EMERGING INFECTIOUS DISEASES®

December 2011

Zoonotic Infections

EMERGING
INFECTIOUS DISEASES®

Emerging Infectious Diseases is published monthly by the Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D61, Atlanta, GA 30333, USA. Telephone 404-639-1960, fax 404-639-1954, email eideditor@cdc.gov.

∞ Emerging Infectious Diseases is printed on acid-free paper that meets the requirements of ANSI/NISO 239.48-1992 (Permanence of Paper)

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On the Cover

Jan Fyt and others (1611–1661)
Atalanta and Meleager Hunt the Calydonian Boar (1648)
Oil on canvas
(243.8 cm × 411.5 cm).
Bequest of John Ringling, 1936, Collection of The John and Mable Ringling Museum of Art http://www.ringling.org/, The State Art Museum of Florida, a division of Florida State University

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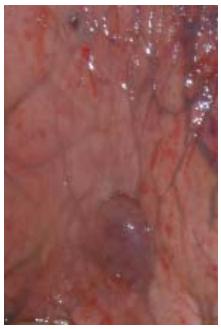

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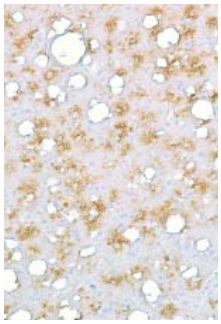

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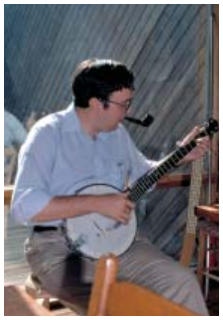

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Risk for Rabies Importation from North Africa

Philippe Gautret, Florence Ribadeau-Dumas, Philippe Parola, Philippe Brouqui, and Hervé Bourhy

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Learning Objectives

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

- Distinguish the animal associated with most cases of fatal rabies
- Evaluate the epidemiology of rabies in North Africa
- Assess how rabies cases are managed in France
- Analyze who should receive strong consideration for preexposure vaccination against rabies prior to travel to North Africa

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Disclosures: ***Philippe Gautret, MD; Florence Ribadeau-Dumas, MD; Philippe Parola, MD, PhD;*** *and* ***Philippe Brouqui, MD, PhD,*** *have disclosed no relevant financial relationships.* ***Hervé Bourhy, MD,*** *has disclosed the following relevant financial relationships: served as a speaker or a member or a speakers bureau for Sanofi Pasteur.*

A retrospective study conducted in France indicated that a large proportion of patients injured by potentially rabid animals while in North Africa did not seek pretravel advice, and some had not received proper rabies postexposure prophylaxis while in North Africa. As a result, imported human rabies cases are still being reported, and the need for postexposure prophylaxis after exposure in North Africa is not declining. Tourists are generally unaware of the danger of importing potentially rabid animals and of the rules governing the movement of pets. In France, for example, rabid dogs have frequently been imported from Morocco to France through Spain. This situation imposes heavy social and economic costs and impedes rabies control in Europe. Rabies surveillance and control should therefore be reinforced in North Africa, and travelers to North Africa should receive appropriate information about rabies risk and prevention.

Among all human deaths from rabies, >99% occur in the developing world and result from bites from rabid dogs (*1*). Although effective and economic control measures are available, rabies remains a neglected disease in most of these countries (*2*). Animal-associated injuries in travelers to rabies-endemic countries are not infrequent (*3*) and pose a serious health threat to persons visiting such areas (*4*). Rabies is a serious public health concern in North Africa (Morocco, Algeria, Tunisia, Libya, Egypt, and Sudan, as defined by the United Nations), causing heavy social

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DOI: http://dx.doi.org/10.3201/eid1712.110300

and economic costs. The possibility of its reintroduction threatens western European countries, where terrestrial carnivores are presently rabies free (*5*,*6*).

Rabies in North Africa

Although rabies is endemic to North Africa (*7*), accurate data on incidence are scarce (*8*), and better surveillance is needed. Initiatives have recently been developed to share information and experiences, provide reliable rabies epidemiologic data, raise rabies awareness, improve access to prevention, and design programs for rabies control. These initiatives include the Africa Rabies Expert Bureau (www.afroreb.info), the global multidisciplinary European Union–funded RABMED CONTROL project (www.rabmedcontrol.org), and the Global Alliance for Rabies Control (www.rabiescontrol.net). Some of these initiatives are expected to provide detailed epidemiologic data about the rabies situation in North African countries. Although rabies incidence in North Africa is certainly underestimated (*2*,*8*,*9*), according to the most recent reports in the World Health Organization (WHO) RabNet database (www.who.int/globalatlas/default.asp), RABMED CONTROL data, and other sources (*5*,*9*–*12*), many human rabies cases are regularly reported in North Africa. The annual incidence varies from 0.02 cases/100,000 population in Tunisia to 0.1 cases/100,000 population in Egypt (Table 1). These disparities may result from differences in the epidemiologic status of animal rabies. More likely, however, they reflect the combined effects of variations in the reliability of epidemiologic data, the implementation of primary wound care, and the accessibility of rabies postexposure prophylaxis (PEP). Indeed, rabies PEP accessibility is subject to large disparities; the lowest rates (0.4 persons receiving rabies PEP/1,000 population) are reported in Sudan, and the highest (3.3 persons receiving rabies PEP/1,000 population) are reported in Tunisia (Table 1).

In Morocco, 90% of human cases of rabies are caused by dog bites. Cases occur mainly in rural areas in Kenitra, Casablanca, and El Jadida (*10*). In Algeria, human deaths from rabies occur mostly in the coastal and northern part of the country (*11*). In Tunisia, human cases are localized in the northern (Bizerte and Béja), central and eastern rural (Siliana, Kairouan, Kasserine, Sidi Bouzid, and Gafsa), and southern coastal (Gabes and Medenine) parts of the country.

Most reported cases of rabies in animals are in dogs, which account for >40% of animals confirmed rabid by laboratory investigations. Rabies cases are also reported for cats, cattle, goats, sheep, and horses. However, data collected for animal rabies (Table 1) are only partial and do not represent the true status of this infection in the field (*8*). In Tunisia, mass vaccination campaigns directed at the canine population, together with the elimination of free-roaming dogs, has proven somewhat effective (*13*). However, rabies in dogs is still prevalent in Algeria, Morocco, and Tunisia (Table 1) because of ineffective implementation of rabies control and vaccination programs for these reservoir animals (*5*,*8*,*14*,*15*).

The information available from Egypt is clearly incomplete (*16*). Elimination of free-roaming cats and dogs has been conducted in Egypt with little effect (*5*). In Sudan, animal rabies is reported mainly in dogs but also in goats and donkeys (*17*,*18*). From Libya, no information about rabies in humans or animals has been available for many years; the country has declared itself free of canine rabies, although rabies is present in all neighboring countries (*19*). This description of the current rabies situation in North Africa is based on reported cases only and does not necessarily represent the real epidemiologic situation in the area, given the underreporting that results from nonexhaustive surveillance.

