
Aspartate aminotransferase, U/L	43	37	ND	65	44	35	5–40
Alanine aminotransferase, U/L	92	102	ND	144	141	97	7–56
Total bilirubin, mg/dL	0.6	0.6	ND	0.5	1.1	0.6	0.1–1.3
Troponin I, ng/mL	ND	ND	ND	0.025	ND	ND	<0.034
Coagulation							
Prothrombin time, s	ND	ND	11.5	11.6	ND	ND	9.0–12.5
International normalized ratio, s	ND	ND	1.1	1.1	ND	ND	0.8–1.2
Partial thromboplastin time, s	ND	ND	ND	27.9	ND	ND	25.0–38.0
Dimerized plasmin fragment, ng/mL	ND	ND	ND	670	ND	ND	<229

*ND, not done.
†Initial admission was to a community hospital; on day 4, the patient was transferred to a tertiary referral center.
‡Reference values during patient's hospitalization at a tertiary referral center.

susceptibility testing using broth microdilution (online Technical Appendix).

In compliance with the investigational new drug protocol for AIG administration, we obtained serial serum samples to assess levels of lethal factor (LF) and anti–protective antigen (PA) IgG. LF levels were determined in additional fluid from the patient's right pleura. LF endoproteinase activity was quantified by using mass spectrometry (*3*). We used an ELISA to determine serum anti-PA IgG levels before and after AIG administration (*4*). The lower limit of quantification (LLOQ) for this assay is 3.7 μg/mL. Seroconversion was defined as a >4-fold increase over the LLOQ (*5*).

Antimicrobial susceptibility testing (online Technical Appendix) performed on the *B. anthracis* isolate showed a MIC of penicillin of ≤0.015 μg/mL and MIC of ciprofloxacin of 0.12 μg/mL. The patient's initial plasma LF level was 58.0 ng/mL, which declined to 1.5 ng/mL before AIG administration: pleural fluid LF was 16.2 ng/mL at initial drainage and declined steadily (Figure 2). Before AIG administration, no anti-PA IgG was discernable because these quantifications were below the LLOQ (Figure 2). Immediately after AIG administration, anti-PA IgG reached maximal value of 160.5 μg/mL and maintained a plateau thereafter.

Conclusions

We describe the second US case of naturally acquired inhalation anthrax since the bioterrorism-related infections of 2001 and the third known case worldwide in which the

Table 2. Antimicrobial drugs administered to patient with inhalational anthrax diagnosed on hospital day 3, Minnesota, USA, 2011*

Antimicrobial drug	Dose	Route, frequency	Hospital and posthospitalization days medication administered
Ceftriaxone	2.0 g	IV, every 24 h	1–4
Azithromycin	500 mg	IV, every 24 h	1–3
Ciprofloxacin	400 mg	IV, every 12 h	2–26
Meropenem	1.0 g	IV, every 8 h	3–4
Vancomycin	2.0 g	IV, once	3
Clindamycin	900 mg	IV, every 8 h	4–14
Rifampin	300 mg	Enteral, every 12 h	4–8
Meropenem	1.0 g	IV, every 8 h	8–22
Ciprofloxacin	500 mg	Oral, every 12 h	26; PH 1-35†

*IV, intravenous; hospital day, number of days in hospital including day of admission; PH, posthospitalization.
†IV medication was discontinued and oral medication was started on day of discharge (hospital day 26) and continued for 35 additional days to complete 60 days of therapy.

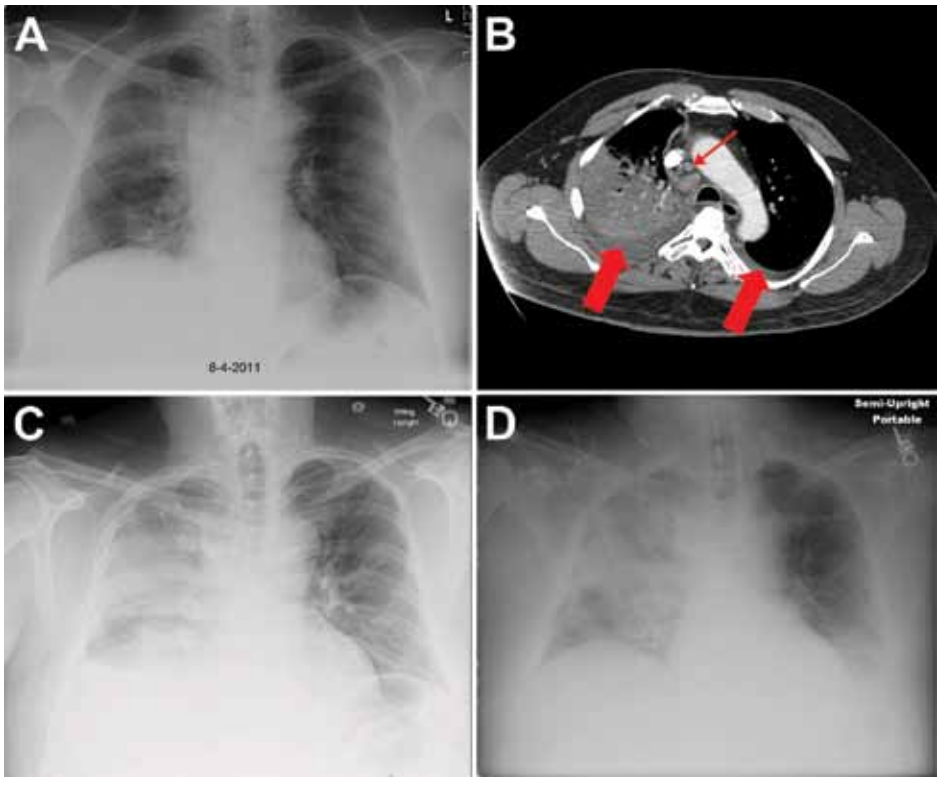

Figure 1. Chest x-ray and computed tomographic scan images for a patient with inhalation anthrax, Minnesota, USA. A) On hospital day 1, the x-ray image revealed a right upper lobe infiltrate and widening of the mediastinum. B) On hospital day 2, computed tomographic scan of the chest with intravenous contrast showed dense consolidation of the right upper lobe, mediastinal adenopathy (small arrow), and bilateral pleural effusions (large arrows). C) By hospital day 4, progressive infiltrates in the right lung were present. D) By day 6, an increasing left pleural effusion was evident.

patient received AIG (*2*,*6*). Before this case, the most recent case in the United States had occurred in Pennsylvania during 2006 (*2*). That patient had a 3-day prodromal illness, and initial plasma LF (294.3 ng/mL) and pleural fluid LF (543.2 ng/mL) levels substantially higher than those reported here. Seroconversion to anti-PA IgG occurred before AIG administration in the 2006 case, possibly because of a longer interval between symptom onset and AIG infusion (10 vs. 6 days). The 2006 patient also survived. A more recent case occurred in London during 2008 (*6*). The patient, who appears to have been in the fulminant phase of illness when tailored antimicrobial drug therapy was initiated on hospital day 4, died on day 7 despite AIG treatment (*6*).

Early recognition of inhalation anthrax is crucial to patient survival. Kuehnert et al. (*7*) proposed a scoring system derived from multivariate analysis to distinguish inhalation anthrax from CAP on the basis of clinical features at disease onset. In the case-patient described here, 3 of 5 identifying variables were present: elevated alanine aminotransferase/aspartate aminotransferase, normal leukocyte count, and tachycardia. The 2 remaining possible variables, low serum sodium level and nausea/vomiting were not present. This correlated with a sensitivity of 82% and specificity of 81% for diagnosing anthrax rather than CAP. Mediastinal widening, exhibited in the patient in this report, has also been proposed as a characteristic that can distinguish anthrax from CAP. When anthrax patients were compared with an age-, sex-, and race-matched control population, mediastinal widening occurred in 82% of anthrax patients and 8% of CAP patients (*8*).

A systematic review of inhalation anthrax cases showed improved survival if antimicrobial drugs were initiated during the prodromal rather than fulminant phase of illness: 75% of patients who survived and 10% of those who died were administered antimicrobial drugs in the prodromal phase (*9*). A new staging system for inhalation anthrax was proposed that divides the prodromal period into early and intermediate progressive stages (*10*). Early diagnosis facilitated implementation of multi–antimicrobial drug therapy during the intermediate progressive stage, which is associated with increased survival rate (67% vs. 21%) (*9*). For the case discussed here, systems to facilitate cooperation between a community hospital and state health agency enabled definitive identification of *B. anthracis* within 24 hours of culture becoming positive, leading to specific interventions including combination antimicrobial therapy, pleural drainage, and AIG administration.

Drainage of pleural fluid has also been associated with increased survival (83% vs. 9%) (*9*); it was performed shortly after the current patient's illness reached the late fulminant stage. Besides elevated LF levels in pleural fluid of recent US case-patients, patients with bioterrorism-related cases had large quantities of *B. anthracis* cell wall antigens, which further supports use of drainage procedures (*11*).

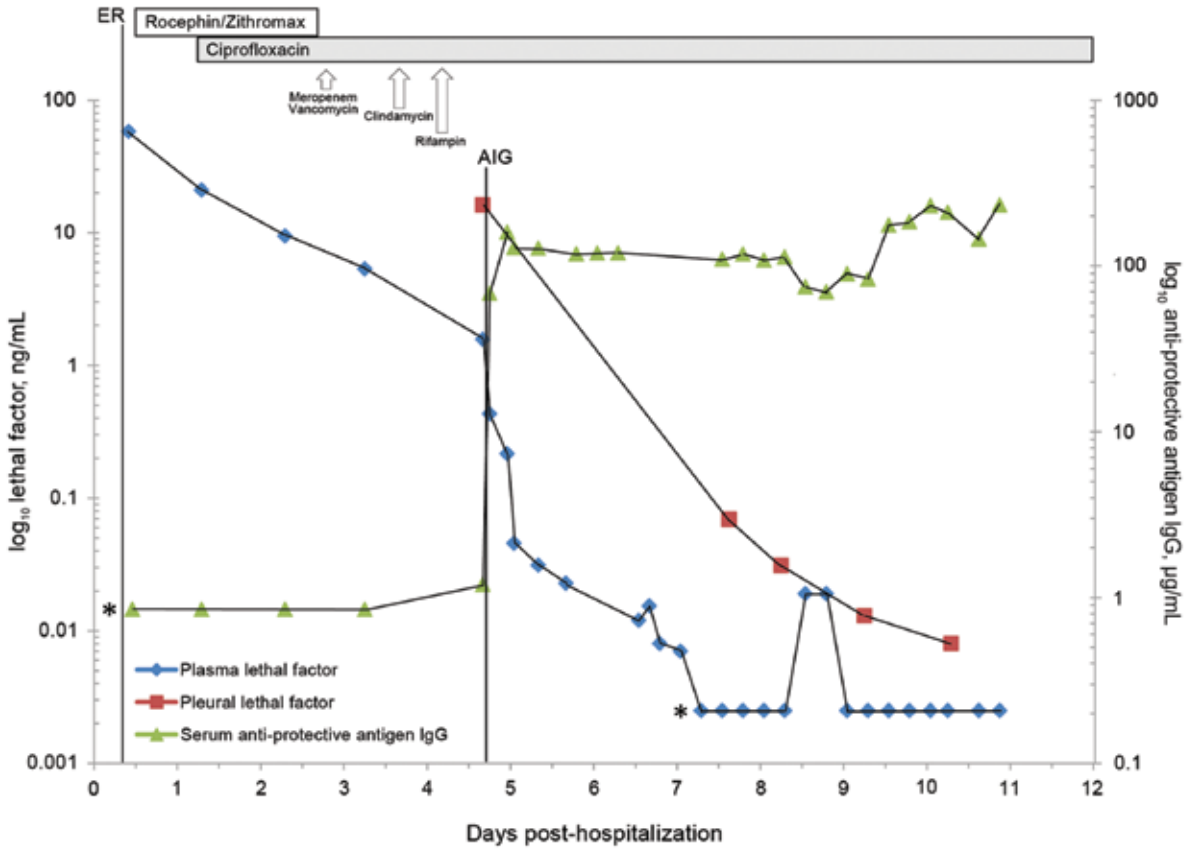

Figure 2. Plasma and pleural fluid lethal factor levels and anti-protective antigen IgG (AIG) levels for a patient from the time of examination in the community hospital emergency department to discharge from the tertiary referral center. Asterisks indicate that anti-protective AIG levels obtained before anthrax immune globulin administration were below the lower limit of quantification. The vertical dashed line represents the time of anthrax immunoglobulin administration. The patient's initial plasma lethal factor level was 58.0 ng/mL and declined to 1.5 ng/mL before AIG administration. Pleural fluid LF was 16.2 ng/mL at initial drainage and declined steadily.

Although no clinical studies have reported on efficacy of passive immunization of humans against anthrax as treatment, mortality rates have been reduced in studies of inhalation anthrax in which animals were given polyclonal antibodies against PA (*12*). AIG administration in the current case was associated with a reduction in toxemia, although the role of passive immunization in anthrax treatment needs further evaluation.

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Dr Sprenkle is the Chief of Pulmonary and Critical Care Medicine at Hennepin County Medical Center, Minneapolis, Minnesota. His primary research interest is in techniques for noninvasive hemodynamic assessment of critically ill patients.

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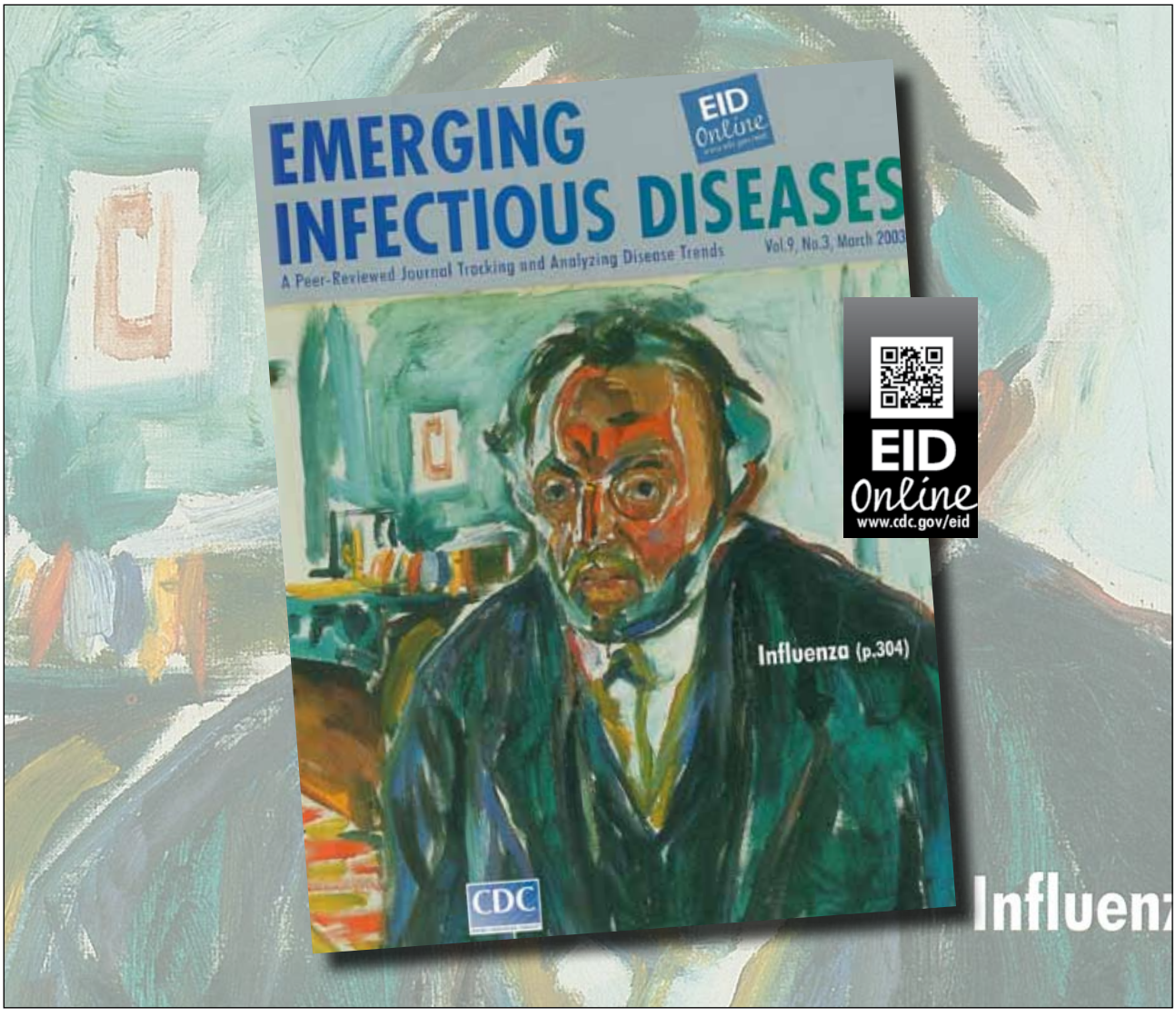
Address for correspondence: Mark D. Sprenkle, University of Minnesota School of Medicine, Pulmonary G-5, 701 Park Ave, Minneapolis, MN 55415, USA; email: spren005@umn.edu



Molecular Detection of *Diphyllobothrium nihonkaiense* in Humans, China

Shanhong Chen,[1] Lin Ai, Yongnian Zhang, Jiaxu Chen, Weizhe Zhang,[1] Yihong Li, Maki Muto,[1] Yasuyuki Morishima, Hiromu Sugiyama, Xuenian Xu,[1] Xiaonong Zhou,[1] and Hiroshi Yamasaki[1]

The cause of diphyllobothriosis in 5 persons in Harbin and Shanghai, China, during 2008–2011, initially attributed to the tapeworm *Diphyllobothrium latum*, was confirmed as *D. nihonkaiense* by using molecular analysis of expelled proglottids. The use of morphologic characteristics alone to identify this organism was inadequate and led to misidentification of the species.