Risk for Importing Human Rabies from North Africa to Europe

North Africa is a popular tourist destination; in 2005, it accounted for 12% of all international travel from France. The most popular destinations were Morocco, Tunisia, and Egypt; these countries together accounted for 89% of travel to North Africa, with 950,000, 920,000, and 590,000 visits, respectively. Algeria accounted for 5% of travel to North Africa, with 140,000 visits. No data are available for Sudan or Libya (*20*). Persons who emigrated from North Africa to

Table 1. Reported rabies cases in humans and animals, North Africa, 2000–2009*

Country	Annual no. human deaths/100,000 population	Annual no. rabies PEP in humans/100,000 population	No. rabies cases in animals (years)
Algeria	0.06†	2.2†	2,206 (2000–2008), 754 (2009)
Egypt	0.1‡	1.9§	5 (2000, 2006, and 2007)¶
Morocco	0.07†	1.6†	3,600 (2000–2008, including 343 in 2007)
Sudan	0.04†	0.4‡	101 (2000–2007, including 38 in 2007)
Tunisia	0.02†	3.3†	1,253 (2000–2007, including 102 in 2007)

*PEP, postexposure prophylaxis. Data sources include World Health Organization RabNet database (www.who.int/globalatlas/default.asp) and RABMEDCONTROL (www.rabmedcontrol.org) (*5*,*9*–*12*).
†Calculated for 2000–2007.
‡Calculated for 2007.
§Calculated for 2000.
¶No data available for other years.

France also travel frequently to their countries of origin to visit friends and relatives. This travel pattern also creates a pathway for rabies reintroduction to France. During the first half of the 20th century, canine rabies progressively disappeared from most countries in western and southern Europe (*21*). At the end of the 1940s, the epizootic of vulpine rabies spread from Poland into the rest of Europe (*22*). Today, oral vaccination of foxes has pushed vulpine rabies back into eastern Europe (*23*). Maintaining rabies-free status among terrestrial carnivores, however, incurs considerable costs. Meanwhile, the legal and illegal importation of live animals imposes a continual risk for reimportation, as does the lack of awareness by travelers visiting rabies-endemic areas (*6*,*24*).

In France, primary health care for patients seeking rabies PEP is delivered through an official network of antirabies medical centers (ARMCs), which facilitate the accurate epidemiologic evaluation of animal-related injuries for which patients require rabies PEP. Since 1970, a total of 21 human deaths from rabies have been recorded in France; these cases resulted in a large number of rabies PEP treatments for contacts (Table 2). Among these fatal cases, 19 were imported (50% originated in North Africa, mainly in Algeria and Morocco), 1 was acquired in French Guiana, and 1 was acquired through a cornea transplant from a donor infected in Egypt. During the past decade, 2 cases were acquired in Morocco by travelers from Austria and Germany (*26*,*27*). Other cases, imported to the United Kingdom, Germany, Sweden, and Finland, were acquired from India, Thailand, the Philippines, Nigeria, and South Africa (*28*–*31*). These data reflect the relevance of specific patterns of international travel between European and rabies-enzootic countries.

Of the 133,852 patients who consulted an ARMC in France during 1996–2009, a total of 6.7% of exposures to animals suspected of having rabies occurred while the patient was traveling outside France (French National Rabies Reference Centre, unpub. data). Persons who acquired animal-related injuries abroad accounted for 11.2% of patients who received rabies PEP during the same period, a figure that rose from 6.8% in 1996 to 17.8% in 2009 (Figure). Among treated patients who were injured abroad, 29.9% had returned from North Africa. This figure underscores the role of North Africa in rabies PEP epidemiology in France (Table 3). Need for rabies PEP seems to be particularly high after travel to Algeria. In 2005, only 5% of international tourists from France visited Algeria, but persons injured in Algeria accounted for 21.4% of patients seeking care in France after possible rabies exposure while abroad. Furthermore, 25% of all human rabies cases observed in mainland France since 1970 resulted from exposure in Algeria. In France, 94.7% of patients injured in North Africa received rabies PEP (Tables 3, 4), compared with 53% of those injured elsewhere, and 16.1% of rabies PEP given included rabies immunoglobulin, compared with only 5.8% given to persons injured elsewhere. This higher rate of rabies PEP among travelers injured in North Africa reflects what

Table 2. Cases of rabies in humans, France, 1970–2010*

Year of death	Patient age, y/sex	Country of exposure	Animal species	Incubation time	Time between illness onset and death	No. contacts who received rabies PEP
1970	3/M	Niger	Cat	10 d	9 d	Unknown
1973	10/M	Gabon	Dog	11 mo or 15 d	20 d	Unknown
1976	5/M	Gabon	Dog	45 d	1 mo	Unknown
1976	18/M	Algeria	Dog	Unknown	23 d	1
1976	28/M	Morocco	Unknown	Unknown	1 mo	Unknown
1976	10/M	Algeria	Dog	1 mo	18 d	Unknown
1977	2/M	Gabon	Dog	18 d	1 d	5
1977	4/M	Morocco	Dog	1 mo	2 d	25
1979	57/F	Egypt	Dog	2 mo	10 d	12
1979	36/M	Egypt	Human (cornea transplant)	1 mo	15 d	128
1980	4/M	Tunisia	Dog	2.5 mo	3 d	66
1982	40/M	Senegal	Dog	122 d	30 d	Unknown
1990	28/M	Mexico	Dog	47 d	10 d	1
1992	3/M	Algeria	Dog	1 mo	3 wk	143
1994	46/M	Mali	Dog	3 mo	11 d	36
1996	3/M	Madagascar	Dog	2 mo	6 d	290
1996	60/M	Algeria	Dog	2 mo	5 d	45
1996	71/M	Algeria	Dog	40 d	3 d	35
1997	50/F	India	Dog	12 d	56 d	36
2003	3/M	Gabon	Dog	>2 mo	10 d	142
2008	42/M	France (French Guiana)	Bat	Unknown	16 d	90

*Adapted from Peigue-Lafeuille et al. (*25*). PEP, postexposure prophylaxis.

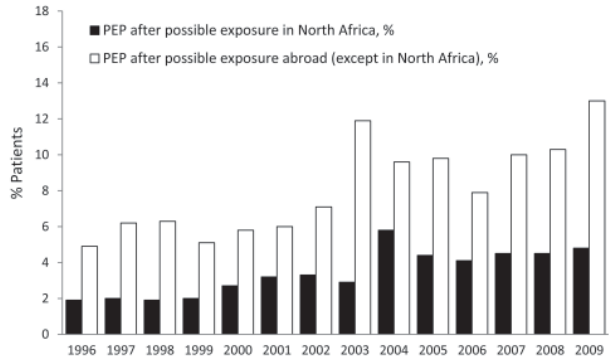

Figure. Proportion of patients injured in countries in North Africa compared with those injured in countries in other foreign regions among all patients who consulted an antirabies medical center and received rabies postexposure prophylaxis (PEP), France, 1996–2009.

is understood about rabies epidemiology in the region and the perception of the risk by physicians, which is largely influenced by the number of rabies infections that developed in France in persons exposed while in North Africa. Children are especially at risk. Since 1970, children ≤15 years of age have accounted for 50% of human rabies cases in mainland France; among these, 8 of 10 cases have occurred in children ≤6 years of age. Moreover, the proportion of children ≤15 of age years receiving rabies PEP in France is higher when their rabies exposure occurred in North Africa (Table 4). Regional variations also exist within France. In a recent study conducted among ≈424 international travelers seeking care for animal-associated injuries at the ARMC in Marseille, southern France, most (41.5%) had traveled to North Africa, and most injuries were from dogs and cats. A correlation was found between the country of exposure and the implicated species; in Algeria, the most implicated animals were dogs and in Tunisia, cats (*32*). During 2004–2010, among 90 patients exposed in North Africa and seeking care in Marseille, 53 (59%) had traveled for tourism and 35 (39%) had visited friends and relatives; however, 26 (69%) travelers injured in Algeria had visited friends and relatives (Table 5). The French-speaking North African community is particularly large in Marseille, where it accounts for one third of the total immigrant population.

Generally, only a few travelers to North Africa seek pretravel advice before departing, which is not surprising because specific vaccination and malaria prophylaxis are not required before travel to North Africa. In 2009 in the Paris ARMC, only 1 (1.2%) of 82 travelers from France who had been injured by animals while in North Africa had been vaccinated before traveling (Paris ARMC, unpub. data). Knowledge about rabies risk and preventive measures among persons traveling from France to rabies-endemic countries is limited (*33*).

Importation of Rabid Animals

Rabid animals have been repeatedly imported by travelers into France; most animals originated in Morocco and were transported through Spain by car (*34*–*37*). During 2000–2009, a total of 8 imported rabies cases were reported. All infected animals were dogs imported from rabies-enzootic countries: 7 from Morocco and 1 from Gambia. Additionally, a dog that died in February 2008 had never traveled out of France. Epidemiologic investigations and molecular typing of the virus confirmed that this case was indirectly linked to an imported dog from Morocco that had died in late 2007; the imported dog had not been examined for rabies. No secondary transmissions to humans resulted from these animal cases. However, the identification of contacts at risk to ensure that they received appropriate care is costly and time-consuming (*6*). The sanitary regulation regarding rabies vaccination of all carnivores entering the European Union is essential for rabies control and must be strictly applied in areas of Europe that have been declared rabies free (*38*). This precaution applies to France in particular because the illegal pet importation route from Morocco through Spain to France is well known.

Availability of Rabies Vaccine and Immune Globulin in North Africa

Modern imported cell culture vaccines are available in Morocco, Egypt, Sudan, and Tunisia. No data are available from Libya. Algeria has been using mouse brain rabies

Table 3. Patients possibly exposed to rabies while visiting North Africa, 1996–2009*

Country of exposure	No. exposed patients visiting ARMC	No. patients receiving rabies PEP	Proportion of all foreign exposures occurring in country, %
Algeria	649	603	7.6
Egypt	117	103	1.3
Libya	10	9	0.1
Morocco	1,036	992	12.5
Sudan	8	8	0.1
Tunisia	687	659	8.3
Total	2,507	2,374	29.9

*Original data from the French National Reference Centre on rabies. ARMC, antirabies medical center; PEP, postexposure prophylaxis.

Table 4. Patients receiving rabies PEP among patients consulting an antirabies medical center, France, 1996–2009*

Location of possible exposure	% Patients age ≤15 y among those who received rabies PEP†	% Patients age ≤6 y among those who received rabies PEP†	% Patients who received rabies PEP among those age ≤15 y†	% Patients who received rabies PEP among those age ≤6 y†	Total no. patients‡		Total no. patients who received rabies PEP
					≤15 y of age	≤6 y of age	
North Africa	29.4	12.3	94.8	95.1	735	306	2,374
All other	24.2	8.8	44.1	37.9	37,895	16,093	69,145

*Original data from the French National Reference Centre for Rabies. PEP, postexposure prophylaxis.
†$p<10^{-6}$.
‡Regardless of rabies PEP status.

vaccine made in Algeria and is starting to use the cell culture vaccine; however, according to the Paris ARMC (in charge of 16.6% of patients receiving rabies PEP in France and 47.4% of patients exposed abroad in 2009 and receiving rabies PEP), 14 (53.8%) of the 26 patients who began their rabies PEP in Algeria still received mouse brain rabies vaccine in 2009. Equine rabies immunoglobulin is available in most prevention centers in Algeria, Morocco, and Tunisia ≈100 centers in Algeria, 147 in Morocco, and 206 in Tunisia) (*9*), but human rabies immunoglobulin is less widely available. Data on the availability of rabies immunoglobulin in Libya and Sudan are not available. However, a substantial proportion of travelers injured in North African countries do not receive adequate rabies PEP. Thus, in the Paris ARMC in 2009, of 32 patients requiring rabies immunoglobulin (patients with grade III exposure who had never previously received preexposure or postexposure rabies vaccine) and whose PEP was started in North Africa, 18 (56.3%) neither received rabies immunoglobulin locally nor consulted with a doctor when they returned to France in time to receive it. Rabies immunoglobulin must be administered <7 days after administration of the first vaccine dose (*39*). In contrast, of 9 (22.2%) patients with grade II exposure and no exposure to bats, 2 received rabies immunoglobulin in Algeria, although this treatment is not recommended by WHO guidelines (*1*). Such deviations were not observed among 26 patients returning from Morocco, Tunisia, or Egypt.

Similarly, in the Marseille ARMC during 1987–2005, among the 34 patients requiring rabies immunoglobulin whose PEP was started in North Africa, only 2 (5.9%) travelers received rabies immunoglobulin in the country of exposure and 23 (67.7%) travelers who received only vaccination in the country of exposure came to home clinics >7 days after receiving the first vaccine injection. After 7 days, administration of rabies immunoglobulin is useless and might even have a negative influence on active immune response (*40*).

Conclusions

We recommend that rabies control measures and, in particular, rabies control and vaccination programs in dogs be further implemented in North African countries. Meanwhile, the persistent risk for rabies in these countries exists for travelers, as shown for France. A large proportion of travelers exposed to potentially rabid animals in North Africa did not seek pretravel advice from travel clinics, thus missing the opportunity to learn about rabies risks and preventive measures. Furthermore, many of these patients did not receive rabies PEP and rabies immunoglobulin in accordance with WHO recommendations in the country of exposure. This problem reinforces the need to inform and train health professionals in these countries with regard to recommended practice for rabies prevention for humans.

Finally, rabies control in Europe is impeded by lack of tourist awareness of the threat of importing rabies from countries where it is enzootic and of the rules governing movement of pets. The sanitary regulations regarding rabies vaccination of all carnivores entering the European Union (*38*) are essential for rabies control and must be strictly applied in European areas that have been declared free of rabies in terrestrial carnivores. These regulations define the requirements that dogs and cats must meet before entry into the European Union, with the aim of preventing entry of an infected but asymptomatic dog or cat from outside Europe.

We suggest that all travelers to North Africa be fully informed about rabies risk, adequate preventive measures, and risk of importing animals. For persons traveling to North Africa to visit relatives and who are at high risk for exposure to potentially rabid animals, rabies preexposure

Table 5. Reasons for travel among 90 patients injured in North Africa and consulting an antirabies medical center, Marseille, France, 2004–2010

Reason for travel	Place of exposure, no. (%) patients				
	Algeria	Morocco	Tunisia	Egypt	Total
Tourism	10 (27.8)	24 (72.7)	17 (89.6)	2 (100)	53 (58.9)
Visit with friends and relatives	26 (72.2)	8 (24.3)	1 (5.2)	0	35 (38.9)
Business	0	1 (3.0)	1 (5.2)	0	2 (2.2)
Total	36	33	19	2	90

vaccination should be discussed; this precaution is especially wise for those undergoing repeated or long visits to places with no modern culture cell rabies vaccine and for groups at risk, such as children ≤15 years of age. As long as these measures and information are not properly implemented, costly capacities for surveillance of rabies in animals and humans, as well as for monitoring rabies exposures in humans, should be maintained.