Diphyllobothriosis is a fishborne cestodiasis caused by infection with adult tapeworms belonging to the genus *Diphyllobothrium* Cobbold, 1858 (*1–5*); the most frequent etiologic agents are *D. latum* and *D. nihonkaiense*. Humans are infected by ingesting raw or undercooked fish infected with larval plerocercoids. Adult tapeworms can grow to ≈2–10 m in length in the human small intestine (*1–6)*. Despite the large size of the tapeworms, clinical symptoms can be absent or mild and include mild abdominal pain, watery diarrhea, and abdominal discomfort (*3–7*). *D. latum* infection can also cause vitamin B_{12}-deficiency anemia (*5*).

Diphyllobothriosis caused by *D. nihonkaiense* has been extensively reported in Japan (*3,4*), but it has also occurred autochthonously in South Korea (*8*) and the Far Eastern Federal District of Russia (originally reported as *D. klebanovskii* infection [*9*]). Sporadic cases have been reported in Europe (*6*), North America (*10*), and New Zealand (*7*) in recent years.

In mainland China, 15 cases of diphyllobothriosis among humans have been reported since the first report in 1927 through 2012; the etiologic species was identified as *D. latum* by morphologic characteristics (*11–13*; Table) and molecular markers (*14,15*). No cases of diphyllobothriosis had been reported in large cities such as Beijing and Shanghai during 1954–2007 (*11*). However, we confirm 4 cases of *D. nihonkaiense* infection in humans in Shanghai, previously identified as *D. latum* infection, during 2008–2011, as well as 1 case in the moderately populous city of Harbin in Heilongjiang Province.

The Study

We examined 5 recent infections of humans with *Diphyllobothrium* spp. (Table, cases 12, 16–19) that occurred in China. Each case had been originally reported as a *D. latum* infection on the basis of morphologic identification only. Case 12 was reported in Harbin City, Heilongjiang Province, in 2009 (*13*). The 4 cases reported in Shanghai were diagnosed at the National Institute for Parasitic Diseases, Shanghai, on the basis of morphologic features of passed strobila. Case-patient 16 lived in Japan, but it was suggested that he acquired the tapeworm in Shanghai where he had frequently eaten raw salmon. Case-patient 17 was a 10-year-old girl from Japan. Whether she became infected in Shanghai or Japan was unclear because of lack of information. Case-patients 18 and 19 acquired the infection in Shanghai because they had never been abroad.

Because all patients in Shanghai had eaten raw salmon, we decided to re-examine how the causative *Diphyllobothrium* spp.were identified. *D. latum* infection is associated with consumption of freshwater fish such as perch (*Perca* spp.), not Pacific salmon (*Oncorhynchus keta*, *O. masou*) and Atlantic salmon (*Salmo salar*) in the Northern Hemisphere (*1–5*). To expand diagnostic parameters and clarify the point of misidentification, we re-identified *Diphyllobothrium* spp. by examining the tapeworms' morphologic features and using a molecular marker. In a sample from case-patient 12, only proglottids stained with acetic acid–carmine were available for testing by both methods (Figure 1, panel A). Proglottids obtained from 4 case-patients in Shanghai were preserved in either 10% formalin (case-patient 16) or 70% ethanol (case-patients 17–19) after collection (Table). Parts of the proglottids were embedded in paraffin, and sagittal sections were prepared for morphologic observation.

For molecular identification of the *Diphyllobothrium* spp., genomic DNA samples were extracted from specimens by using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). In specimens from case-patients 17–19, the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*, 1,566 bp) was amplified by PCR by using *Ex Taq* DNA polymerase (Takara Bio, Shiga, Japan) (*7*). In formalin-fixed samples of proglottids from case-patients 12

Author affiliations: Chinese Center for Disease Control and Prevention, Shanghai, China (S. Chen, L. Ai, Y. Zhang, J. Chen, X. Xu, X. Zhou); Harbin Medical University, Harbin, Heilongjiang Province, China (W. Zhang, Y. Li); and National Institute of Infectious Diseases, Tokyo, Japan (M. Muto, Y. Morishima, H. Sugiyama, H. Yamasaki)

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[1]These authors contributed equally to this article.

Table. Cases of infection with *Diphyllobothrium* species in humans reported in mainland China, 1927–2012*

Case no.	Residence/place of eating fish, if different	Proglottids expelled	Suspected source of infection	Proglottid specimen fixative	Morphologic identification	Molecular identification	GenBank accession no. for *cox1* gene	Reference
1–4	Heilongjiang	NA	NA	NA	*D. latum*	NT	NA	(*11*)
5	Beijing	NA	NA	NA	*D. latum*	NT	NA	(*11*)
6	Shanghai	NA	NA	NA	*D. latum*	NT	NA	(*11*)
7	Beijing (returned from United States)	NA	NA	NA	*D. latum*	NT	NA	(*11*)
8	Guangzhou (returned from Argentina)	NA	NA	NA	*D. latum*	NT	NA	(*11*)
9	Heilongjiang	NA	Raw fish	NA	*D. latum*	NT	NA	(*11*)
10	Jilin	NA	Raw fish	NA	*D. latum*	NT	NA	(*11*)
11	Fujian (lived in Yokohama, Japan, until 1996)	2003 Jan	*Plecoglossus altivelis*	NA	*D. latum*	NT	NA	(*12*)
12	Heilongjiang	2009 Jan	Raw fish	10% formalin	*D. latum*	*D. nihonkaiense*	AB684625	(*13*) and this study
13	Heilongjiang	NA	Salmon	NA	*D. latum*	*D. latum*	NA	(*14*)
14	Jilin	NA	Salmon	NA	*D. latum*	*D. latum*	NA	(*14*)
15	Shanghai, 2008-2011/Japan, China	2011 Dec	Raw sea and freshwater fish	NA	*D. latum*	*D. latum*	NA	(*15*)
16	Shanghai/Japan (returned from Japan in June 2008; ate raw salmon in Shanghai)	2008 Oct	Raw salmon	10% formalin	*D. latum*	*D. nihonkaiense*	AB684624	This study
17	Shanghai	2011 Sep	Raw salmon	70% ethanol	*D. latum*	*D. nihonkaiense*	AB684621	This study
18	Shanghai. Ate raw salmon in April 2011	2011 Jun	Raw salmon	70% ethanol	*D. latum*	*D. nihonkaiense*	AB684622	This study
19	Shanghai. Ate raw salmon in 2011	2011 Jul	Raw salmon	70% ethanol	*D. latum*	*D. nihonkaiense*	AB684623	This study

*Identification of *Diphyllobothrium* spp. was performed by morphologic identification alone in cases 1–11; organisms in each case were identified as *D. latum*. Cases 12–19 were assessed by morphologic and molecular identification; morphologic identification of all specimens was *D. latum*. Molecular identification varied from morphologic findings in 5 of 8 tested specimens. NA, not available; NT, not tested.

and 16, DNA degradation caused by the fixative meant that only shorter *cox1* fragments (249 bp, corresponding to sites 880–1128 of *cox1*) could be amplified successfully by PCR by using KOD FX DNA polymerase (Toyobo, Osaka, Japan). DNA sequencing of amplicons was performed with a 3100-*Advant* Genetic Analyzer or 3730 xl DNA Analyzer (Life Technologies, Foster City, CA, USA). Phylogenetic analysis was performed by the maximum likelihood method (MEGA 5.05, http://megasoftware.net/mega.php) and Bayesian inference (MrBayes version 3.1.2, http://mrbayes.sourceforge.net/). Clades were assessed by bootstrap resampling (1,000 replicates) and a posterior probability (10^6 generations) for the maximum likelihood and Bayesian inference trees, respectively. *Diphyllobothrium* spp. isolated from case-patients 12 and 16 were identified on the basis of sequence identity (%) by performing a BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis of a DNA Data Bank of Japan (www.ddbj.nig.ac.jp).

Accurately identifying the *Diphyllobothrium* spp. isolated from case-patient 12 on the basis of morphologic features alone was difficult (Figure 1, panel A). In Figure 1, panels B–E show the sagittal sections of the proglottids from case-patients 16–19. The angle formed by the cirrus sac and the anterior–posterior axis of the proglottids was used as a criterion for differentiating *D. latum* from *D. nihonkaiense* (*1*), even though this criterion is not considered definitive: the angle is usually horizontal in *D. latum*, but oblique in *D. nihonkaiense*. Nonetheless, in this study, on the basis of morphologic criteria, tapeworms from case-patients 16, 17, and 19 were identified as *D. latum* (Figure 1, panels B, C, and E) and the tapeworm found in case-patient 18 was identified as *D. nihonkaiense* (Figure 1, panel D).

Phylogenetic trees based on the complete *cox1* nucleotide sequences showed the same topologies in maximum likelihood and Bayesian inference analyses, implying that the 3 isolates from persons in China (case-patients 17–19; GenBank accession numbers AB684621–AB648623) are *D. nihonkaiense* (Figure 2). The 2 isolates (AB684625 and AB684624) from case-patients 12 and 16, respectively, were excluded from the analysis because they produced smaller PCR products, but they were identified as *D. nihonkaiense* on the basis of their 99%–100% sequence identity to *D. nihonkaiense*.

The 5 *Diphyllobothrium* spp. tapeworms examined in this study were previously identified as *D. latum* on the basis of morphologic characteristics, as were 3 of the 5 when we re-examined their morphologic characteristics. However, the 5 etiologic agents were confirmed as *D. nihonkaiense*

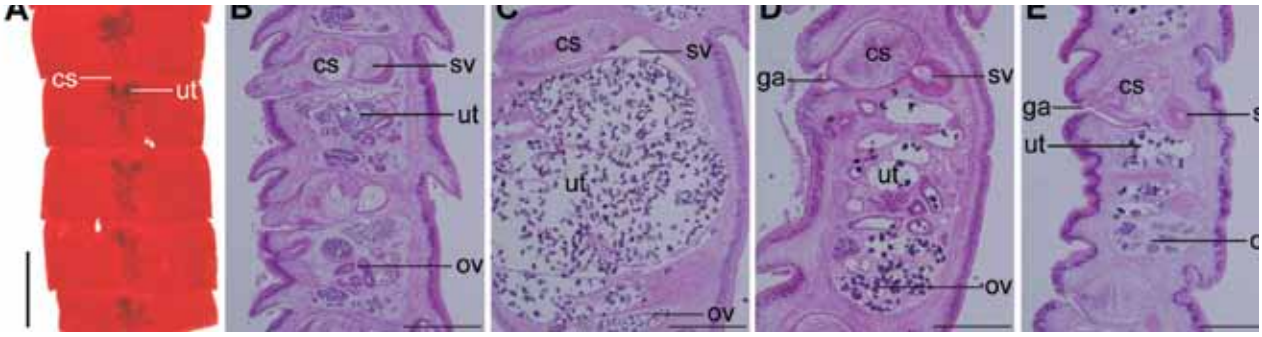

Figure 1. Diphyllobothriid samples examined in the present study, China, 2008–2012. A) Proglottids stained with acetic acid–carmine from case-patient 12. B–E) Sagittal sections of proglottids stained with hematoxylin-eosin from case-patients 16–19. cs, cirrus sac; ut, uterus; sv, seminal vesicle; ov, ovary; ga, genital atrium. Scale bar in panel A represents 2 mm; scale bars in panels B–E represent 500 μm.

by molecular analysis. This discrepancy in the identity of these agents may be attributed to the morphologic similarities between the species and the century-long confusion between the parasite *D. latum* and the parasite that caused human diphyllobothriosis associated with the consumption of Pacific salmon in Japan (*1*–*3*). Diphyllobothriosis caused by *D. nihonkaiense* has also been reported in South Korea (*8*) and in the Far Eastern Federal District of Russia (*9*) and is considered to be autochthonous and linked to the consumption of wild Pacific salmon in these regions. Therefore, some cases of diphyllobothriosis reported in mainland China were probably caused by infections with *D. nihonkaiense*; case-patient 12 (*13*) in this study is considered to have had such a case. However, a recent report stating that the causative species of 2 diphyllobothriosis cases in northeastern China was *D. latum,* which suggests that *D. latum* is also indigenous to mainland China (*14*).

Conclusions

We confirmed human diphyllobothriosis caused by *D. nihonkaiense* in mainland China by using a mitochondrial DNA marker. Reassessment of a case in Harbin revealed that some, if not all, of the autochthonous diphyllobothriosis cases were likely initially misdiagnosed as *D. latum* infection because of morphologic similarities between *D. nihonkaiense* and *D. latum* tapeworms. Consequently, molecular analysis is indispensable not only for avoiding diagnostic confusion among *Diphyllobothrium* spp., but also for facilitating the acquisition of reliable epidemiologic and epizootic information and improving clinical relevance and preventive controls for diphyllobothriosis.

Information on diphyllobothriosis and warnings of the potential risks associated with infection by its local species should be disseminated to food handlers, restaurant owners, physicians, and consumers. Because

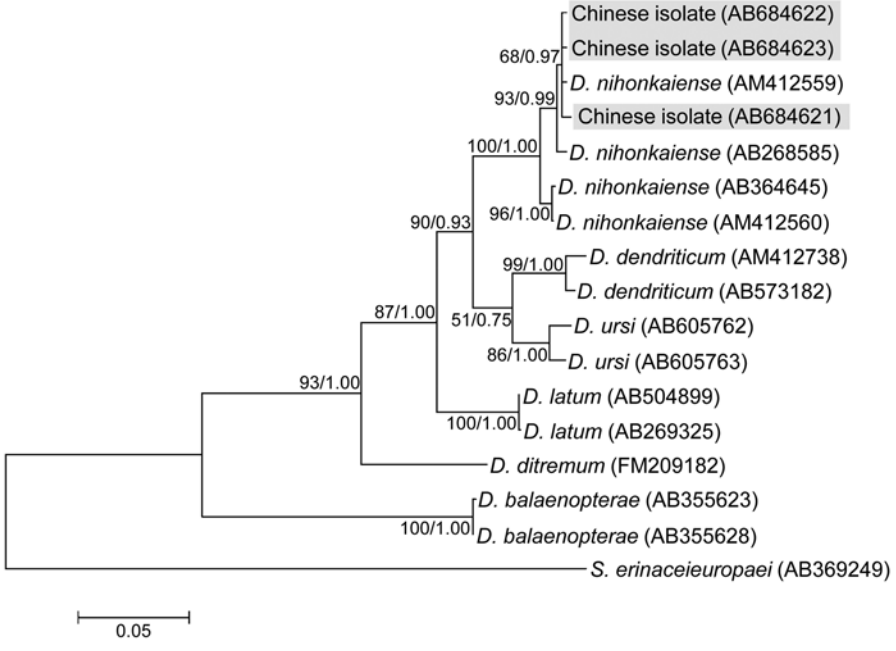

Figure 2. Phylogenetic tree constructed by using the maximum likelihood algorithm (Kimura's 2-parameter model) on the basis of the complete *cox1* sequences of isolates from *Diphyllobothrium* species found in persons in China and related *Diphyllobothrium* species. Numbers at nodes are bootstrap values (1,000 replicates) and posterior probabilities (10^6 generations) for maximum likelihood and Bayesian inference, respectively. *Spirometra erinaceieuropaei* was used as an outgroup. Scale bar indicates the number of base substitutions per site.

we cannot determine with certainty whether previous diphyllobothriosis cases in mainland China were caused by *D. latum* or *D. nihonkaiense*, identification of *Diphyllobothrium* spp. should be performed with care. In addition, studies on the distribution and sources of infection of *D. latum* and *D. nihonkaiense* on mainland China should be undertaken.

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Dr Chen is a professor at the National Institute for Parasitic Diseases in the Chinese Center for Disease Control and Prevention in Shanghai, China. Her research interests include parasite biology and immunodiagnosis of parasitic diseases.