Dr Gautret is a physician, parasitologist, and director of the Rabies Treatment Centre and of the Travel Clinic at Marseille University Hospital. He coordinates the Epidemic Intelligence and Response program for EuroTravNet. His professional interests include zoonoses, tropical and travel medicine, Hajj medicine, and medical parasitology.

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Worldwide Occurrence and Impact of Human Trichinellosis, 1986–2009

K. Darwin Murrell and Edoardo Pozio

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Learning Objectives

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

- Evaluate epidemiologic patterns of trichinellosis
- Analyze the clinical presentation of trichinellosis
- Distinguish the most common animal source of trichinellosis

Editor

Caran R. Wilbanks, Technical Writer/Editor, *Emerging Infectious Diseases. Disclosure: Caran R. Wilbanks has disclosed the following relevant financial relationship: partner is employed by McKesson Corporation.*

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Disclosures: ***K. Darwin Murrell, PhD;*** *and* ***Edoardo Pozio, PhD,*** *have disclosed no relevant financial relationships.*

To assess the global incidence and clinical effects of human trichinellosis, we analyzed outbreak report data for 1986–2009. Searches of 6 international databases yielded 494 reports. After applying strict criteria for relevance and reliability, we selected 261 reports for data extraction. From 1986 through 2009, there were 65,818 cases and 42 deaths reported from 41 countries. The World Health Organization European Region accounted for 87% of cases; 50% of those occurred in Romania, mainly during 1990–1999. Incidence in the region ranged from 1.1 to 8.5 cases per 100,000 population. Trichinellosis affected primarily adults (median age 33.1 years) and about equally affected men (51%) and women. Major clinical effects, according to 5,377 well-described cases, were myalgia, diarrhea, fever, facial edema, and headaches. Pork was the major source of infection; wild game sources were also frequently reported. These data will be valuable for estimating the illness worldwide.

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DOI: http://dx.doi.org/10.3201/eid1712.110896

Since the mid-19th century, trichinellosis has been a well-recognized meat-borne zoonosis; however, despite concerted control efforts, it remains a threat in many countries. Veterinary control over the slaughter of food animals to ensure food safety, particularly meat inspection, was introduced in Germany in 1866 specifically to prevent trichinellosis from pork infected with the muscle larvae of *Trichinella spiralis* (*1*). In the European Union, the estimated annual cost incurred from meat inspection of 167 million pigs (*2*) ranges from €25 million to €400 million (*3*). Even in countries without mandatory meat inspection (e.g., United States), the economic cost of selling pork in international and national markets is substantial (*4*).

The epidemiology and systematics (i.e., the study of the diversification) of this zoonosis are now recognized to involve, in addition to *T. spiralis*, 7 other species in 4 genotypes, all of which are more commonly found in wild animals than in domestic pigs (*5*). *Trichinella* spp. have been found in domestic and wild animals in 66 countries (*6*). Human trichinellosis has been documented in 55 countries, particularly those with well-established food behavior that includes consuming meat dishes with raw or undercooked meat (*6*). Whether trichinellosis is a low-prevalence disease or is frequently misdiagnosed is not clearly understood; its detection can be difficult in low-level infections and its clinical manifestations overlap those of other diseases, such as influenza and chronic fatigue syndrome (*7*). Human infection is classically characterized by gastroenteritis; myalgia; malaise; facial edema; headache; subungual or conjunctival hemorrhages; and increased eosinophils, leukocytes, and muscle enzymes (*7*).

Reliable estimates of the incidence of trichinellosis among humans and its effect on health are not available; these estimates are necessary for setting priorities. In the 1990s, the global prevalence of trichinellosis was ≈10 million, and a recent incidence estimate suggested ≈10,000 infections per year (*6*). However, because of problems related to incomplete data from some regions and to the quality of diagnostic criteria for infection, the Foodborne Disease Burden Epidemiology Reference Group of the World Health Organization (WHO) requested a systematic review of the global incidence, burden of disease, and major sources of infection that used strict criteria for data selection and extraction. Our analyses and summaries of the epidemiologic and clinical data selected provide a basis for an assessment of trichinellosis as a public health problem.

Data Sources and Selection Criteria

We retrospectively reviewed trichinellosis outbreak investigations conducted worldwide during 1986–2009. The data analysis focused on incidence, age and sex of patients, infection rates, major clinical aspects including sequelae, and meat sources of infection. The database we developed was geographically organized according to the WHO regions (www.who.int/choice/demography/regions/en): African Region, 46 countries; Region of the Americas, 12 countries; Eastern Mediterranean Region, 22 north African and Middle Eastern countries; European Region, 44 European and 6 Asian countries; South-East Asian Region, 11 east Asian countries; and Western Pacific Region, 27 countries. Data searches of literature included PubMed, Centres for Agricultural Bioscience International (CABI) abstracts, WHO library, System for Information on Gray Literature, Pan America Health Organization Virtual Library, and Index Medicus for South-East Asian Region.

The search terms used were trichinosis, trichinellosis, trichinelliasis, and trichinella. These were combined with the terms prevalence, outbreaks, epidemiology, clinical symptoms, and duration. The search terms were also combined with pork, pig, wild boar, wild pig, warthog, horse, badger, jackal, cougar, walrus, armadillo, turtle, and bear meat. Published abstracts were screened for retention by using the criteria of relevance to human outbreaks or single cases occurring from 1986 through 2009 and by determining whether the report was based on original data (primary source or unpublished data managed by national government agencies). The full paper versions of selected abstracts were then obtained where possible and further screened and evaluated. Outbreak reports published >1 time were occasionally encountered, and care was taken to prevent duplication of data in the database; preference was given to published international, peer-reviewed versions. In some instances, data were obtained through contact with scientists in countries of interest who had access to unpublished and detailed information about outbreaks; these sources are indicated in the reference lists in the online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/11-0896-Techapp.pdf). In addition, information about isolated outbreaks maintained by a national health system was obtained for some countries through personal contacts (see Acknowledgments). For reports in which English versions were not available, translations were obtained through the generous help of colleagues (see Acknowledgments).

Definitions

Reports, published or unpublished, were excluded from the database if the diagnosis of *Trichinella* spp. infection was not based on a diagnostic procedure that we defined as confirmatory. Although direct demonstration of muscle larvae infection in biopsy samples is now infrequent, reliance solely on serologic testing to confirm infection can be problematic (*8*). Therefore, a serologically positive case was included in the database only if the sample was confirmed by a Western blot test or if the patient's illness could be classified as highly probable according to the clinical diagnostic algorithm published by Dupouy-Camet and Bruschi (*7*), in which a patient with a positive serologic test result must also exhibit ≥1 classical trichinellosis signs and symptoms (e.g., myalgia, facial edema, headaches, diarrhea, eosinophilia).

We took a conservative approach in extracting clinical data for the analysis of frequency of major signs and symptoms in patients with *Trichinella* spp. infections and excluded report data if there was lack of clarity and reliability of clinical procedures and laboratory tests used to confirm infection. However, in some instances of inadequate clinical descriptions, the report was retained if it otherwise presented useful epidemiologic data.