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Address for correspondence: Hiroshi Yamasaki, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan; email: hyamasak@niid.go.jp

Low-Incidence, High-Consequence Pathogens

Ermias D. Belay and Stephan S. Monroe

Infectious diseases that have epidemic or pandemic potential and spread rapidly through a population within a short time are an ongoing public health concern in industrialized and developing countries. Frequent exposure to infectious sources (e.g., food, infected animals, and vectors) or a high rate of person-to-person spread facilitates spread of these diseases. Foodborne illnesses and seasonal influenza are notable examples. These diseases typically are associated with high rates of illness and substantial societal and economic cost but relatively low rates of death in otherwise healthy persons. Other infectious diseases, in contrast, may occur infrequently but are associated with high rates of death. The low incidence of some of these diseases reflects effective public health prevention measures, such as vaccinations. For a select group of zoonotic infectious diseases with high death rates, the low incidence reflects infrequent spillover from an animal reservoir into humans. Often, humans represent a dead-end host for these pathogens, and person-to-person transmission is rare if appropriate infection control practices are followed. Many of the pathogens highlighted in the current issue of Emerging Infectious Diseases can collectively be described as low-incidence, high-consequence pathogens. Selected diseases caused by these pathogens are described below.

Examples of High-Consequence Pathogens

Rabies

Rabies, one of the oldest known infectious diseases, is nearly 100% fatal and continues to cause tens of thousands of human deaths globally (*1*). Canine rabies has been eliminated in North America and many South American and European countries, but it is still the source of most human rabies cases in other areas, primarily in many African and Asian countries (*2*,*3*). Urbanization and lack of aggressive rabies elimination programs may have contributed to resurgence of canine rabies–associated human deaths in several provinces in China (*4*,*5*). In the United States, the number of human deaths from rabies has declined to an average of 3 cases per year during the last several decades (*1*). Apart from a few imported canine rabies cases, most human cases in the United States resulted primarily from bat rabies virus variants. Nonetheless, suspected or confirmed human exposures in the United States result in tens of thousands of postexposure prophylaxis regimens every year (*6*).

Smallpox

Despite the eradication of smallpox in 1980, concerns about intentional or accidental release of variola virus and its potential for severe disease and high rates of death (average 30%) have fueled research into the development of new diagnostic tests, therapies, and vaccines. Recent advances in biosynthetic technologies risking possible reconstitution of the virus have heightened these concerns. To bolster preparedness efforts, some countries have procured or retained smallpox vaccine supplies in their national stockpiles. Detection in the 1970s of a related orthopoxvirus that causes monkeypox (*7*), a similar but milder illness in humans that can be fatal in up to 10% of patients, raised concerns that this virus may replace the ecologic and immunologic niche created by the eradication of smallpox (*8*–*10*). Waning herd immunity after cessation of smallpox vaccination, which appears to cross-protect against monkeypox, might have facilitated spread of the virus in areas to which it is endemic (*10*). Ecologic factors, including changes in the environment and agent reservoirs, also might have contributed to changes in the incidence of monkeypox (*11*). However, in humans, monkeypox virus is less virulent and less transmissible than variola virus (*9*). Monkeypox can spread from person to person after prolonged contact with a patient or indirectly through exposure to body fluids or fomites contaminated with the virus (*12*). Monkeypox occurs endemically and in occasional outbreaks in central and western Africa, where the presumed natural reservoirs of the virus exist (*9*,*10*). The 2003 monkeypox outbreak in the United States clearly illustrated the potential for monkeypox virus or other zoonotic viruses to be transported great distances and spread quickly among immunologically naive populations (*9*).

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

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Hemorrhagic Fever Diseases

Hemorrhagic fever disease can be caused by several families of viruses, including arenaviruses, bunyaviruses, filoviruses, and flaviviruses. Filoviruses, which comprise 4 Ebola viruses pathogenic to humans and 1 Marburg virus species, have caused multiple outbreaks of hemorrhagic fever primarily in central and eastern Africa (*13*,*14*). Since 1976, 10 large filovirus outbreaks involving >100 persons have been documented from the Congo Basin, Gabon, Sudan, and Uganda (*15*). During these outbreaks, transmission chains resulted from direct person-to-person spread in households and nosocomial transmissions through contact with body fluids, dead bodies, or infectious fomites. Rigorous attention to appropriate infection control practices has proven to be effective in interrupting transmission. Filovirus outbreaks are associated with high case-fatality rates, generally ranging from 25% to 90% (*16*). Exposure to imported animals and subsequent person-to-person spread caused a cluster of Marburg hemorrhagic fever cases in several European cities in 1967 (*13*). Although filovirus infection is primarily limited to sporadic outbreaks in the African continent, recent infections of tourists from Europe and the United States have been documented (*13*,*17*). Despite the low incidence of filovirus infections, their occurrence in outbreaks with high rates of death and the potential to spread to places away from the original focus has prompted investments in enhancing surveillance mechanisms and focused research in developing effective therapies and vaccines. Basic research on filoviruses is limited by the need to conduct the work in high-containment laboratories.

Anthrax

In the United States, naturally occurring anthrax infection is more commonly reported in animals than in humans (*18*,*19*). Naturally occurring anthrax cases have been associated with direct contact with infected animals, occupational exposure during industrial processing of infected animal products, and production or use of drums made from contaminated hides. Anthrax cases resulting from these forms of exposure are very rare in much of the industrialized world because of improvements in hygiene and use of modern animal husbandry practices and reduced use of contaminated imported raw materials in industrial processing of animal products (*19*,*20*). Inhalation anthrax is the rarest of the 3 common forms of anthrax (cutaneous, gastrointestinal, and inhalation), but it has the highest case-fatality rate. During 2009–2010, anthrax among persons injecting heroin was reported primarily in Scotland but also in other European countries, adding a new route of infection (*20*). Spore formation, persistence in the environment, ease of dissemination, inhalation route of transmission, and associated high rates of death make *Bacillis anthracis* one of the most serious bioterrorism agents. The 2001 anthrax outbreak in the United States from the mailing of spore-laden envelopes highlighted the need for preparedness and countermeasure efforts to mitigate the effects of intentionally released *B. anthracis* (*21*).

Role of Infectious Disease Pathology

Unexplained sudden illnesses and deaths can be sentinels for the recognition of newly emerging infections and for the early detection of outbreaks of naturally occurring or intentionally released infectious agents. If laboratory tests are negative or inconclusive and a patient dies, thorough pathologic investigation aides in identifying the etiologic agent. Over the past several decades, the Centers for Disease Control and Prevention (CDC) has effectively used infectious disease pathology to diagnose the causes of sudden illness and death and to assist in identifying sources of multiple high-profile outbreaks, many of which were caused by new and reemerging etiologic agents.

When hantavirus pulmonary syndrome was first identified in 1993 in the Four Corners area of the southwestern United States, pathologic examination was critical for characterizing the illness and contributed to discovery of the etiologic agent (*22*). Autopsy and examination of biopsied tissues played a major role in the investigation of the bioterrorism-related anthrax cases in 2001 in the United States. During the early phase of the epidemic of severe acute respiratory syndrome (SARS) in 2003, investigations focused on characterizing the etiologic agent of what appeared to be a severe respiratory illness spreading among household contacts and to health care workers. Attempts to identify an infectious agent by standard laboratory testing failed to produce consistent results. As the number of SARS-related deaths increased, specimens examined by virus isolation techniques, electron microscopy, and pathologic examination led to identification of the causative agent of SARS as a novel coronavirus (*23*). More recently, infectious disease pathology has been instrumental in the investigation of a multistate outbreak of fungal meningitis associated with epidural injection of steroid preparations and in the identification of several organ transplant–associated infections, such as lymphocytic choriomeningitis virus, *Balamuthia* disease, and rabies.

Conclusions

We briefly described only selected examples of low-incidence, high-consequence pathogens. Many other similar infectious diseases with relatively low incidence but high-rates of death occur in many parts of the world. Besides those mentioned above, other low-incidence, high-consequence pathogens described in this issue of Emerging Infectious Diseases include Crimean-Congo hemorrhagic fever and Rift Valley fever. Ongoing surveillance and public health research of high-consequence pathogens are

critical for identifying their natural reservoirs, developing diagnostic tests, and devising appropriate control and prevention measures. Studying the molecular characteristics of these pathogens is critical to understanding their pathogenesis and ultimately to developing vaccines and antimicrobial drugs. Despite low incidence of these diseases, maintaining a preparedness posture to tackle the challenges posed by the emergence or reemergence of some of these pathogens should remain a priority. Public health resources are wisely spent by adequately preparing for the inevitable emergence or reemergence of infectious diseases that might currently be of low incidence but have the potential to spread to immunologically naive populations. The application of the age-old tools of pathology bolstered with a wide array of bioassays, developed by using modern advances in molecular diagnostics, has helped CDC tackle old infectious disease challenges and newly emerging and reemerging diseases. Advanced molecular detection approaches in concert with infectious disease pathology can play a prominent role in emergency preparedness and in addressing the public health challenges of the future.

Dr Belay is Acting Director of the Division of High-Consequence Pathogens and Pathology at CDC. His primary areas of research include prion diseases, Kawasaki disease, and the biology and epidemiology of high-consequence pathogens and public health policy regarding these pathogens.

Dr Monroe is Deputy Director of the National Center for Emerging and Zoonotic Infectious Diseases at CDC. His primary areas of research interest include high-consequence pathogens, foodborne and vector-borne illnesses, health care–associated infections, and public health policy related to them.

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Address for correspondence: Ermias D. Belay, Division of High-Consequence Pathogens and Pathology, Centers for Disease Control and Prevention, 1600 Clifton Rd, Mailstop A30, Atlanta, GA 30333 USA; email: ebelay@cdc.gov

Injectional Anthrax in Heroin Users, Europe, 2000–2012

To the Editor: Anthrax is a global zoonotic disease, but human infections are rare in countries of Western Europe. During 2009–2010, a total of 119 (47 laboratory-confirmed) drug-abuse–related cases of anthrax were reported in the United Kingdom and Germany (*1*). In these patients, the disease had an unusual manifestation. In contrast to acquiring the disease through typical routes of infection, leading to cutaneous, inhalation, or gastrointestinal anthrax, these patients became infected by injecting heroin (*1*–*3*). The term injectional anthrax was then coined to describe this new mode of infection. Patients with injectional anthrax show severe symptoms, and death rates are high. Of the 47 patients with confirmed cases of injectional anthrax acquired during the 2009–2010 outbreak, 19 died from the disease (*1*). However, this outbreak was not the first report of death caused by *B. anthracis* in an injectional drug (heroin) user; the disease was described in 1 person who died in Norway in 2000 (*3*).

Attempts to directly connect the United Kingdom cases to batches of anthrax-contaminated heroin were unsuccessful. No viable *B. anthracis* or DNA could be retrieved from the investigation's drug samples. However, PCR- or culture-positive samples were obtained from some patients and used for genotyping (*2*). Later, researchers from Arizona and the United Kingdom worked together to use a high-resolution molecular approach to genotype the 2009–2010 outbreak strains. This information was used to obtain insight into the epidemiology and likely geographic origin of the European outbreak strains.

All patients were infected by a single *B. anthracis* strain type (*2*,*4*) that belonged to the large Trans-Eurasian clade of *B. anthracis*. The whole genome of a representative strain of this type, Ba4599, had previously been sequenced (*2*). Strains related to strains associated with those isolated from European drug users, which belong to the A.Br. 008/011 canSNP cluster but are still genetically distinct, have so far only been identified from Turkey (*2*). However, more isolates from other relevant regions need to be investigated to confirm the likely geographic source.

In June 2012, after a 20-month gap, 2 new cases of injectional anthrax in heroin consumers were reported in Bavaria (*5*,*6*). Additional cases have been reported since then from Germany, Denmark, the United Kingdom, and France, leading to 26 deaths as of August 2013 (*7*).

Molecular phylogenetic methods were used to determine the genetic relatedness of these strains with Ba4599 (*8*,*9*). Genotyping results using canonical single nucleotide polymorphisms (SNPs) (Figure) (*2*,*4*) placed all of these strains along branch A.Br.008 within the Trans-Eurasian group of *B. anthracis* (*10*). Further hierarchical fine-scale typing of the isolates by interrogating SNPs that were discovered from the heroin-associated strain Ba4599 (*2*) indicated that all isolates are identical at these SNPs (Figure) (*7*). The initial strain isolated in Norway in 2000 also falls into this group (*7*). In addition, analysis by multiple locus variable number tandem repeats suggested that all investigated strains are closely related, differing at just 2 markers (*7*). Thus, we conclude that all injectional anthrax isolates likely came from the same source.

This reemergence of drug-related anthrax in Europe strengthens the view that heroin may provide a continuing route of entry for *B. anthracis* into Western Europe (*2*). Ideally, this unfortunate deadly incident could offer an opportunity to sensitize heroin users to the risks for severe infection and to educate public health officials to be vigilant for this rare disease. This study also shows the power of molecular genotyping approaches for trace-back analysis of infectious disease agents.

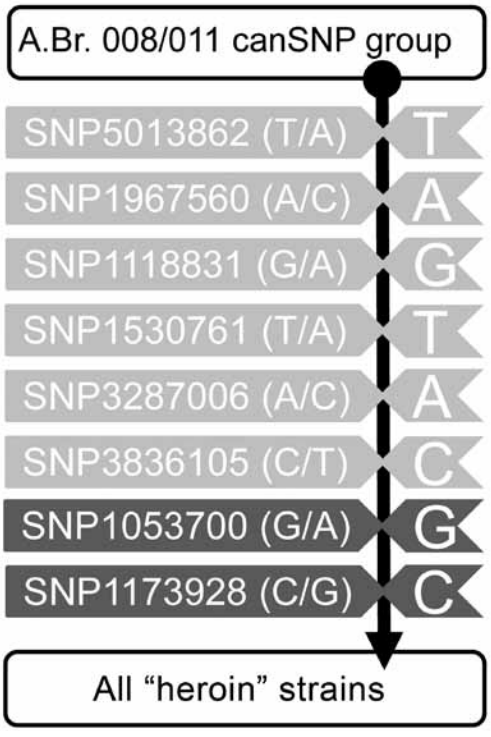

Figure. Diagram of single nucleotide polymorphism (SNP) assays used for bioforensic genotyping of heroin-associated *Bacillus anthracis* strains. Shown are the results of PCR-based SNP assays performed to elucidate the phylogenetic position of strains. Indicated at the top of the column is the whole strain pool of *B. anthracis* genotype A.Br. 008/011; the vertical black line indicates the assays in a direction of revealing increasing proximity to the heroin-associated strains. SNPs common to those of some strains from Turkey (*2*) are shown in light gray, and SNPs unique for the heroin-associated strains (*2*), including the isolate of 2000 from Norway, are depicted in dark gray (SNP designations and alleles are indicated).

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Matthias Hanczaruk, Udo Reischl, Thomas Holzmann, Dimitrios Frangoulidis, David M. Wagner, Paul S. Keim, Markus H. Antwerpen, Hermann Meyer, and Gregor Grass

Author affiliations: Bundeswehr Institute of Microbiology, Munich, Germany (M. Hanczaruk, D. Frangoulidis, M.H. Antwerpen, H. Meyer, G. Grass); University Hospital Regensburg, Regensburg, Germany (U. Reischl, T. Holzmann); and Northern Arizona University, Flagstaff, Arizona, USA (D. Wagner, P.S. Keim)

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Address for correspondence: Gregor Grass, Bundeswehr Institute of Microbiology, Neuherbergstrasse 11, 80937 Munich, Germany; email: gregorgrass@bundeswehr.org

Nasopharyngeal Bacterial Interactions in Children

To the Editor: Xu and colleagues (*1*) examined the nasopharyngeal bacterial colonization rates in children with acute otitis media (AOM) and in healthy children. They found that *Haemophilus influenzae* colonization was competitively associated with *Streptococcus pneumoniae* and *Morexella catarrhalis* colonization in children with AOM but was not associated with *S. pneumoniae* and *M. catarrhalis* colonization in healthy children. We have a serious concern regarding their analysis.

The authors calculated odds ratios (ORs) by considering a bacterial colonization as an outcome variable and another bacterial colonization as an exposure variable. They considered an OR >1 as the presence of synergistic associations between bacteria (i.e., co-colonization is more likely to occur than it would by chance) and OR <1 as the presence of competitive associations (i.e., co-colonization is less likely to occur than it would by chance). This inference may be justified in a population-based cross-sectional study. If 2 bacterial colonizations occur independently in a stationary population, the prevalence of co-colonization will be the product (multiplication) of each prevalence, and the OR between 2 bacterial colonizations in the population (OR_{pop}) will be 1 (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/2/12-1724-Techapp1.pdf).

However, the authors enrolled their case-patients according to clinical signs (i.e., AOM or healthy). Let us assume that case-patients are enrolled from a population of children during a time period of *t*. Let r_c be the risk for enrollment (that is, of developing the disease) among colonized children and r_n be the risk for enrollment

among noncolonized children. The OR among the enrolled case-patients ($OR_{case\text{-}patient}$) becomes r_n/r_c, which is the reciprocal of the risk ratio (RR) of developing the disease ($RR = r_c/r_n$) (online Technical Appendix). Usually, RR is >1 for diseased children and <1 for healthy children. Therefore, even in this independent colonization scenario, $OR_{case\text{-}patient}$ becomes <1 ("pseudo-competitive associations") in diseased children, and $OR_{case\text{-}patient}$ becomes >1 ("pseudo-synergistic associations") in healthy children. This is probably what the authors have observed in the study.

We cannot infer an association of multiple bacterial colonizations in a population despite an observed association in the diseased (or healthy) children, and this association is widely misunderstood (*2–4*). The authors' discussion regarding a potential emergence of *H. influenzae*, associated with AOM, after the introduction of pneumococcal conjugate vaccine is thus unjustifiable.

Motoi Suzuki, Bhim Gopal Dhoubhadel, Lay Myint Yoshida, and Koya Ariyoshi

Author affiliation: Institute of Tropical Medicine, Nagasaki University, Japan

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Address for correspondence: Motoi Suzuki, Department of Clinical Medicine, Institute of Tropical Medicine, Nagasaki University, Sakamoto 1-12-4, Nagasaki 852-8523, Japan; email: mosuzuki@nagasaki-u.ac.jp

In Response: The point made by Suzuki et al. (*1*) is an interesting one, but it is not directly relevant to our conclusions (*2*). To summarize, given bacterium 1 and bacterium 2, suppose OR[pop] is the odds ratio (OR) between bacterium 1 and bacterium 2 in the population. If r[c] and r[n] are the risk of enrollment among colonization-positive and colonization-negative children, respectively, then OR[case] = (r[n]/r[c]) OR[pop], so that OR[case] <1 can be attributed to a higher risk among colonized children.

The underlying assumption of the analysis is that enrollment risk is constant for both bacteria (alone or in combination). In fact, differential risk does exist, and it cannot be separated from the issue of the relative aggressiveness of the bacterium. This can be seen by considering the ORs in the Table.

When we compared the ORs between bacteria pairs for all subjects and children with acute otitis media (AOM), we found a large decrease for pairs involving nontypeable *Haemophilus influenzae* (NTHi), in contrast to the remaining pair, *Streptococcus pneumoniae*/ *Moraxella catarrhailis* (*Spn*/*Mcat*). We do not believe that these values are explainable by the effect described by Suzuki et al., especially when (as is done in that analysis) we assume a colonization-positive risk of enrollment r[c] that is independent of bacterium distribution. We also point out that these estimates are instructive, but not sufficient, because each pairwise comparison may depend on interactions with the third bacterium. We therefore used a statistical model (*2,3*) that permits the isolation of third-order effects by modeling co-occurrence rates of 2 bacteria while controlling for a third. This allowed us to reach our conclusion, which is primarily concerned with the specific role played by NTHi, and follows from the existence of a pattern in the reported ORs, rather than the absolute value of any single OR.

It is also instructive to examine the colonization rates for AOM versus number of AOM events (nAOM) subjects: p(*Spn*-nAOM) = 0.30; p(*Spn*-AOM) = 0.53; p(NTHi-nAOM) = 0.12; p(NTHi-AOM) = 0.48; p(*Mcat*-nAOM) = 0.36; p(*Mcat*-AOM) = 0.43. As we would expect, colonization rates of each bacterium are higher for AOM patients. What is of interest is that the colonization distribution is different for children with AOM, which suggests that NTHi is more aggressive than other bacteria in some sense, and this effect is made more precise by the statistical model we used. The essential point is that the issue of competitive association cannot be isolated from differential enrollment risks, which is what our analysis reports.

Table. Comparison of the odds ratios between bacterium pairs for all subjects and children with acute otitis media*

	Odds ratios, p values	
Bacteria pair	All subjects	Acute otitis media
NTHi/*Mcat*	0.94, p = 0.6935	0.47, p = 0.0006
NTHi/*Spn*	1.58, p = 0.0004	0.50, p = 0.001
Spn/*Mcat*	1.71, p<0.0001	1.53, p = 0.05

*NTHi, nontypeable *Haemophilus influenzae*; Mcat, *Moraxella catarrhailis*; Spn, *Streptococcus pneumoniae*.

Anthony Almudevar[1]
Author affiliation: University of Rochester Medical Center, Rochester, New York, USA

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Address for correspondence: Michael Pichichero, Rochester General Hospital Research Institute, 1425 Portland Ave, Rochester, NY 14621, USA: email: michael.pichichero@rochestergeneral.org

[1]On behalf of Qingfu Xu, Janet Casey, and Michael Pichichero.

Laboratory-acquired Buffalopox Virus Infection, India

To the Editor: In India, buffalopox virus (BPXV), a variant of vaccinia virus, is associated with severe disease outbreaks among buffaloes (*1,2*), cattle (*3*), and humans in contact with these animals (*1,4*). Most human BPXV infections occur in animal attendants and milkers (*1,4*). A similar type of vaccinia virus infection has also been reported from rural areas in Brazil (*5*). We report a case of laboratory-acquired infection with BPXV in a researcher in India. Clinical signs, symptoms, diagnosis, and management of this case highlight the need for observance and enforcement of strict biosafety measures within the laboratory.

A 28-year-old man (researcher) who was freeze-drying BPXV isolates in a laboratory in Hisar, India sustained a cut on his right palm through nitrile gloves by accidental piercing of shrapnel from a broken ampule. The virus being freeze-dried ($10^{5.5}$ 50% tissue culture infectious doses/mL) was isolated from a buffalo in Jalgaon, India, in 2010. The injured site on the palm was immediately cleaned with 70% ethanol and treated with povidone–iodine solution. No untoward reaction was observed ≤2 days postinjury. Erythema appeared at the injury site on postinjury day 3. Subsequently, a small vesicle developed on postinjury day 5 (Figure, panel A). This vesicle progressed into a pustule with a central area of necrosis by postinjury day 7 (Figure, panel B). On postinjury day 9, symptoms worsened (onset of high fever and general malaise and pain at the affected site), and the researcher sought medical care.

Physical examination showed high fever (104°F), unilateral axillary lymphadenopathy, and edema of the palm (Figure, panel C). Amoxicillin (500 mg, 2×/d), cephalexin (500 mg, 2×/d), and analgesic/antipyretic (paracetamol, 500 mg, 2×/d) were prescribed to control secondary complications caused by bacterial infection and pain.

The next day, the entire palm became cyanotic and edema increased (Figure, panel D). The researcher was then referred to a specialty hospital where the lesion was surgically excised on postinjury day 11 (Figure, panel E) under axial block anesthesia. Blood, necrotic tissue, pustular material, and swab specimens were obtained for laboratory examination. Postsurgery treatment included cleaning of the surgical site on alternate days and oral medication (amoxicillin/clavulanic acid, 625 mg; ibuprofen, 400 mg; paracetamol, 325 mg; and rabeprazole, 20 mg) for 5 days.

On postinjury day 19, the surgeon advised the patient to take cefuroxime (500 mg/d for 5 days) and use a topical ointment containing mupirocin to prevent a delay in healing (Figure, panel F). The lesion healed slowly, and by postinjury day 30, thickening and blackening of the skin was observed (Figure, panel G) that extended to a wider area by postinjury day 38, and the skin started to peel off by postinjury day 50. The entire skin of the palm sloughed off with complete healing by postinjury day 85, leaving a 20-mm blackened eschar over the area (Figure, panel H).

Clinical samples were subjected to laboratory examination. Virus was isolated from tissue samples in a Vero cell line during the first passage. BPXV infection was confirmed by PCR amplification of orthopoxvirus-specific A type inclusion gene (552 bp) and a BPXV-specific C18L gene (368 bp) from tissue material and the laboratory-isolated virus (BPXV/Human/Lab/11), according to procedures described by Singh et al. (*6*). Sequences of these 2 genes were submitted to GenBank under accession nos. JN653284.1 and JN653278.1, respectively. Phylogenetic analysis showed 95%–100% nt similarity of the laboratory isolate (BPXV/Human/Lab/11) with other BPXVs from India (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/20/2/13-0358-Techapp1.pdf). Antibodies against BPXV were detected in patient serum samples by using an indirect immunoperoxidase test and a 50% plaque-reduction neutralization test according to methods reported by Bera et al. (*7*). Serum of the infected patient showed 50% plaque-reduction neutralization test titers of 256 and 512 on postinjury days 11 and 28, respectively. These findings confirmed BPXV infection because the patient had not been vaccinated against smallpox.

Pre-freezing of the glass ampule to –80°C caused a hairline crack in

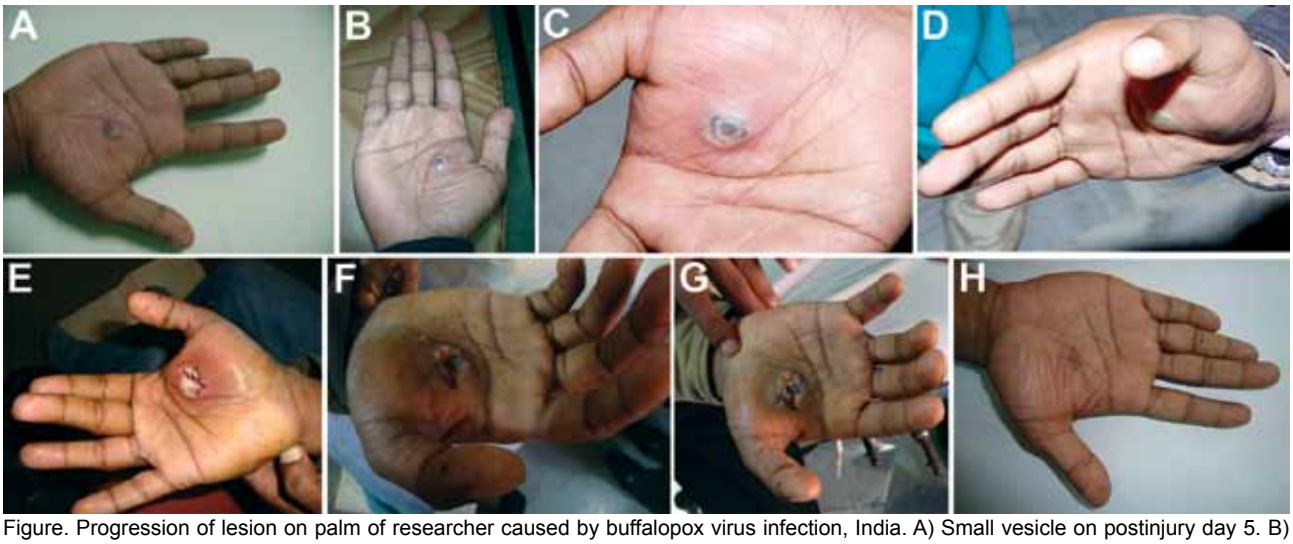

Figure. Progression of lesion on palm of researcher caused by buffalopox virus infection, India. A) Small vesicle on postinjury day 5. B) Pustule with a central area of necrosis on postinjury day 7. C) Pustule with edema on postinjury day 9. D) Increase in edema on postinjury day 10 (cyanosis is not apparent). E) Lesion after surgical excision on postinjury day 11. F) Lesion on postinjury day 19 (healing was erratic). G) Blackening and thickening around surgical site on postinjury day 30, which then extended to a wider circumference. H) Healed lesion with a black eschar on postinjury day 85.

the glass. The ampule broke while being introduced into the freeze-drying manifold and pierced the palm of the researcher. As a follow-up measure, the freeze drying procedure was reviewed and the pre-freezing temperature was reduced to –60°C. Measures were also taken to ensure use of better-quality ampules. Surgical and contact material associated with the lesion was placed in biohazard bags for autoclaving before disposal. In addition, laboratory and hospital staff was apprised of the risk associated with BPXV transmission.

Reporting of laboratory-acquired infections is crucial because infections could also spread to other personnel. Strict biosafety practices and laboratory guidelines are useful in minimizing laboratory-acquired infections. Guidelines, no matter how stringent, are not sufficient on their own. Laboratory-acquired infections occur because humans or machines are not infallible. Thus, laboratories should have emergency procedures in place to deal with such situations.

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Thachamvally Riyesh,[1] Shanmugasundaram Karuppusamy,[1] Bidhan C. Bera, Sanjay Barua, Nitin Virmani, Sarita Yadav, Rajesh K. Vaid, Taruna Anand, Manish Bansal, Praveen Malik, Inderjeet Pahuja, and Raj K. Singh

Author affiliations: Veterinary Culture Collection, Hisar, India (T. Riyesh, S. Karuppusamy, B.C. Bera, S. Barua, R.K. Vaid, T. Anand, M. Bansal, P. Malik); National Research Centre on Equines, Hisar (N. Virmani, I. Pahuja, R.K. Singh); and Central Institute for Research of Buffaloes, Hisar (S. Yadav)

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[1]These authors contributed equally to this article.

Address for correspondence: Thachamvally Riyesh, National Research Centre on Equines, Sirsa Rd, Hisar, Haryana, India; email: riyeshvet@gmail.com

Recurrence of Animal Rabies, Greece, 2012

To the Editor: Rabies is caused by 12 recognized virus species within the *Lyssavirus* genus (family *Rhabdoviridae*) (*1*) and each year causes 55,000 deaths worldwide among humans. In Europe, the main reservoir and vector of rabies is the red fox (*Vulpes vulpes*), followed by the raccoon dog (*Nyctereutes procyonoides*) in central and Baltic Europe (*2*). Among these virus species, the rabies virus (former genotype 1, classical rabies virus) is maintained in reservoir mammals, mainly carnivores. Despite the successful oral vaccination campaigns of wildlife that eliminated rabies in large parts of Europe (*2*), the disease still occurs in Europe; 4,884 cases in animals were recorded in 2012.

Until 2012, Greece had been free of rabies since 1987; the last case occurred in a dog. During 1971–1987, a total of 248 cases were recorded in domestic animals, of which only 6 occurred during 1981–1987 (*3*). The wide compulsory vaccination of dogs, along with control of stray dogs, were the main measures leading to the elimination of rabies in Greece.

Greece shares land borders with Turkey, Former Yugoslav Republic of Macedonia (FYROM), and Bulgaria, where rabies is reported in wild and domestic animals (*4*; www.who-rabies-bulletin.org/). After the fox rabies case reported in FYROM in 2011, ≈0.3 km from Greece (*4*), rabies surveillance was improved along the northern and eastern land borders of Greece, and wild and domestic animals found dead or suspected of having rabies were collected. This program was approved by the European Commission. The first rabies case was diagnosed on October 19, 2012, in a red fox in Palaiokastro, 60 km from the Albanian border. The animal was wandering in the village during daytime and attacked a dog before being killed by local hunters and sent to the National Reference Laboratory for Animal Rabies (Athens, Greece) for rabies testing. The second case, isolated in Ieropigi, 4 km from the Albanian border, was a shepherd dog demonstrating aggressive behavior against dogs and sheep of the flock. Seven additional cases (6 foxes, 1 dog) were reported in December 2012 in 2 prefectures of northern Greece that share borders with FYROM (*5*).

In 2012, a total of 237 domestic and wild animals were submitted to the National Reference Laboratory for Animal Rabies for rabies testing. Samples were tested by the fluorescent antibody test, rabies tissue culture infection test, real-time reverse transcription PCR, and heminested reverse transcription PCR, as described (*6*,*7*). In brief, viral RNA was extracted (Viral RNA Mini Kit, QIAGEN, Hilden, Germany) from 140 μL of homogenized brain suspension supernatant and subjected to the partial nucleoprotein (N) gene amplification (positions 71–644 compared with PV strain genome). The PCR products were bidirectionally sequenced by using the same primers in a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The phylogenetic tree was constructed by using the neighbor-joining method with 1,000 replicates using MEGA5 (*8*).

Of 237 animals tested, 9 (7 foxes, 2 dogs) were rabies positive by fluorescent antibody test, rabies tissue culture infection test, and PCR. Positive samples were subjected to sequencing analysis of the N gene. The sequence analysis of the first 567 nt of the N gene of the 3 isolates (GR64C/12, GR112C/12, GR238C/12) showed 99.8% nt identity with the N gene of a rabies virus (GenBank accession no. JQ973884) isolated from a red fox in FYROM in 2011. Nucleotide identity was 100% between the 6 isolates from Greece (GR177C/12, GR187C/12, GR217C/12, GR231C/12, GR236C/12, GR242C/12) and isolate JQ973884, as well as a Serbia strain (GenBank accession no. JF973785). This perfect nucleotide identity shown with the Serbia isolate collected >15 years ago suggests the persistence of some viral strains over time in the Balkans, in accordance with previous studies (*4*,*9*). Amino acid identity was perfect among all 9 isolates from Greece.