Consequently, the total number of human infections (Tables 1–3) exceeds the number of cases used to summarize the frequency of major signs and symptoms (Table 4). Except in rare cases, clinical data were extracted from outbreak reports only if the data were from multiple cases that met these criteria; exceptions were reports of single cases from countries with rare occurrences of trichinellosis but that had good clinical and laboratory confirmation data (e.g., Korea, Japan, India).

From the original 494 abstracts identified from literature searches, 378 were judged to potentially meet the criteria for data extraction, and full articles were obtained for most abstracts. From these, 261 reports were retained for data extraction and inclusion. A major reason for rejection of articles was failure to meet the criteria for confirmation of infection, especially interpretation of serologic results.

Incidence

In Table 2, the incidence (100,000 person-years) is reported for specific periods because the data were obtained over a shorter period than the formal study interval (1986–2009). For some countries, when incidence figures were not reported in published papers or national health reports, we calculated incidence from data available in the referenced reports by using the WHO World Population Prospects (the 2008 revision) (http://esa.un.org/wpp/unpp/p2k0data.asp). Overall, from 1986 through 2009, there were 65,818 cases and 42 deaths from trichinellosis reported from 41 countries (Tables 1–3). The European Region accounted for 86% of cases (56,912), of which 28,564 (50%) occurred in Romania, mainly during 1990–1999. The full references for specific country reports summarized in Tables 2–5 are available in the online Technical Appendix.

Of 46 countries in the African Region, trichinellosis has been documented only among soldiers in the Gojjam region and policemen in the Arsi region of Ethiopia, a country where the Christian population accounts for ≈60% of the total population. In the Eastern Mediterranean Region, trichinellosis was documented only in the Christian population of Lebanon and in Iran from the consumption of wild boar meat (Table 2). In Algeria and Senegal, where most of the population is Muslim, trichinellosis has been documented only in Europeans (*6*).

In the European Region, 4 epidemiologic patterns are discernable: 1) countries of eastern Europe where incidence rates are >1 case/100,000 inhabitants (Bosnia-Herzegovina, 4.1; Bulgaria, 2.4–2.9; Croatia, 1.7–4.8; Latvia, 1.1–1.3; Lithuania, 1.2–6.6; Romania, 2.9–8.5; and Serbia, 5.0); 2) countries with a low number of inhabitants where the occurrence of a large outbreak results in a high incidence rate (e.g., Israel, 3.0; Slovakia, 6.2; and Slovenia, 10.5); 3) 19 countries with a low incidence rate caused either by sporadic infections or by a large general population that reduces the incidence per 100,000 inhabitants even when a large outbreak occurs; and 4) 21 countries where no autochthonous infections were reported during the period. Incidence in eastern Europe spiked during the late 1980s and early 1990s and then decreased over the past decade. This pattern may be linked to the political, social, and economic changes that occurred with the breakup of the former Soviet Union as described by Djordjevic et al. (*9*). The gradual restoration of infrastructure related to food safety (e.g., meat inspection, pig production management, veterinary services) probably contributed substantially to the decrease in incidence in these countries.

The number of cases in the Region of the Americas was comparatively low (Table 1), except in Argentina (Table 2). National incidence estimates are limited for Region of the Americas countries and published only for the United States, Chile, and Argentina; data from Canada, Mexico, and Argentina pertain only to selected states, provinces, or districts that had large outbreaks. In Canada during the period, a few large outbreaks in northern communities among native people who consumed wild game accounted for most of the outbreaks in the country. As an example of the problem of informal or clandestine meat transportation, 2 outbreaks occurred among foreign hunters; in 1 outbreak, the hunters transported infected bear home (France) and unknowingly exposed friends and family (17 total cases). For Canada and Greenland, trichinellosis was caused by consumption of wild game harboring *T. nativa*, which does not infect swine; no pork-transmitted *Trichinella* spp. have been recorded in Canada for many years.

The risk for trichinellosis has decreased markedly in the United States and Chile since the 1990s. The large number of cases associated with Argentina contrasts with

Table 1. Clinically confirmed cases of trichinellosis in humans documented in World Health Organization regions, 1986–2009

Region (no. countries)	No. (%) countries with trichinellosis	No. (%) documented human infections	No. (%) deaths
African Region (46)	1 (2.17)	28 (0.04)	1 (3.57)
Region of the Americas (12)	5 (42.67)	7,179 (10.90)	10 (0.10)
Eastern Mediterranean Region (22)	2 (9.09)	50 (0.07)	0
European Region (50)	29 (58.00)	56,912 (86.47)	24 (0.04)
South-East Asian Region (11)	1 (9.69)	219 (0.33)	1 (0.50)
Western Pacific Region (27)	3 (11.11)	1,344 (2.04)	6 (0.40)
Other*	NA	86 (0.13)	0
Total (168)	41 (24)	65,818 (100.00)	42 (0.40)

*Infections acquired in countries other than the one in which diagnosis occurred. NA, not applicable

the situation among other countries in South America. The cases in Argentina may be related to the European origins of persons immigrating there and the risky food behavior they brought with them (*10*). Although incidence data from Mexico and Argentina are limited, trichinellosis outbreaks are reported frequently in Argentina from domestic pork,

Table 2. Total cases and incidence of *Trichinella* spp. infections, by World Health Organization region and country, 1986–2009*

Region/country	Years	No. cases	Average incidence†
African Region, Ethiopia	1986	8	0.02
	1990	20	0.04
Region of the Americas		7,179	
Argentina	1990–2005	5,221	1.48
Canada	1987–2009	257	0.03
Chile	1991–2004	698	0.36
Mexico	1986–2001	351	0.02
United States	1987–2007	652	0.016–0.004
Eastern Mediterranean Region		50	
Iran	2007	6	0.008
Lebanon	1995	44	1.25
European Region		56,912	
Belarus	1988, 1989	16	0.08, 0.55
Bosnia and Herzegovina	1993–2003	1,600	0.1–8.0
Bulgaria	1990–2006	4,108	2.9
Croatia	1994–2009	2,110	0.02–12.3
Czech Republic	1986–2009	31	0.01
Estonia	1986–2009	91	0.0–2.9
France	1986–2009	1,203	0.00–0.95
Georgia	1988	3	0.05
Germany	1986–2009	185	0.00–0.01
Greece	2009	1	0.008
Hungary	1986–2009	158	0.18–0.057
Ireland	2007	2	0.04
Israel	2002, 2004	230	0.5, 3.0
Italy	1986–2009	1,181	0.0–0.9
Kyrgyzstan	1996	10	0.2
Latvia	1986–2009	636	0.07–3.8
Lithuania	1989–2009	3,979	0.4–21.8
Macedonia	1992	6	0.3
Poland	1986–2007	3,084	0.05–1.5
Romania	1986–2007	28,564	1.7–16.1
Russia	1996–2002	971	0.3–0.6
Serbia	1994–2003	5,210	1.8–7.8
Slovakia	1986–2008	440	0.0–6.2
Slovenia	1989–2006	203	0.00–10.5
Spain	1986–2009	1,244	0.0–0.4
Switzerland	1994, 2009	4	0.01, 0.04
Turkey	2003, 2004	425	0.01, 0.59
United Kingdom	1999	7	0.01
Ukraine	1986–2009	1,210	0.00–0.30
South-East Asian Region		219	
India	1996–2002	3	0.0003
Thailand	1993–2007	216	0.35
Western Pacific Region		1,344	
Japan	1999–2005	4	NA
South Korea	1999–2003	8	0.016
Laos	2004–2006	123	2.09
People's Republic of China	1995–2009	1,137	NA
Singapore	1998	25	0.64
Vietnam	2001–2004	47	0.058

*The detailed data and references for each country are available in the online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/11-0896-Techapp.pdf), section A. NA, insufficient data for incidence calculation.
†Incidence/100,000 person-years. For some countries, incidence was not reported and was calculated from data available in the report referenced.