Phylogenetic analysis of the partial sequences of the N gene of isolates from Greece compared with representative sequences from the Balkans (Figure) showed that isolates from Greece resolved within the East Europe (EE) group of the cosmopolitan lineage. The EE group encompasses the 9 Greek isolates with referenced viral sequences from FYROM, Bulgaria, Serbia, Bosnia Herzegovina, and Montenegro. Within the EE group, <1.5%-nt divergence exists between all analyzed N gene sequences (567 nt).

The perfect nucleotide identity shown between the 6 isolates from Greece and 2 strains from Serbia and FYROM demonstrates a close genetic relationship between them. This finding supports the hypothesis of movement of rabies-infected hosts in the western Balkan countries.

Greece was rabies free for 25 years. In the 2012 outbreak, the rabies cycle appears to be sylvatic, whereas until 1987, dogs were the main reservoir of rabies. Measures including public awareness campaigns and intensified vaccination of stray animals and shepherd dogs, combined with control of stray dogs and cats, already have been implemented. In accordance with the

Figure. Neighbor-joining phylogenetic tree comparing 9 isolates (7 red foxes, 2 dogs) from Greece with isolates from Former Yugoslav Republic of Macedonia, Bulgaria, and Serbia. All 9 samples were isolated in Greece during October 19, 2012–December 28, 2012. Representative isolates from central, western, and northeastern Europe and from Russia, extracted from GenBank, also were included in the phylogenetic tree. The phylogenetic analysis was based on analysis of the first 567 nt of the N gene by using the neighbor-joining method (Kimura 2-parameter model). Bootstrap values >70% were regarded as significant support of tree topology. The GenBank accession numbers of the sequences are included for each taxon within the tree, as is the country of origin. Abbreviations for the phylogenetic groups (NEE, C, D, SF) are indicated on the tree. Scale bar indicates nucleotide substitutions per site.

current European Commission recommendations, Greece continued a rabies surveillance and eradication program throughout the country in 2013, including the implementation of an oral rabies vaccination program for foxes in autumn 2013 (*10*).

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Konstantia E. Tasioudi, Peristera Iliadou, Eirini I. Agianniotaki, Emmanuelle Robardet, Emmanouil Liandris, Spiridon Doudounakis, Mirsini Tzani, Paraskevi Tsaroucha, Evelyne Picard-Meyer, Florence Cliquet, and Olga Mangana-Vougiouka

Author affiliations: Ministry of Rural Development and Food, Athens, Greece (K.E. Tasioudi, P. Iliadou, E.I.Agianniotaki, E. Liandris, S. Doudounakis, M. Tzani, P. Tsaroucha, O. Mangana-Vougiouka); WHO Collaborating Centre for Research and Management in Zoonoses Control, Malzéville, France (E. Robardet, E. Picard-Meyer, F. Cliquet); OIE Reference Laboratory for Rabies, Malzéville (E. Robardet, E. Picard-Meyer, F. Cliquet); European Union Reference Laboratory for Rabies, Malzéville (E. Robardet, E. Picard-Meyer, F. Cliquet); European Union Reference Laboratory for Rabies Serology, Malzéville (E. Robardet, E. Picard-Meyer, F. Cliquet); and Technopôle Agricole et Vétérinaire, Malzéville (E. Robardet, E. Picard-Meyer, F. Cliquet)

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Address for correspondence: Konstantia E. Tasioudi, Department of Virology, Institute of Infectious and Parasitic Diseases, Athens Center of Veterinary Institutes, 25 Neapoleos str, Agia Paraskevi, 15310, Athens, Greece; email: ntasioudi@gmail.com

Stable Transmission of *Dirofilaria repens* Nematodes, Northern Germany

To the Editor: Dirofilariosis caused by infection with the filarial nematode *Dirofilaria repens* is considered an emerging zoonosis in Europe (*1*). The main reservoirs for this parasite are dogs and other carnivores. As is the case for other filarial species, mosquitoes transmit infectious third-stage larvae, which develop into fertile adults in their definitive vertebrate hosts. Humans may become infected as aberrant hosts, and, in most cases, these worms remain infertile (*1*,*2*). Infections in humans usually manifest as subcutaneous nodules, which are caused by developing worms that are trapped by immune mechanisms (*2*). Subcutaneous migration of a worm may result in local swelling. Severe clinical manifestations have also been reported and may affect various organs, including the brain, lung, and eye (*2*,*3*). Eye infections are found in particular during the migratory phase of the parasite.

Transmission of *D. repens* occurs in various regions of the Old World, including Europe, Africa, and Asia. The primary areas in Europe to which *D. repens* is endemic are countries of the Mediterranean region, where warm temperatures enable development of infectious third-stage larvae in mosquitoes. However, during the past decade, several autochthonous cases of canine and human dirofilariasis have been reported from countries farther north, including Austria, the Czech Republic, and Poland (*4*–*6*). Several factors might be responsible for this spread to the north, including climate change and increased translocation of dogs from southern to central Europe.

Until recently, central Europe, including Germany, was not considered a region to which *D. repens* was endemic. However, a survey in 44 hunting dogs from the Upper Rhine region showed that 3 animals were positive for *D. repens* microfilariae (*7*). In addition, testing of a kennel of sled dogs located in the federal state of Brandenburg, near Berlin, found 8 of 28 animals were infected with the parasite (*8*).

To determine whether local transmission of *D. repens* is taking place in Germany, we retrospectively analyzed 74,547 mosquitoes from a large-scale German mosquito-borne virus surveillance program for the presence of filarial DNA. Mosquitoes were collected during the main animal-trapping seasons (May–September) 2011 and 2012 by using carbon dioxide–baited encephalitis virus surveillance or gravid traps (*9*). Mosquitoes were collected from 55 trapping sites located in 9 federal states in the southwest and northeastern parts of Germany (Figure), frozen at –70°C, and transported to the laboratory at the Bernhard Nocht Institute, where they were classified morphologically to the species level. Up to 25 mosquitoes of the same species from individual collection sites were subsequently combined to create a total of 4,113 pools. These pools comprised a broad range of ornithophilic and mammalophilic mosquitoes common in Germany; *Culex* spp. and *Aedes vexans* were the most abundant species.

Nucleic acids were extracted from each mosquito pool, and the presence of filarial DNA was determined by PCR using a set of generic primers that amplify DNA from a broad range of filarial species, including those affecting birds and other animals (*9*). A total of 1,050 (25.5%) of the 4,113 pools were found to be positive for filarial DNA. To further determine if any of these pools contained DNA from *D. repens*, we analyzed all 1,050 pools by PCR using the *D. repens*–specific primers 5′-GAGATGGCGTTTCCTCGTG-3′ and 5′-GACCATCAACACTTAAAG-3′.

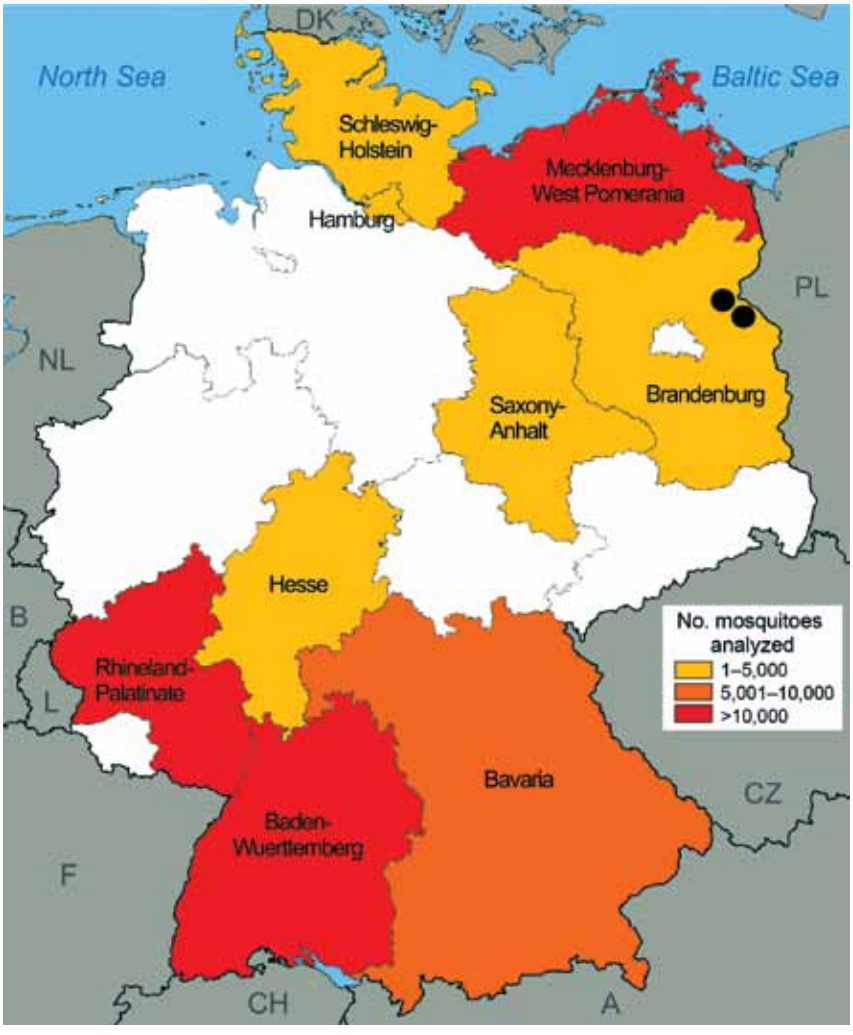

Figure. Origin of mosquito samples analyzed for infection with *Dirofilaria repens*, by federal state, Germany, May–September 2011 and 2012 (n = 74,547). Black dots indicate collection locations of mosquitoes that tested positive for *D. repens* DNA.

Four pools were positive for *D. repens* DNA; all of these pools consisted of mammalophilic mosquitoes collected at 2 trapping sites in the federal state of Brandenburg, near Oder Valley, at a latitude of 52°N (global positioning satellite coordinates 52°47′N, 14°14′E, and 52°51′N, 14°7′E). One pool, consisting of *Culiseta annulata* mosquitoes, was collected in August 2011; the other 3 pools, 2 of *Anopheles maculipennis* sensu lato and 1 of *Ae. vexans* mosquitoes, were trapped in July and August 2012. The identification of *D. repens* DNA in all 4 pools was confirmed by DNA sequencing of a 538-bp fragment of the *g*ene encoding cytochrome oxidase 1 (GenBank accession no. KF410864). Sequence comparisons indicated 99.8% identity to the same gene in a *D. repens* nematode isolate from Italy (accession no. AM749234).

In conclusion, we found mosquitoes that were positive for *D. repens* DNA at a latitude of 52°N in northern Germany; these mosquitoes, from 3 species, were collected at 2 locations in 2011 and 2012. Our findings support recent projections that have suggested increases in average temperatures could affect climatic conditions during summer sufficiently to enable development of *D. repens* larvae in central Europe at latitudes of up to 56°N (*10*). In the federal state of Brandenburg in Germany, conditions for larval development were predicted to be sufficient in each of the past 12 years. These projections, together with our finding of mosquitoes carrying *D. repens* in Brandenburg in 2 successive years and the recurrent detection of infected dogs in this area (*8*), present a strong case for the existence of stable local transmission of *D. repens* in this region of Germany.

Christina Czajka, Norbert Becker, Hanna Jöst, Sven Poppert, Jonas Schmidt-Chanasit, Andreas Krüger, and Egbert Tannich

Author affiliations: Kommunale Aktionsgemeinschft zur Bekämpfung der Stechmückenplage, Waldsee, Germany (C. Czajka, N. Becker, H. Jöst); Bernhard Nocht Institute for Tropical Medicine/World Health Organization Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research, Hamburg, Germany (C. Czajka, H. Jöst, S. Poppert, J. Schmidt-Chanasit, E. Tannich); German Centre for Infection Research, Hamburg (H. Jöst, J. Schmidt-Chanasit, E. Tannich); and Bundeswehr Hospital Hamburg, Hamburg (A. Krüger)

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Address for correspondence: Egbert Tannich, Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Str. 74, 20359 Hamburg, Germany; email: tannich@bnitm.de

Rabies in Henan Province, China, 2010–2012

To the Editor: Rabies is considered a reemerging zoonosis in China because many cases have been reported in recent years (*1*). The first case of rabies in Henan Province was reported in 1951. No more than 10 cases were reported per year during 1995–2001. However, beginning in 2002, the number of cases increased exponentially each year, and reached >100 in 2005 (*2*). To identify the epidemic characteristics of rabies in Henan Province, we examined the archived data of cases during 2010–2012. The surveillance data were collected by the Henan Center for Disease Control and Prevention (CDC) through systematic reporting and reports from sentinel hospitals.

Henan Province is situated in the mid-eastern region of China between northern latitudes 31°23′–36°22′ and eastern longitudes 110°21′–116°39′. The climate zone spans from warm temperate to subtropical, is humid to semi-humid with risk for monsoons, and has average annual temperatures ranging from 12°C to 16°C. The province occupies an area of 165,994 km^2 divided into 18 municipalities, which are subdivided into 159 county-level divisions. Its population was reported to be ≈94 million in 2010 (*3*).

During 2010–2012, a total of 94 cases of rabies in humans were reported in Henan Province. Rabies was diagnosed in almost all of those cases in sentinel hospitals on the basis of clinical features of the disease. Rabies was

etymologia

Dirofilaria [di-ro-fĭ-lar′e-ə]

From the Latin dīrus (“fearful” or “ominous”) + fīlum (“thread”), *Dirofilaria* is a genus of nematodes of the superfamily Filarioidea. The first known description of *Dirofilaria* may have been by Italian nobleman Francesco Birago in 1626 in his Treatise on Hunting: “The dog generates two worms, which are half an arm’s length long and thicker than a finger and red like fire.” Birago erroneously identified the worms as a larval stage of another parasite, *Dioctophyme renale*. The dog heartworm was named *Filaria* by American parasitologist Joseph Leidy in 1856, and the genus was renamed *Dirofilaria* by French parasitologists Railliet and Henry in 1911.

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Address for correspondence: Ronnie Henry, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E03, Atlanta, GA 30333, USA; email: boq3@cdc.gov

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confirmed in <10 patients by using a rapid fluorescent focus inhibition test in the Henan CDC. This test is performed by mixing different dilutions of test serum samples with a constant number of rabies virus isolates in a multichambered slide (*4*).

The number of patients in whom rabies was diagnosed in 2010, 2011, and 2012 were 31, 31, and 32, respectively. Thus, the prevalence of the disease during this period was 0.031 cases/100,000 inhabitants. There was no significant difference in occurrence of the disease during each year of the study period ($\chi^2 = 0.021$, p = 0.989). Rabies was reported in 13 (72.2%) of 18 municipalities and 53 (33.3%) of 159 counties (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/2012/13-1056-Techapp1.pdf).

Patients who tested positive for rabies were reported during all seasons, but more were reported during summer. Of the 94 cases, 33 (35%) were reported in summer months; the largest number of cases in 1 month (n = 15) was reported during September.

The age of the patients with rabies ranged from 2 to 83 years. The disease was most often found in persons 45–60 years of age (n = 36) and was reported least frequently in persons 15–30 years of age (n = 4). The male-to-female ratio of persons in whom rabies was diagnosed was 1.9:1.0 (n = 62 males, 32 females). The age and sex of these patients were similar to those of rabies-infected persons reported in the past in Henan or in other parts of China (*5*,*6*). Nearly all the patients with rabies were exposed to the virus through dog bites; 1 patient was exposed by being scratched by a cat. Most patients (n = 87 [92.5%]) died within 2 days of being exposed; 3 patients (3.2%) died within 9 days. The date of death was not recorded for 4 patients (4.3%).Three patients who died had received rabies vaccine after being bitten by dogs.

Rabies is a fatal infectious disease and remains a public health problem in China (*1*). In Henan Province, the increase in the number of rabies cases parallels the increase in the number of dogs (*7*). Nearly one third of China's provinces reported rabies in this survey; the number of cases reported in the central region was much higher than that in other regions (online Technical Appendix Figure). This may be related to the growing economy in the central region and a resulting increase in the number of people who keep dogs.

With regard to patient characteristics, the proportions of farmers, children, and students who had rabies were 72.3%, 9.6%, and 12.8%, respectively. The larger number of infected farmers could have been affected by many factors. The high cost of rabies vaccine and the lack of prompt treatment were related to many additional deaths. The number of children and student case-patients was 21 (22.4%). This high percentage may indicate that these groups need additional protection. Regarding the patient who was infected by cat scratch, there had been no reports of rabies in cats in China before this incident (*8*). Thus, we should be aware of this possible route of infection.

In summary, the severity of rabies and its increased incidence present a public health threat, and appropriate control strategies in Henan province are needed. A new rabies control system should be established that includes cooperation of the various health care sectors to provide protection to the public.

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Guo-Wei Li[1], Zhen-Yu Qu[1], Alfred King-Yin Lam, Jian-Guo Wang, Feng-Lan Gao, Tong-Xing Deng, and Jia-Hai Lu

Author affiliations: Zhengzhou Center for Disease Control and Prevention, Zhengzhou, China (G.-W. Li); Luohe Medical College, Luohe, China (Z.-Y. Qu, J.-G. Wang, F.-L. Gao,T.-X. Deng); School of Public Health, Sun Yat-sen University, Guangzhou, China (G.-W. Li, J.-H. Lu); and School of Medicine and Griffith Health Institute, Griffith University, Gold Coast, Queensland, Australia (A.K.-Y. Lam).

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[1]These authors contributed equally to this article.

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Address for correspondence: Jia-Hai Lu, School of Public Health, State Ministry of Education Key Laboratory of Tropical Diseases Control Research, Sun Yat-sen University, 74 Zhongshan 2 Rd, Guangzhou, Guangdong Province, 510080, People's Republic of China; email: lujiahai@mail.sysu.edu.cn

Avian Influenza A(H7N9) Virus Infection in Pregnant Woman, China, 2013

To the Editor: In February 2013, human infection with reassortant avian influenza A(H7N9) virus occurred in eastern China. A total of 135 laboratory-confirmed cases and 44 deaths among case-patients have been reported as of August 11, 2013. Unlike infection with other H7 subtype viruses (e.g., H7N2, H7N3, and H7N7), which often cause mild-to-moderate-human disease (*1*), infection with H7N9 subtype virus caused severe pneumonia and acute respiratory distress syndrome in most laboratory-confirmed case-patients (*2,3*). Pregnant women are particularly susceptible to severe complications from influenza (seasonal and pandemic), and have an increased risk for maternal death (*4*).

On March 30, 2013, a 25-year-old pregnant woman came to the outpatient department of a hospital in Zhenjiang, Jiangsu Province, China. She had cough and fever (temperature 38.0°C), which had begun 2 days earlier. She also reported mild myalgia and mild sore throat. The patient had no any underlying medical conditions and was at 17 weeks gestation, as estimated by ultrasound. On April 5, she was admitted to the respiratory department of the hospital with a temperature of 39.9°C, a leukocyte count of 7.9 $\times 10^9$ cells/L, and a lymphocyte count of 0.7 $\times 10^9$ cells/L.

On April 6, she was transferred to the intensive care unit because of shortness of breath, respiratory failure, and loss of consciousness. She was given mechanical ventilation, broad-spectrum antimicrobial drugs, oseltamivir, gamma-globulin, antifibrotic therapy (glutathione), and nutritional support. Oseltamivir (150 mg/d, 2 times/d) had been administered during April 6–12. A chest radiograph showed extensive infiltrates of both lungs.

On April 21, she regained consciousness, and her condition stabilized over the next few days. On April 23, she was extubated, transferred to the common ward, and given nasal oxygen supplementation and antimicrobial and antifibrotic drug therapy. Her condition improved gradually, and on May 14 she was discharged in good health without fetal abnormality.

The fetus was monitored daily by using ultrasound to check the heart rate; fetal heart rate and activity were normal during hospitalization. The fetus continued to grow appropriately and was delivered by cesarean section at 35 weeks' gestation on July 17 (length 48 cm, weight 3,300 g, and Apgars scores of 9 at 1 min and 10 at 5 min). The clinical timeline for the case-patient is shown in the Figure.

The patient and her husband lived in a house with her husband's parents. No live poultry were present in the residential district, but the husband's parents worked as pork butchers in a live animal market ≈500 m from the residential district. Several kinds of live poultry (e.g., chicken, duck, pigeon, and quail) were sold in the market. During the 2 weeks before illness onset, the patient did not have contact with persons known to be febrile. However, during that time, she visited the live animal market once. Eighteen potential close contacts of the patient were identified (15 health care workers and 3 household members). Respiratory symptoms did not develop in any of these contacts during a 7-day surveillance period.

Four methods were used for laboratory diagnosis: real-time reverse transcription PCR, virus isolation, full-genome sequencing, and modified hemagglutination inhibition assays. Clinical samples tested were 2 throat swab specimens obtained from the patient on April 6 and 7, 38 paired serum samples obtained from the patient and close contacts during the acute and convalescent phases of infection, and 6 environmental samples (2 avian feces samples and 4 poultry cage specimens obtained from the live animal market that the patient visited). Throat swab specimens from patient

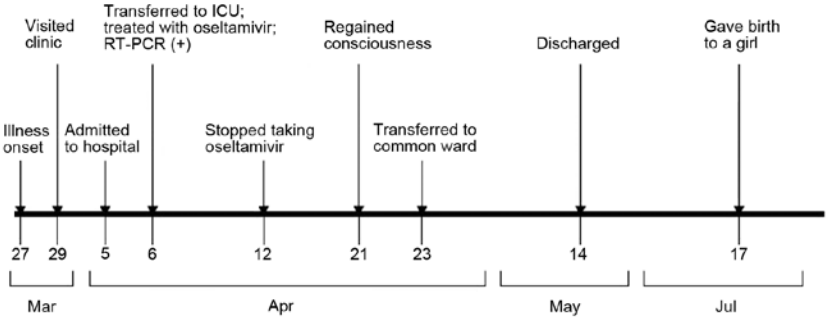

Figure. Clinical timeline for a pregnant woman infected with avian influenza A(H7N9) virus, China, 2013. ICU, intensive care unit; RT-PCR, reverse transcription PCR.

were positive for the hemagglutinin (HA) and neuraminidase genes of avian influenza A (H7N9) virus. Of 6 environmental samples, 5 were positive for (H7N9) virus HA genes. No (H7N9) virus HA antibodies were detected from paired serum samples from all 18 close contacts.

Two virus strains were isolated: 1 from a patient specimen (A/Zhenjiang/1/2013) and 1 from a chicken cage specimen (A/environment/Zhenjiang/4/2013) (GenBank accession nos. KF007057–KF007064 and KF007009–KF007016, respectively). Genome comparison showed that isolates had a nucleotide identity of 96.8%–99.8%, indicating an amino acid identity of 98.2%–99.6%. Phylogenetic analysis showed that 5 genes (HA, nucleoprotein, neuraminidase, matrix, and nonstructural protein) of the 2 isolates belonged to the same clade. However, the 3 polymerase genes (polymerase basic 1, polymerase basic 2, and polymerase acidic) clustered in a different clade. These results suggested that the 2 strains originated from an independent reassortment mechanism and that the H7N9 subtype viruses had undergone genetic reassortment to generate multiple novel genotypes in China.

According to epidemiologic and clinical data for infections with avian influenza A(H7N9) virus, most patients with severe illness, including severe pneumonia and acute respiratory distress syndrome, were elderly men with underlying medical conditions (*2*,*3*). Our findings suggest that pregnancy might be a risk factor for clinically severe influenza in young women infected with H7N9 subtype virus.

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Xian Qi,[1] Lunbiao Cui,[1] Ke Xu,[1] Bin Wu, Fenyang Tang, Changjun Bao, Yefei Zhu, Ming-hao Zhou, and Hua Wang

Author affiliation: Jiangsu Provincial Center for Disease Control and Prevention, Nanjing, China

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Address for correspondence: Hua Wang, Jiangsu Provincial Center for Disease Control and Prevention, 172 Jiangsu Rd, Nanjing 210009, China; email: hua@jscdc.cn

[1]These authors contributed equally to this article.

Peste des Petits Ruminants Virus, Mauritania

To the Editor: Peste des petits ruminants virus (PPRV; genus *Morbillivirus*, family *Paramyxoviridae*) causes severe infectious disease in sheep and goats in Africa and Asia. Pneumo-enteritis clinical signs are dominated by ocular and nasal discharge, and mortality rates are high (*1*). Four distinct lineages of PPRV have been described on the basis of a phylogenetic analysis of a cDNA fragment of the nucleoprotein (NP) gene (*2*): lineages I and II are found in western Africa (*1*,*3*,*4*), lineage III in eastern Africa and the Middle East, and lineage IV in Asia. Recent studies have shown changes in this distribution (*1*,*5*), including the emergence of PPRV lineage IV in northeastern and northern Africa (*5*). Sparse serologic results (*6*,*7*) are available regarding PPRV spread in Mauritania or genetic features of circulating PPRV strains.

A seroprevalence survey was implemented in October 2010 to assess PPRV spatial distribution in Mauritania. The study was limited to 8 southern provinces (*wilayas*), which covered 99.3% of the national sheep and goat stocks (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/20/2/13-1345-Techapp1.pdf). Of 40 districts (*mougataas*), 21 were randomly selected. A single geographic point was randomly sampled within each of the selected *mougataas*, and 100 small ruminants were sampled in a 7-km radius around the coordinates. None of the *mougataas* in which sampling occurred had a PPRV vaccination program.

In addition, field veterinary officers from Trarza and Tagant Provinces were asked to report suspected outbreaks of peste des petits ruminants disease (PPR) during January–March 2012. All outbreaks were investigated, and biologic samples were collected for laboratory diagnostics.

All serum samples from the 2010 and 2012 surveys were analyzed by using antibody ELISA ID Screen PPR competition (IDvet Innovative Diagnostics, Grabels, France). Optical density values were converted to inhibition percentages; according to the ELISA cutoff value, inhibition percentages of ≤45% were considered positive. A logistic beta-binomial regression model was used to analyze prevalence rates within mougataas. Swab samples were tested by using reverse transcription PCR (RT-PCR) adapted to a 1-step format (OneStep RT-PCR Kit; QIAGEN, Hilden, Germany) and based on nucleoprotein (NP) 3–NP4 PPRV-specific primers targeting the 3′ end of the NP gene (*8*). Amplicons of 351 nt were extracted, and after sequencing, nucleic acid segments were aligned with PPRV sequences stored in the database of the Centre de coopération internationale en recherche agronomique pour le développement (Montpellier, France) or retrieved from GenBank (Figure).

A total of 1,190 sheep and 714 goat serum samples were collected during the 2010 survey; the estimated serologic PPRV prevalence rate was 43% (n = 1,904; 95% CI 38%–47%). PPRV infection was widespread: prevalence rates ranged from 3% (Guerou) to 98% (Kobeni) (online Technical Appendix Figure 2). No significant difference was found by species or animal age (p = 0.28 and p = 0.92, respectively), but an increasing gradient in prevalence rates was observed from north to south; the effect of latitude was significant ($p<10^{-6}$) (online Technical Appendix Table). The increasing prevalence rates moving from the north to the south might be related to higher small ruminant density in southern Mauritania (pastoral resources), which might increase between-herd transmission. Moreover, the movement of livestock between Mauritania and 2 countries to the south, Mali and Senegal (online Technical Appendix Figure 2),

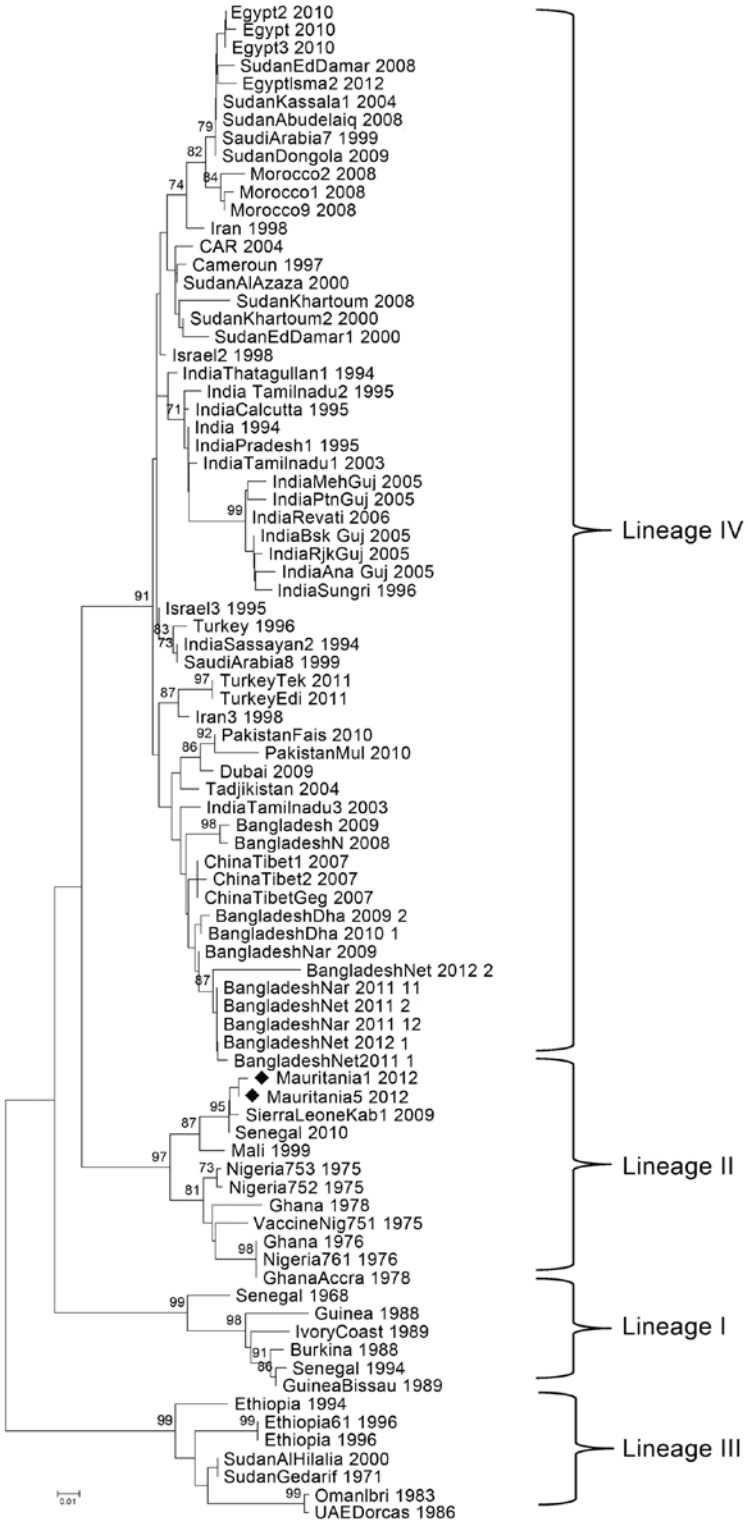

Figure. Phylogenetic tree based on the nucleoprotein gene of peste des petits ruminants viruses identified in Mauritania (black diamonds) and selected comparison sequences from GenBank. The neighbor-joining method was used for phylogenetic analysis; evolutionary distances were computed by using the Tamura 3-parameter method and a gamma distribution parameter with a value of 4 (*9*). CAR, Central African Republic; Nig, Nigeria; UAE, United Arab Emirates. Scale bar indicates nucleotide substitutions per site.

was favorable for PPRV exchanges over the years.

Three suspected outbreaks of PPR were reported during January–March 2012 (online Technical Appendix Figure 2). Both sheep and goats were affected; the animals, particularly young animals, had signs typical of acute PPR. Illness rate ranged from 11% to 17% and case-fatality rates from 39% to 58%. Clinical signs lasted 27–39 days. A total of 43 animals were sampled for virus detection, and 12 animals from 2 sites tested positive by RT-PCR. Seroprevalence rates were estimated on larger samples, including recovering animals in the 3 outbreak locations; these rates were high for all 3 sites: 61%, 70%, and 75% (n = 87, 31, and 12, respectively).

N-gene sequences were obtained from 2 sheep swab specimens collected in Trarza during the outbreak survey in early 2012 (deposited in the GenBank under accession nos. KF483658 [Mauritania1_2012] and KF483659 [Mauritania5_2012]). These isolates were placed in a phylogenetic tree built from PPRV sequences recently collected in western (Senegal, Mali) and northern Africa (Morocco), as well as isolates from other parts of the world retrieved from GenBank. Phylogenetic analysis involved 255 nt located on the C terminus end of the NP gene of the virus (84 aa). The PPRV strain from Mauritania belonged to lineage II (Figure). Sequences were close to, but distinct from, those collected in Senegal and distinct from those identified in Morocco and northern Africa (lineage IV).

Our study results highlight 2 PPRV epidemiologic systems: northern Africa, where all identified PPRVs belonged to lineage IV and were closely related to PPRV initially identified in Sudan (*5*); and western Africa, where all identified PPRVs belonged to lineages I and II (*3*,*4*). This information might be useful for the design of regional control strategies. Ongoing monitoring of PPRV in Mauritania is needed to watch for the possible spread of PPRV lineage IV from northern Africa.