Table 3. Trichinellosis acquired in locations different from those where the disease developed and was diagnosed, 1986–2009*

Country where infection developed and was diagnosed	Country where infection was acquired (no. clinical cases)
Austria	Yugoslavia (10)
Belgium	Canada (1)
Czech Republic	Poland (2), Ukraine (2), France (1)
Denmark	Poland (12)
France	Algeria (6), Cameroon (1), Canada (13), Croatia (1), Greenland (2), Kenya (2), Laos (5), Senegal (5), Spain (1), Thailand (1), Turkey (3), Yugoslavia (1)
Germany	Canada (1), Poland (3)
Italy	Romania (4)
The Netherlands	Yugoslavia (3), Montenegro (5)
Hong Kong	Canada (1)

*Complete data and references are available in the online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/11-0896-Techapp.pdf), section B.

indicating that a substantial pig husbandry risk persists in that country.

The Asian countries of the Western Pacific Region and the South-East Asian Region reported few outbreaks during the period (Tables 1, 2). Although large outbreaks in the People's Republic of China have been reported (*11*), the criteria for selection of reports and data extraction eliminated some reports because of insufficient diagnostic detail to meet the confirmation criteria. Most of the outbreaks reported from Thailand, Laos, and Vietnam, occurred in the northern mountainous regions among the indigenous people who practice free-roaming pig husbandry (*12*–*14*). After a 30-year period of no reports of trichinellosis cases, Laos recently experienced several outbreaks (*12*). The estimated incidence in rural areas of that country is high, which suggests a possible emerging problem there.

Globally, reporting of trichinellosis varies greatly. A major factor affecting the collection of epidemiologic and clinical data is an absent or inadequate national reporting system. For example, in some countries of eastern Europe (e.g., Bosnia-Herzegovina, Byelorussia, Georgia, Moldavia, Romania, Russia, Ukraine) trichinellosis occurs frequently in villages during the winter, and infection might not be diagnosed and subsequently reported unless infection is sufficiently severe to require hospitalization or the cases are part of a larger outbreak that requires attention from public health authorities (A. Marinculic, M.C. Cretu, W. Kociecka, N. Iashvili, N. Bogatko, pers. comm.). For example, in Romania, most of the 20,059 cases documented during 1990–1999 pertain to hospitalized persons only. However, for each hospitalized person, there are probably others in whom a moderate or mild infection developed that did not justify the travel and costs that would be incurred in seeking attention for diagnosis and treatment. Consequently, they are not usually officially recorded as having trichinellosis (M.C. Cretu, pers. comm.). In countries where most of the population is Muslim, *Trichinella* spp. infection is rare and may not be reported at all because of a scarcity of physicians, lack of good diagnostic tools, and occurrence in remote areas. In contrast, in industrialized countries such as those of Western Europe, United States, and Canada, nearly all cases are more likely to be detected and recorded, including asymptomatic cases associated with large outbreaks. For these reasons, the data we present may underrepresent the incidence of trichinellosis in lesser developed countries in comparison to that in industrialized and affluent countries.

Sex- and Age-specific Infection

Data from clinical reports (Table 5) demonstrate that trichinellosis is a disease primarily of adults, occurring about equally among both sexes (2,631 [51%] of 5,154 infections occurred in male patients). Infection in male patients did occur more frequently, however, in Ethiopia (100%), Vietnam (91%), Japan and South Korea (75%), Thailand (64%), and China (57%). Age-specific infection data (Table 5) show the highest proportion of cases, for both sexes, was among persons 20–50 years of age (median 33.1 years). Data on age-specific prevalence rates were rarely reported; however, recent improvements in diagnosis of trichinellosis, particularly immunodiagnostic methods, may encourage more human prevalence surveys

Table 4. Frequency of major clinical signs associated with trichinellosis among World Health Organization regions, 1986–2009*

Region	Total no. cases†	Clinical sign, no. cases						
		Diarrhea	Myalgia	Fever	Facial and/or eyelid edema	Headache	Eosinophilia	Deaths
African Region	28	28	8	11	8	6	6	0
Region of the Americas	1,229	400	969	821	790	410	606	10
Eastern Mediterranean Region	45	43	42	41	Not reported	30	0	4
European Region	3,118	798	1,971	1,387	1,617	351	1,850	24
South-East Asian Region	210	82	206	103	102	71	97	1
Western Pacific Region	747	79	409	474	429	104	180	8
Total no. (%)	5,377 (100.0)	1,430 (27.0)	3,605 (67.0)	2,837 (53.0)	2,946 (55.0)	972 (18.0)	2,739 (51.0)	35 (1.0)

*Report references are available in the online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/11-0896-Techapp.pdf), section C.
†Cases included in this table were selected from all reports on the basis of detailed descriptions of clinical data in the reports.

Table 5. Demographic data on trichinellosis patients, by World Health Organization region and country, 1986–2009*

Region/country	% Male patients (total no. cases)†	Age, y, of infected persons (no. cases)
African Region: Ethiopia	100 (28)	Range 23–25 (3); mean 24 (3)
Region of the Americas		
Canada	62.1 (150)	Range 21–67 (85); mean 34.4 (65)
Chile	60 (667)	Range 5–70 (667)
Mexico	35 (59)	Range 25–44 (59)
United States	57.5 (632)	Range 1–87 (412); mean 42.0 (126); median 37.1 (232)
Eastern Mediterranean Region: Lebanon	54 (44)	Range 10–70 (44); mean 33 (44)
European Region		
Bulgaria	49 (228)	Range 1–70 (228)
Croatia	57 (200)	Range 3–67 (200); mean 35 (200)
Czech Republic	41.9 (31)	Range 9–68 (31); mean 35.9 (31)
France	51.4 (586)	Range 1–84 (581); mean 43.8 (581)
Germany	51.9 (104)	Range 1–73 (101); mean 34.8 (101)
Israel	100 (26)	Mean 32 (26)
Italy	50.3 (382)	Range 1–90 (368); mean 36.7 (368)
Romania	53.2 (521)	Range 1- >60 (521); mean 31.4 (521)
Slovakia	63.6 (11)	Range 16–80 (21); mean 40.5 (21)
Spain	57.5 (237)	Range 2–86 (140); mean 40.7 (177)
Turkey	52.6 (418)	Range 1.5–73 (418); mean 31.1 (418)
South-East Asian Region: Thailand	71 (165)	Range 7–70 (210); mean 35.6 (208); median 34.5 (140)
Western Pacific Region		
Laos	47 (111)	Range 5–69 (111); mean 30.4 (90); median 34 (21)
People's Republic of China	58.2 (802)	Range 1–90 (482)
Vietnam	92 (42)	Range 20–60 (42); mean 45.4 (42)
Singapore	56 (25)	Mean 22.5 (25)

*Clinical details and report references are available in the online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/11-0896-Techapp.pdf), section D.
†Data are from reports that presented adequate sex and age data on ≥10 cases during 1986–2009.

and surveillance for trichinellosis that could yield better information about sex- and age-specific rates.