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Ahmed Salem El Arbi, Ahmed Bezeid El Mamy, Habib Salami, Ekatarina Isselmou, Olivier Kwiatek, Geneviève Libeau, Yaghouba Kane, and Renaud Lancelot

Author affiliations: Ministère du Développement Rural, Nouakchott, Mauritania (A.S. El Arbi); Centre National d'Elevage et de Recherche Vétérinaire, Nouakchott (A.B. El Mamy, E. Isselmou); Centre de coopération internationale en recherche agronomique pour le développement, Montpellier, France (H. Salami, O. Kwiatek, G. Libeau, R. Lancelot); Institut National de la Recherche Agronomique, Montpellier (H. Salami, O. Kwiatek, G. Libeau, R. Lancelot);and Ecole Inter-Etats des Sciences et Médecine Vétérinaires de Dakar, Dakar, Senegal (Y. Kane)

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Address for correspondence: Renaud Lancelot, UMR CMAEE, CIRAD, Campus International de Baillarguet, TA A-DIR/B, 34398 Montpellier, France; email: renaud.lancelot@cirad.fr

Novel Bunyavirus in Domestic and Captive Farmed Animals, Minnesota, USA

To the Editor: Xing et al. (*1*) conclude that evidence of infection with a severe fever with thrombocytopenia syndrome (SFTS)–like virus or Heartland-like virus (HRTV) was found in many captive large mammals from much of Minnesota, raising the specter of widespread distribution of a novel pathogen. Although it is likely that HRTV can be found beyond the areas in northwestern Missouri, where it was discovered (*2*), we contend that this conclusion is not substantiated by the data presented by Xing et al., which were generated by an assay that was developed to diagnose SFTS virus infections in China (*1*,*3*). The study used an ELISA developed for an SFTS virus recombinant nucleocapsid protein that detects SFTS-reactive antibodies (*3*). The conclusions reached by Xing et al. are based on the assumption that the SFTS assay developed in China will cross-react with HRTV antibodies (*1*). This assumption remains unsupported because the SFTS assay has not been evaluated for cross-reaction with antibodies to other non-SFTS members of the genus *Phlebovirus* (*1*,*3*). In addition, it is well recognized that serologic tests, like the ELISA, are often group reactive (*4*), requiring neutralization tests to confirm antibody presence and provide specificity. Alternative explanations include the possibility that positive results from testing by Xing et al. may have been caused by cross-reaction with antibodies directed against other known tick-associated phleboviruses endemic to North America, such as Lone Star virus (*5*), which is not known to be pathogenic. In the absence of confirmatory data generated by an independent method, the report by Xing et al. (*1*) should be considered speculative. Reports suggesting substantial expansion in the geographic range of a pathogenic organism should be based on rigorously validated laboratory methods.

Roger S. Nasci, Aaron C. Brault, Amy J. Lambert, and Harry M. Savage

Author affiliation: Centers for Disease Control and Prevention, Fort Collins, Colorado, USA

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Address for correspondence: Roger S. Nasci, Arboviral Diseases Branch, Division of Vector-Borne Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, 3156 Rampart Rd, Fort Collins, CO 80521, USA; email: RNasci@cdc.gov

In Response: We welcome the critiques of Nasci et al. (*1*), who may have misinterpreted the point of our Dispatch. Regarding identification of Heartland virus (HLV) in farm animals, in our article (*2*), we stated that "the viruses detected in this region are most likely HLV or close relatives of HLV," which indicates that the exact identification of the viruses in the animals in Minnesota will not be confirmed until the viruses are isolated and/or the genomic sequence data are available.

The underlying data were obtained with an ELISA specific to severe fever with thrombocytopenia syndrome virus (SFTSV). The conclusion was based on our knowledge, at the time our manuscript was submitted, that in North America no other known phleboviruses of this expanded Uukuniemi group that contains SFTSV and HLV were reported to be cross-reactive with SFTSV. When tested by using our reagent, SFTSV was not cross-reactive with Rift Valley fever virus. Related phleboviruses of this group (e.g., Bhanja, Palma, Forecariah, and Kismayo viruses) have not been reported in North America (*3*). Phleboviruses of this group, such as Murre virus and RML-105355 virus, and Sunday Canyon virus, were isolated in Alaska and Texas, respectively, but are not cross-reactive with SFTSV (*4*).

Other bunyaviruses in North America (e.g., Cache Valley virus and California serogroup viruses) are distantly related and have ≈11% amino acid sequence homology to SFTSV. The recently characterized Lone Star virus appears to be the closest relative to SFTSV and HLV and may cross-react with SFTSV and HLV, as also suggested by Nasci et al., but this virus is apparently known only from 1 isolate obtained in 1967 (*5*). These data suggest that SFTSV is not serologically cross-reactive with the known Uukuniemi group viruses that are currently being transmitted in North America. Our report shows that tickborne

phleboviruses, which are closely related to SFTSV and HLV, may be more generally distributed in the midwestern United States and emphasizes the need to substantiate our serologic evidence with virus isolation and genomic characterization, which are underway.

Zheng Xing and Michael Murtaugh

Author affiliations: College of Veterinary Medicine, University of Minnesota at Twin Cities, St. Paul, Minnesota, USA (Z. Xing, M. Murtaugh); and Medical School, Nanjing University, Nanjing, China (Z. Xing)

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Address for correspondence: Zheng Xing, 300D Veterinary Science Building, College of Veterinary Medicine, University of Minnesota at Twin Cities, 1972 Commonwealth Ave, Saint Paul, MN, 55108; email: zxing@umn.edu

Infectious Schmallenberg Virus from Bovine Semen, Germany

To the Editor: The teratogenic Schmallenberg virus (SBV) (genus *Orthobunyavirus*) was detected in bovine semen in a recent German field study (*1*). Vector-borne transmission by *Culicoides* spp. biting midges is most common (*2*), but venereal transmission of SBV might contribute to the spread of this virus to previously unaffected regions. We investigated the infectivity of SBV RNA–positive semen by experimental subcutaneous injection of cattle and interferon α/β receptor–deficient ($IFNAR^{-/-}$) mice (*3*).

Commercially produced semen straws with egg yolk–based diluent were used for the injection of 6- to 9-month-old heifers. The straws originated from 6 semen batches (quantification cycle [C_q] values 26.4–36.4) collected from 6 bulls (designated A–C and E–G) during August and September 2012 (*1*). To increase the probability of SBV infection of injected cattle, 5 straws of semen (≈220 μL each) from 1 batch from an individual bull were pooled and diluted in minimal essential medium with antibiotics to 4 mL. Six cattle (C1–6) were subcutaneously inoculated, each with a pool from 1 of the 6 bulls. To investigate the infectivity of a single insemination dose (1 straw), 5 cattle (C7–C11) were subcutaneously injected with single straws from bull F that had been confirmed to contain infectious SBV. Serum samples were obtained on several days (Figure), and clinical signs and rectal body temperatures for the injected cattle were monitored daily.

In addition, 20 SBV RNA–positive semen batches (C_q 25.9 to 36.5) collected from 11 bulls (A–K) during August–November 2012 (*1*) were subcutaneously injected into 40 $IFNAR^{-/-}$ mice (4–6 weeks old). For each batch, 2 mice were each injected with half of a semen straw (80–120 μL). All mice were monitored clinically and weighed daily. Samples of serum, liver, and spleen were harvested immediately after euthanasia at 22 days postinjection (dpi).

All serum samples and organ homogenates were tested for SBV RNA by using small segment–specific quantitative reverse transcription PCR (*4*). Serum samples were tested for SBV-specific antibodies by using the ID Screen Schmallenberg Virus Competition ELISA (IDvet, Montpellier, France), according to the manufacturer's instructions; selected serum samples were also tested by neutralization test against an original SBV isolate from Germany, as described (*5*).

SBV infection was confirmed in 5 of 11 injected cattle: C3, C5, and C9–C11. SBV RNA (C_q 25.0–29.3) was first detected in serum at 3 to 6 dpi and persisted for 2–4 days. Seroconversion occurred at 8–12 dpi (Figure). None of the SBV-infected animals showed obvious clinical signs or fever; this finding is in accordance with reports of subclinical SBV infection in adult cattle (*5*–*7*). Samples from the other 6 cattle and all $IFNAR^{-/-}$ mice had negative results (data not shown).

The 2 infectious semen batches contained moderate (C_q 26.4) or low (C_q 34.2) viral loads of SBV RNA, indicating that a high sensitivity is required for reliable SBV RNA detection in semen samples (*1*). The onset of SBV infection in the 3 animals injected with single semen straws ranged from 3 to 5 dpi, and not every straw was infectious, although biologic and technical replicates of straws from 1 semen batch showed similar PCR results (data not shown) (*1*). Possible explanations for differences in the infectivity of individual straws are that the viral RNA load of an SBV-containing straw does not necessarily correlate with infectivity or that the infectivity of 1 straw is lower than the minimal cattle

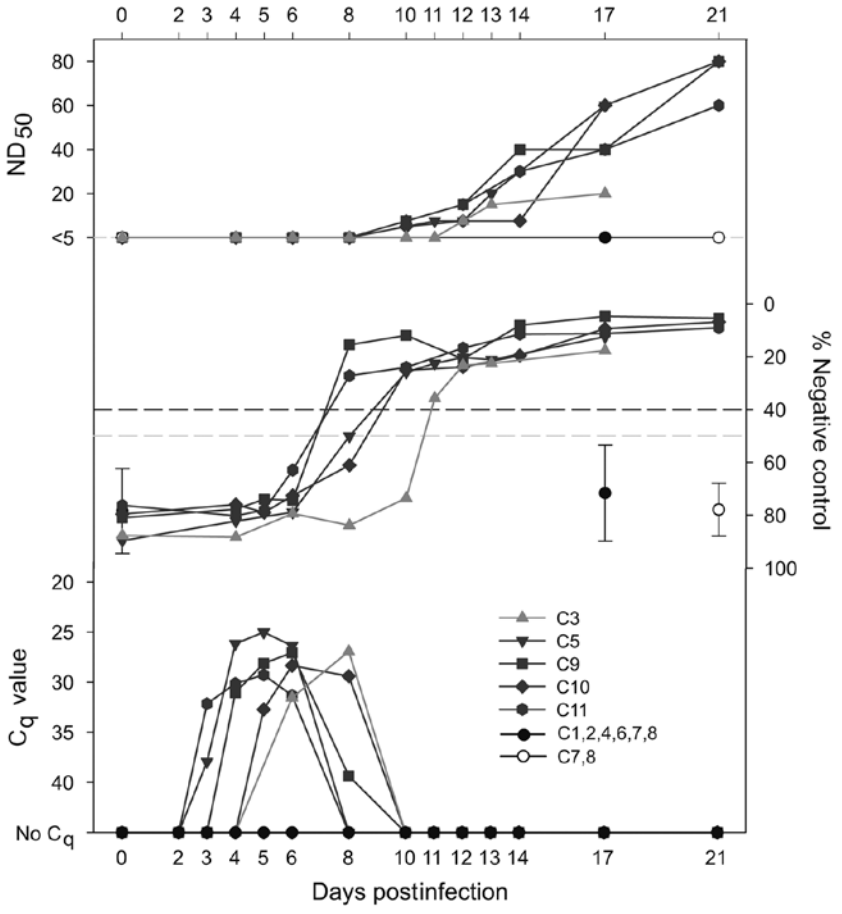

Figure. Detection of Schmallenberg virus (SBV) RNA and antibodies in serum of 5 of 11 cattle (C) injected with SBV RNA–positive bovine semen (quantification cycle [C_q] values 26.4–36.4). Infectivity was measured by using reverse transcription PCR (C_q), competitive ELISA (% negative control), and serum neutralization test (ND_{50}, serum dilution that caused virus neutralization in 50% of the replicates). RNAemia and seroconversion occurred in C3, injected with pooled semen from bull C (C_q 34.2), and in C5 and C9–C11, injected with pooled or single semen straws from bull F (C_q 26.4). The onset of infection in cattle injected with semen from bull F varied between 3 and 5 days postinfection. Black lines indicate virologic and serologic results for cattle that remained uninfected. Dotted lines indicate positive (top) and negative (bottom) cutoff values; whiskers indicate SD of the mean.

infectious dose for SBV. Cattle might be more susceptible than IFNAR$^{-/-}$ mice to infection, particularly when SBV titers are low or borderline (*7, 8*). Therefore, we cannot exclude the possibility that the semen batches tested only in IFNAR$^{-/-}$ mice might be infectious for cattle or that semen samples with higher SBV titers might be infectious in the mice.

We used subcutaneous injection of SBV RNA–positive semen to demonstrate infectivity because this transmission route has a high sensitivity for proving infectivity of SBV-containing samples (*7*). However, the possibility of intrauterine SBV infection of dams is unknown. Oronasal inoculation of 2 calves did not result in SBV infection of the animals (*5*), which suggests that mucosal in utero infection with SBV-containing semen is unlikely. In contrast, viremia was detected in most cows that were artificially inseminated and simultaneously inoculated in the uterus with cell culture–passaged Akabane virus, a teratogenic orthobunyavirus closely related to SBV (*9*). Intrauterine lesions caused by insemination or breeding might therefore increase the risk for SBV infection.

In conclusion, we demonstrated that SBV RNA–positive bovine semen could contain infectious SBV. However, the actual risk for transmission of SBV by insemination of dams with SBV-containing semen remains to be evaluated. Although SBV infection of the developing embryo is unlikely, venereal transmission would lead at worst to viremia of the dam, facilitating vector transmission. To prevent venereal SBV transmission, sensitive PCR testing of semen batches from SBV-infected bulls is the method of choice (*1*,*10*).

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Claudia Schulz,[1] Kerstin Wernike,[1] Martin Beer, and Bernd Hoffmann

Author affiliation: Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

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[1]These authors contributed equally to this article.

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Address for correspondence: Martin Beer, Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald-Insel Riems, Germany; email: martin.beer@fli.bund.de

NDM-1–producing Strains, Family *Enterobacteriaceae*, in Hospital, Beijing, China

To the Editor: The prevalence of New Delhi metallo-β-lactamase-1 (NDM-1)–producing strains (family *Enterobacteriaceae*) in China remains unclear. Recently, to clarify the prevalence of $bla_{\text{NDM-1}}$ in *Enterobacteriaceae* strains, we carried out retrospective surveillance for $bla_{\text{NDM-1}}$ among carbapenem-resistant enterobacterial strains isolated from patients at the Chinese PLA General Hospital in Beijing. This tertiary teaching hospital has 4,000 beds and 12,000 daily outpatient visits. More than 50% of patients admitted to the hospital are from areas outside Beijing. During January 2009–June 2013, a total of 8,586 enterobacterial isolates were obtained from routine clinical samples that had been passively sent to the microbiology department. Of these, 242 (2.8%) strains exhibited resistance to carbapenems.

In this study, we used PCR amplification to screen the carbapenem-resistant strains for the $bla_{\text{NDM-1}}$ gene and other common resistance determinants. The MICs of various antimicrobial drugs were measured by E-test (AB bioMérieux, Solna, Sweden). S1 nuclease pulsed-field gel electrophoresis and Southern blot analysis were used to identify the sizes of $bla_{\text{NDM-1}}$-carrying plasmids. The incompatibility (Inc) groups of the plasmids were detected by several multiplex and simplex PCRs. Multilocus sequence typing (MLST) was carried out for *Klebsiella pneumoniae* and *Escherichia coli* isolates, according to protocols provided on MLST websites (www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html and http://mlst.ucc.ie/mlst/dbs/Ecoli). The transferability of plasmids was identified by conjugation experiments.

Five $bla_{\text{NDM-1}}$-positive enterobacterial isolates of the following species were identified: *E. coli* (1 isolate in October2010), *K. pneumoniae* (1 isolate in August 2012), *Providencia rettgeri* (1 isolate in October 2012), *Enterobacter cloacae* (1 isolate in November 2012), and *Raoultella ornithinolytica* (1 isolate in March 2013). According to the 2013 Clinical and Laboratory Standards Institute performance standard M100-S23 (www.clsi.org/), the NDM-1-producing *K. pneumoniae* (IR5047) isolate exhibited low-level resistance to imipenem and meropenem, whereas other isolates showed high-level resistance to carbapenems. Only *E. coli* and *Providencia rettgeri,* which carry 16S rRNA methylase genes, exhibited high-level resistance to amikacin (Table). S1 nuclease pulsed-field gel electrophoresis and Southern blot analysis showed that the $bla_{\text{NDM-1}}$ gene was located on plasmids of various sizes belonging to different Inc groups. The *K. pneumoniae* isolate was defined as a novel ST1240 with the allelic profile 2–1-1–1-1–3-24, and the *E. coli* isolate was identified as ST167.

In China, various $bla_{\text{NDM-1}}$-carrying strains of the *Enterobacteriaceae* have been sporadically identified, including *K. pneumoniae*, *K. oxytoca*, *Escherichia coli*, *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Citrobacter freundii* (*1–4*). We identified a *P. rettgeri* isolate and an *R. ornithinolytica* isolate that produced NDM-1. The $bla_{\text{NDM-1}}$-positive *P. rettgeri* isolates have also been identified in Pakistan, India, Canada, and Mexico, whereas the NDM-1-producing *R. ornithinolytica* strain has only been detected in India (*5–9*). In this study, all 5 NDM-1–producing strains were isolated only once, and no dissemination of NDM-1–producing strains of *Enterobacteriaceae* has been found. Two strains (*K. pneumoniae* and *Enterobacter cloacae*) were isolated within 48 hours of the patient's hospital admission,

Table. Phenotype and molecular characteristics of NDM-1–producing strains isolated from Chinese PLA General Hospital, Beijing, China, 2009–2013*

Isolate no.	Organism	Source	ST†	bla_{NDM-1} plasmid size, type	Prevalence of RDs	MICs, mg/L							
						CTX	FEP	TZP	IPM	MEM	ETP	AK	LVX
IR5028	*Escherichia coli*	Urine	167	≈190 kb, A/C	bla_{TEM}, bla_{OXA-1},‡ $bla_{CTX-group\ 1,\ 2,\ 9}$, *aac*(6′)-*Ib-cr*,‡ *armA*‡	>256	>256	>256	>32	>32	>32	>256	>32.0
IR5047	*Klebsiella pneumoniae*	Blood	1,240	≈50 kb, X3	bla_{TEM}, bla_{SHV}, bla_{OXA-1}, *qnrS*,‡ *oqxAB*‡	>256	64	>256	8	6	>32	2	0.38
IR5337	*Providencia rettgeri*	Urine	NA‡	≈190 kb, A/C	bla_{PER}, bla_{CMY}, *mtC*‡	>256	>256	>256	>32	>32	4	>256	>32.0
IR5338	*Enterobacter cloacae*	Urine	NA	≈50 kb, X3	bla_{SHV},‡ bla_{OXA-1}, $bla_{CTX-group\ 2}$	>256	>256	>256	>32	>32	>32	1.5	2.0
IR5343	*Raoultella ornithinolytica*	Abscess	NA	≈70 kb, N	*qnrS*‡	>256	32	>256	>32	>32	>32	2	0.75

*NDM-1, New Delhi metallo-β-lactamase-1–producing; ST, sequence type; RDs, resistance determinants; CTX, cefotaxime; FEP: cefepime; TZP, piperacillin-tazobactam; IMP, imipenem; MEM, meropenem; ETP, ertapenem; AK, amikacin; LVX, levofloxacin; NA, not applicable.
†The alleles at each of the multi-locus ST loci for a given isolate are combined into an allelic profile and assigned an ST designation.
‡Shown to be transferred by conjugation experiments.

indicating the infections were imported (from Shandong and Hebei Provinces, respectively). *Escherichia coli*, *P. rettgeri,* and *R. ornithinolytica* were isolated 48 hours after admission of patients (from Henan and Hebei Provinces). Therefore, the patients might have acquired the NDM-1–producing *Enterobacteriaceae* strains at the hospital.

However, the source of the bla_{NDM-1} determinant remains unclear. The possibility that the strains were imported cannot be excluded for the several reasons. First, examination to determine the infectious agent had not been performed for a considerable number of patients within 48 hours of their admission. Second, NDM-1–producing *Enterobacteriaceae* species have not spread in this hospital. Third, the bla_{NDM-1}-carrying plasmids in the same Inc group and of similar size exhibited substantial differences in resistance determinants (Table), which suggests a different evolutionary origin for these isolates. In an additional survey of clinical data, we found no epidemiologic relationship between the patients who were infected by NDM-1–producing pathogens. These data suggest a sporadic pattern of NDM-1–producing enterobacteria in the hospital.

Sequencing analysis (data not shown) indicated that the bla_{NDM-1}-carrying plasmid carried by *K. pneumoniae* (≈50 kb, IncX3) was different from the plasmid found in the *Acinetobacter pitti* isolate that was disseminated in an intensive care unit of the Chinese PLA General Hospital in 2008 (*10*). This finding suggests that the 2 plasmids had a different evolutionary origin. The IncX3 plasmid that we found was highly homologous (>99%) to the plasmid pNDM-HN380 (GenBank accession no. JX104760), which has been identified in several *Enterobacteriaceae* strains isolated from patients in southern China (*1*). This finding showed that the IncX3 plasmid that was 50 kb in size acted as the main factor mediating the transmission of the bla_{NDM-1} gene across China. IncA/C plasmids are the leading group of bla_{NDM-1}-carrying plasmids and have been detected in *E. coli* isolated from China (*1*). In this study, *E. coli* (IR5028) and *P. rettgeri* (IR5337) carried IncA/C plasmids. However, these 2 strains exhibited diverse resistant determinants on these plasmids (Table). This observation suggested that the 2 plasmids have different integrating processes. For *R. ornithinolytica*, the bla_{NDM-1} gene was located on an IncN plasmid of ≈70 kb, which is very different from other plasmids.

In conclusion, we identified various NDM-1–producing enterobacterial isolates at the Chinese PLA Hospital in Beijing and the emergence of novel bla_{NDM-1}-carrying clones among common species of *Enterobacteriaceae*, such as *K. pneumoniae* ST1240 and *E. coli* ST167. There is an urgent need for monitoring and surveillance of epidemiologic and genotypic profiles of NDM-1–producing *Enterobacteriaceae* species in China.

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Guang Zhou,[1] Si Guo,[1] Yanping Luo, Liyan Ye, Yang Song, Guangwei Sun, Ling Guo, Yong Chen, Li Han, and Jiyong Yang

Author affiliations: Chinese PLA General Hospital, Beijing, China (G. Zhou, Y. Luo, L. Ye, Y. Song, G. Sun, L. Guo, J. Yang); Henan Provincial People's Hospital, Zhengzhou, China (S. Guo); and Chinese PLA Institute for Disease Control and Prevention, Academy of Military Medical Sciences, Beijing (Y. Chen, L. Han)

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[1]These authors contributed equally to this work.

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Address for correspondence: Jiyong Yang, Microbiology Department, 301 Hospital, 28# Fuxing Rd, Beijing 100853, China; email: yangjy301@hotmail.com

Deadly Outbreaks: How Medical Detectives Save Lives Threatened by Killer Pandemics, Exotic Viruses, and Drug-Resistant Parasites

Alexandra M. Levitt

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As a reader of epidemiology literature and teaching materials since 1958, when a professor dropped the book Eleven Blue Men on my desk, I have tried to keep up with interesting outbreaks and studies. I bring to the attention of other epidemiologists the book, Deadly Outbreaks: How Medical Detectives Save Lives Threatened by Killer Pandemics, Exotic Viruses, and Drug-Resistant Parasites, by Alexandra M. Levitt, a recent collection of real and different outbreaks. It should be of interest not only to infectious diseases control specialists, but also to laboratory personnel and persons involved in studies that support epidemiologists in the field and in academic institutions.

Some of the outbreaks reported in this book, such as Legionnaires disease in Philadelphia in 1976, are relatively well known. Other outbreaks, such as West Nile fever in Queens, New York, in 1999 or drug-resistant malaria in Thailand in 1982, are not as well known to the general public.

The 7 sections in this book contain some of the latest laboratory advances and developments in subspecialties of virology, bacteriology, laboratory medicine, and environmental medicine. For example, who among us knew that channelopathy (progressive inflammatory neuropathy) would be a disease in humans working in pig slaughter houses?

This book is a reminder to all of us working in public health that our areas of interest are evolving in a variety of directions in the field and in research laboratories. This evolution is to be expected and is welcomed as we struggle to control new diseases that knock on our doors and open our eyes as we promote the interests of the rest of the world. It also reminds us that it takes all sorts of persons with a wide variety of backgrounds, interests, and abilities to respond to these new diseases and their associated problems and challenges. Our job is to respond quickly to limit these problems so that they have a minimal effect.

This book is a worthy addition to the libraries of health departments and schools of public health and medicine. It merits a place not too far away from where copies of Eleven Blue Men are shelved. Welcome to the modern epidemiology of the twenty-first century!

J. Lyle Conrad

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (retired)

DOI: http://dx.doi.org/10.3201/eid2002.131476

Address for correspondence: J. Lyle Conrad, 1069 Burton Dr NE, Atlanta, GA 30329, USA; email: jconrad@bellsouth.net

Nellie Mae Rowe (1900–1982) *Picking Cotton* (1981) (detail) Crayon, felt-tip pen, ballpoint pen, on paper (19 × 24.5 in) Arnett Collection of the Souls Grown Deep Foundation. Photo: Gamma One Conversions. © The estate of Nellie Mae Rowe

Seeing Things Differently

Sharon Bloom and Alexandra M. Levitt

> "You can draw a mule, dog, cat, or a human person, I'm going to draw it different. 'Cause you always see things different. Each person's different."
>
> —Nellie Mae Rowe

Picking Cotton, by Nellie Mae Rowe (1900–1982), is a work of folk art that captures Rowe's experiences as an African American woman living in rural Georgia, United States. Nellie Mae Rowe was born on July 4, 1900, as the ninth of ten children. The family lived on a rented farm 20 miles south of Atlanta. Her father, a farmer and blacksmith, was born a slave in 1854; her mother, born the year after President Lincoln issued the Emancipation Proclamation (1864), was a gospel singer and talented quilter.

Early childhood was pleasant for Nellie Mae, who had a natural affinity for drawing and making dolls. However, after 4 years of elementary school, she was put to work on the family farm and in the neighbors' fields. At 16, she married Ben Wheat, and in 1930, the couple moved to Vinings, Georgia. Wheat died in 1936, and after a year, Nellie Mae married an older widower, Henry Rowe.

During and after marriage, Nellie Mae Rowe worked as a domestic servant. When her second husband died in 1948, she felt liberated to resurrect her early creative urges. She converted her 4-room Vinings home into her "playhouse"—decorating all available space inside and out in the yard with dolls, drawings, and other fanciful creations. A devout Christian, she attributed her talent to God.

Rowe worked with humble materials such as crayon, cardboard, chewing gum, and felt-tip markers, using bright colors and unexpected juxtapositions to create a sense of

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (S. Bloom); and Centers for Disease Control and Prevention, New York, New York, USA (A.M. Levitt)

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energy and movement. Her early works involved single figures, but her later drawings combined memories and fantasy into narrative themes of the southern farm—incorporating people, plants, birds, expressive animals and speckled fish. Drawings made during the last years of her life (including *Picking Cotton*) are largely autobiographical. After receiving a diagnosis of multiple myeloma in 1981, Rowe continued to draw until her last months of life. With the assistance of an Atlanta-based art dealer, Rowe's work gained national recognition. In 1982 (the year of her death), Rowe's drawings were included in the Corcoran Gallery of Art's exhibition "Black Folk Art in America, 1930–1980," which is regarded as the beginning of American recognition of self-taught artists.

According to its owner, William Arnett, *Picking Cotton* (1981) could have been entitled "What I don't like about my life as I look back on it." The picking of cotton refers to the most degrading form of manual labor the artist knew. Rowe is seen in a lush field in an orange party dress (a statement that this work was not meant for her), bending over with a tool in her hand and a cotton-sack on her back in the shape of a vulnerable woman. Imposed on the artist, and central to the composition, is a black mule, symbolizing the forced labor of the black woman. The seated white woman scrutinizing Rowe may represent her employers during her years as a domestic servant; the red rodent beneath her chair is Rowe's symbol for an unpleasant situation. In addition, the blue-green man sitting in the lower left corner, who grins while he watches her work, may represent her former husbands. The picture also includes brightly colored birds with pointed beaks, one pecking from a curved bush whose pointed petals suggest a ravenous set of jaws.

Picking Cotton, with its animated and unsettling themes, evokes the complex and ever-changing interactions among people, animals, and the environment that can lead to the emergence or resurgence of human infectious diseases. Many of Rowe's wild and domestic animals are associated with high-consequence zoonotic pathogens addressed in this issue. For example, her bird pecking at the "jaws of death" could serve as a symbol of the role birds play in spreading West Nile and avian influenza (H7N9) viruses. Her rat brings to mind rodent-borne pathogens, including a novel hantavirus and lymphocytic choriomeningitis virus, and her dogs bring to mind reports of the resurgence of rabies virus.

Rowe's multifaceted work illustrates her personal mythology, her response to life experiences, and an assimilation of African American spiritual and narrative traditions. The words and drawings of the free-spirited Nellie Mae Rowe—born to a former slave on Independence Day—remind us how much we can learn from people who see things differently, including both artists and scientists.

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Address for correspondence: Sharon Bloom, Center for Global Health, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E41, Atlanta, GA 30333, USA; email: sbloom@cdc.gov

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Invasive Fungal Infections after Natural Disasters

High Relatedness among *Mycobacterium abscessus* subsp. *massiliense* Strains from Geographically Distant Outbreaks

Comparison of Patients with Imported *Plasmodium ovale curtisi* or *P. ovale wallikeri* Infections, Spain

Drug-Susceptibility Testing and Management of Drug-Resistant Tuberculosis, Thailand, 2004–2008

Hendra Virus Vaccine, a One-Health Approach to Protecting Horse, Human, and Environmental Health

Drought and Epidemic Typhus, Central Mexico, 1655–1918

Bovine *Waddlia chondrophila* in Human Placenta and Miscarriage

Influenza A(H1N1)pdm09 Virus in Giant Pandas, China

Infective Endocarditis in Northeastern Thailand

Candidatus Neoehrlichia mikurensis and *Anaplasma phagocytophilum* in Hedgehogs

Septic Arthritis in Pig Farmer Caused by *Streptococcus suis* Serotype 5

Cyclospora spp. in Drills, Bioko Island, Equatorial Guinea

Rift Valley Fever in Kedougou, Southeastern Senegal, 2012

Atypical *Streptococcus suis* in Man, Argentina

Mixed Scrub Typhus Genotype, Shandong, China, 2011

Detection of *Bartonella henselae* and *B. koehlerae* DNA in Birds

Tick-borne Pathogens in Northwestern California, USA

Novel Cetacean Morbillivirus in Guiana Dolphin, Brazil

Complete list of articles in the March issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

April 2–5, 2014
16th International Congress on Infectious Diseases
Cape Town, South Africa
http://www.isid.org/icid/

April 9–11, 2014
9th Conference Louis Pasteur Emerging Infectious Diseases
Paris, France
http://www.clp2014.org/

May 17–20, 2014
114th General Meeting
American Society for Microbiology
Boston, Massachusetts
http://www.asm.org/asm2014/

June 24–27, 2014
EMBO conference on "Microbiology after the genomics revolution - Genomes 2014"
Institut Pasteur, Paris
http://www.genomes-2014.org

Oct 31–Nov 3, 2014
IMED 2014
Vienna, Austria
http://imed.isid.org

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EMERGING INFECTIOUS DISEASES®

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
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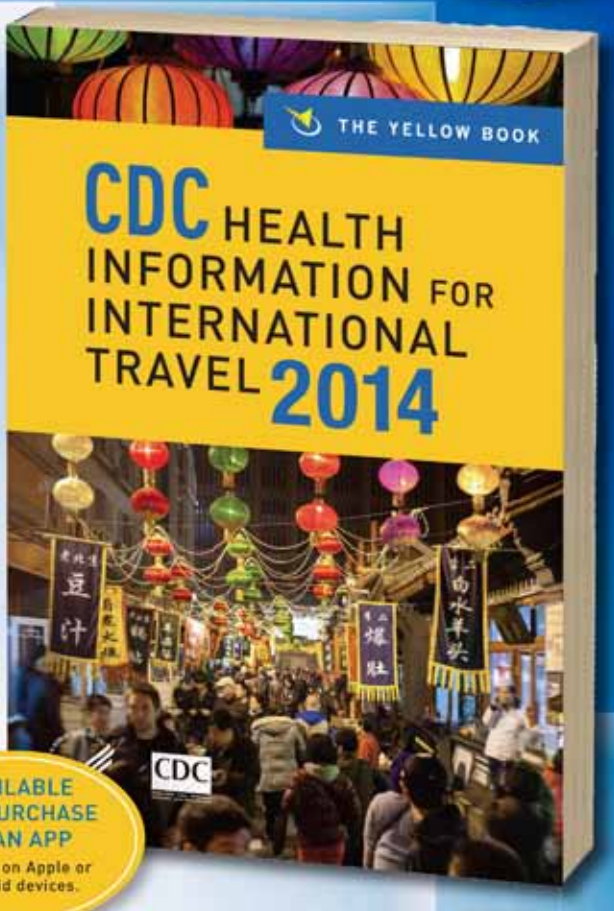

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Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical 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.

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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.

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