Although infections also occur in children and teenagers, the predominance of infection in adults probably results from culture-driven food behavior. Improperly cooked or prepared meat dishes may be more commonly eaten at adult-oriented events, particularly if alcohol is consumed. There are only a few published studies on the link between food behavior and trichinellosis (*15*,*16*), but this potential behavioral risk factor is similar to that that occurs in other foodborne parasites, such as fish-borne parasites (*17*).

Clinical Signs and Sequelae

For 5,377 cases, the chief clinical signs of trichinellosis were compatible in type and frequency with the classical trichinellosis syndrome (*7*), i.e., myalgia, diarrhea, fever, facial edema, and headaches that, after treatment, disappeared within 2–8 weeks (Table 4). Their rapid recovery reflects improvements in diagnostic methods, drug therapy, and public health education. The more rapid diagnosis and treatment in recent decades may also account for the low death rate; 42 deaths occurred worldwide during the 24-year period. Determining the disease burden of trichinellosis, however, is hampered by lack of data on the long-term sequelae of infection; few clinical reports included posttreatment follow-up evaluations, particularly beyond 1 month. The few studies that included follow-up over a longer time span indicate that myalgia and fatigue can persist for 4 months and, in a substantial proportion of cases, for up to 2 years (*18*–*20*). There is a need for internationally recognized epidemiologic and clinical protocols for trichinellosis outbreaks that include follow-up investigations that would facilitate reliable calculations of disease estimates.

Sources of Infection

Domestic pigs and wild boars were the major sources of *Trichinella* spp. infection for humans, but in recent years new infection sources, particularly from exotic hosts, have emerged (Table 6). An example is the cause of outbreaks in France, where in addition to wild boar sources, most trichinellosis cases for the past 2 decades have resulted from consumption of raw horse meat, a strong food preference in French culture (*21*). In Italy, human infections from consumption of horse meat have also been documented in 2 areas (Emilia Romagna and Lombardy regions in northern Italy and the Apulia region in southern Italy), where the French fondness for raw horse meat was introduced centuries ago (*16*). In China and the Slovak Republic, dog meat was the source of infection in several outbreaks (*22*,*23*). Although Judaic and Muslim religions forbid the consumption of pork, in Israel, Lebanon, and

Table 6. Types of meat linked to trichinellosis cases and outbreaks in the world, by World Health Organization region and country, 1986–2009*

Region/country	Meat source, % cases or outbreaks		
	Domestic pig	Wild game	Other
African Region: Ethiopia	0	100	0
Region of the Americas			
Argentina, Chile	100	0	0
Canada	0	100	0
United States	57	43	0
Mexico	86	0	14 (horse)
Eastern Mediterranean Region: Iran and Lebanon	0	100	0
European Region			
Belarus, Croatia, Georgia, Macedonia, Serbia, United Kingdom	100	0	0
Estonia, Turkey, Ukraine	50	50	0
France	0	65	35 (horse)
Germany	83	17	0
Greece, Israel	0	100	0
Hungary	52	48	0
Italy	38	38	24 (horse)
Lithuania	48	52	0
Poland	41	59	0
Romania	95	5	0
Slovakia	50	25	25 (dog)
Spain	60	40	0
South-East Asian Region: Thailand	50	50	0
Western Pacific Region			
People's Republic of China	86	13	1 (dog and others)
Japan	25	75	0
South Korea	0	100	0
India, Laos, Papua New Guinea	50	50	0
Singapore, Vietnam	100	0	0

*Data for each country aggregated from our database of studies. Data are from reports cited in the online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/11-0896-Techapp.pdf), section A.

Syria human outbreaks of trichinellosis have occurred after consumption of meat from wild boars among the Christian Arab population and immigrant laborers (*24–27*). Muslim populations are not entirely protected from acquiring trichinellosis, however, as demonstrated by a large outbreak in Turkey from the consumption of minced beef illegally mixed with pork of unknown origin (Table 2) (*28*).

The demographic movements of humans with culturally unique food practices, the illegal importation of uncontrolled meat from trichinellosis-endemic to -nonendemic countries, and the introduction of risky new food practices have resulted in cases in Denmark, Germany, Italy, Spain, Sweden, and the United Kingdom (Tables 2, 3) (*29–33*). Many cases of trichinellosis have occurred among international travelers who acquired *Trichinella* spp. infections while visiting or hunting in disease-endemic areas and the disease developed after they returned to their home countries (Table 3) (*34–38*).

Issues Affecting the Effective Control of Trichinellosis

Human behavior is the biggest determinant in the persistence of trichinellosis in the face of increasing regulations directed at ensuring the safety of meat and the enhancement of good management practices in farming, especially in areas in which trichinellosis is highly endemic, such as the European and the Americas regions. Of particular concern is an increase in the association of wild animals with domestic pigs. For example, in the United States, the expansion of the range of feral pigs (wild boars) into major areas of pig production, including free-range systems, may increase the risk for incursion of *Trichinella* spp. into the human food chain (*39*). The increased frequency of outbreaks from eating pork from wild boars in Europe is believed to be related to the great increase in wild boar populations (*40*). As with other foodborne zoonoses, cultural traditions in food behavior and practices in the use of domestic and wild animals are not easily altered, and trichinellosis can be expected to remain a food-safety risk in many areas of the world for the foreseeable future.

Acknowledgments

We thank the following persons for their willingness to provide articles, national data on human trichinellosis incidence, or translations for key articles and abstracts: N. Akkoc, H. Auer, R. Beck, S. Boutsini, J.Y. Chai, G. Deksne, Do Dung, P. Dubinsky, J. Dupouy-Camet, J. Epshtein, T. Garate, E. Golab,

M.A. Gómez Morales, B. Gottstein, L. Kolarova, T. Kortbeek, A. Malakauskas, Y. Nawa, Nguyen De, K. Nöckler, Ming-Bao Quiang, M. Ribicich, and J. Waikagul.

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Sealpox Virus in Marine Mammal Rehabilitation Facilities, North America, 2007–2009

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Sealpox, a zoonotic disease affecting pinnipeds (seals and sea lions), can occur among captive and convalescing animals. We surveyed 1 worker each from 11 marine mammal centers and interviewed 31 other marine mammal workers to ascertain their knowledge of and experience with sealpox virus and to identify factors associated with sealpox virus outbreaks among pinnipeds in marine rehabilitation facilities. Demographic and health data were obtained for 1,423 pinnipeds at the 11 facilities. Among the 23 animals in which sealpox was clinically diagnosed, 4 arrived at the facility ill, 11 became ill <5 weeks after arrival, and 2 became ill ≥5 weeks after arrival; the timing of illness onset was unknown for 6 animals. Most infections occurred in pinnipeds <1 year of age. Nine affected animals were malnourished; 4 had additional illnesses. Sealpox had also occurred among workers at 2 facilities. Sealpox is a noteworthy zoonosis of rehabilitating convalescing pinnipeds; workplace education can help to minimize risks for human infection.

Sealpox is a zoonotic disease of seals and sea lions (pinnipeds) and can be a complication of animals undergoing rehabilitation (*1*–*4*). The virus has been confirmed in free-ranging pinnipeds in the northern and southern Atlantic and Pacific Oceans (*5*,*6*), and infections have been observed in animals off the coast of Queen Maud's Land, Antarctica (*7*). Sea lionpox virus is taxonomically and genetically distinct from other sealpox viruses found in Pacific Ocean pinnipeds (*8*); however, for convenience, hereafter we will refer to sea lionpox virus as sealpox virus.

Eight pinniped species are known to be susceptible to infection with sealpox viruses: *Halichoerus grypus* (gray seals), *Phoca vitulina* (harbor seals), *P. groenlandica* (harp seals), *Callorhinus ursinus* (northern fur seals), *Mirounga angustirostris* (northern elephant seals), *Zalophus californianus* (California sea lions), *Eumetopias jubatus* (steller sea lions), and *Otaria flavescens* (South American sea lions) (*9*–*13*). Many of the reported infections have been in young animals brought into rehabilitation environments (*1*,*11*), but infections have also been observed among animals in colonies undergoing exogenous stress (pollution, food scarcity, other underlying infection) and, on occasion, in otherwise seemingly healthy adults. Sealpox virus can spread easily among confined animals and can increase the costs and length of rehabilitation. Studies conducted in pinniped rehabilitation centers have suggested that underlying health conditions and a history of veterinary care are risk factors for sealpox virus infection among California sea lions (*1*,*14*).

Animals infected with sealpox virus typically show development of firm skin nodules (1–3 cm) on the head, neck, and thorax. Solitary clusters of nodules may also be found on the abdomen, flippers, and mucosa or oral cavity (*6*,*13*). The nodules frequently are inflamed or necrotic but usually heal spontaneously within a few weeks, leaving a slightly raised, gray, furless scar. Illness levels can be substantial, but death rates generally are low except among juveniles, for which infections can interfere with feeding (*1*,*2*,*14*). The transmission dynamics of sealpox virus have not been thoroughly investigated, but virus transmission is thought to occur directly by skin-to-skin contact.

Sealpox viruses are tentatively classified in the genus *Parapoxvirus* (*15*), which comprises multiple species of virus that can infect humans. Other members of this genus—namely, orf virus, pseudocowpox virus, and bovine papular

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DOI: http://dx.doi.org/10.3201/eid1712.101945

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stomatitis virus—commonly infect domestic ruminants and pose a minor occupational hazard to producers of sheep, goat, and cattle. The typical manifestations of infection in humans with any of these agents, including sealpox virus, are similar, usually culminating in a single nodular lesion with a diameter of ≈3 mm–1 cm. The nodule is often painful and evolves slowly over the course of several weeks. Infected persons may also briefly experience systemic signs and symptoms (fever, myalgia, fatigue) during the initial stages of lesion formation.

Sealpox virus is likely transmitted to humans when broken skin comes into contact with virus shed from lesions (skin or oral) on infected pinnipeds. Two epidemiologically linked and 1 molecularly confirmed case of sealpox virus infection in marine mammal handlers have been reported (*16*,*17*). In each case, the handler reported cutting himself while handling pinnipeds that had lesions indicative of sealpox virus infection. Although few sealpox virus infections in humans have been reported, and risks to marine mammal workers remain undefined, data indicate that ≈50% of marine mammal handlers have been injured by a marine mammal (*18*,*19*). The prevalence of human sealpox virus infections may therefore be underestimated.

Since the Marine Mammal Protection Act was enacted in 1972, researchers have documented sizable increases in the abundance of animals of several pinniped species in North America (*20*,*21*). Moreover, the number of live-stranded pinnipeds brought to rehabilitation facilities in the United States since the early 1970s has steadily increased over an order of magnitude and now represents hundreds of animals each year (*22*). Thus, compared with 40 years ago, today more opportunities exist for encounters between humans and convalescing pinnipeds.

To better understand the risks for sealpox virus infection in humans, we conducted a study of marine mammal workers at 11 marine mammal centers with rehabilitation capacity for species in North American waters; the objectives of the study were to ascertain the workers' knowledge of and experience with sealpox virus and to identify factors associated with sealpox virus outbreaks among pinnipeds in rehabilitation centers. In addition, we also performed a survey of 31 marine mammal workers attending a professional conference to learn about their knowledge of and experience with sealpox infection in humans and animals.

Methods

Study Population

During 2007–2009, we contacted 25 marine mammal facilities in North America by phone or email to determine whether they met our inclusion criteria and were willing to participate in the survey. We invited facilities to participate that receive wild pinnipeds for research or rehabilitation purposes. Only facilities that had housed ≥1 pinnipeds during the previous year were asked to participate. Of the 25 marine mammal facilities contacted, 11 met the criteria and agreed to participate. In addition, we asked marine mammal workers attending a professional conference to fill out a separate study survey; 31 agreed to participate.

Study Design

We asked each of the 11 facilities that met the inclusion criteria to complete a questionnaire. From each facility, 1 person with knowledge of the center (the facility informant) provided the following information about pinnipeds maintained at the facility during the past 12 months: number and species of animals; demographic and health data; and information pertaining to quarantine practices, animal housing, and medical monitoring. We obtained written consent and sent surveys to participants by email or fax. Surveys were designed by using LiveCycle Enterprise Suite 2.5 software (Adobe, San Jose, CA, USA). Completed surveys were returned to us by mail, fax, or email.

Statistical Analyses

Data were entered in a spreadsheet and analyzed by using IBM SPSS Statistics 17.0 (www-01.ibm.com/software/analytics/spss). Frequencies and proportions were calculated for the following categorical variables: species, facility location, demographics, disinfectants used for cleaning cages, and housing characteristics. Pinnipeds were divided into 2 age groups, 1 comprising those ≤1 year of age (pups) and the other comprising those >1 year of age (adults). Free-text responses were coded into categorical variables when appropriate. Odds ratios and 95% confidence intervals were calculated for potential risk factors (age, location, sex) associated with animals who had sealpox cases, where appropriate.

Results

Of the 11 marine mammal facilities surveyed, 5 were located on the eastern (Atlantic) coast and 6 were located on the western (Pacific) coast of North America (Table 1). Survey informants who represented these facilities were staff veterinarians or veterinary technicians (55%), self-described facility directors (18%), or researchers and rehabilitation trainers (27%). Survey informants provided demographic and health information for 1,423 pinnipeds, representing 14 species (Table 1). Of these pinnipeds, 47% were California sea lions and 27% were harbor seals, and 84% of the animals were housed in facilities located along the Pacific coast (Table 1). Five species were represented on both coasts. Fifty-five percent of pinnipeds described were male, and 83% were <1 year of age.

Table 1. Characteristics and infection status for 1,423 pinnipeds at 11 marine mammal centers in the 12 months before survey of marine mammal centers, North America, 2007–2009

Species, age group, and residential status	No. animals					
	Sex		Facility location *		With sealpox†, n = 23	Total, N = 1,423
	M, n = 780‡	F, n = 643§	Pacific coast, n = 1,198¶	Atlantic coast, n = 227#		
Animal (species)						
California sea lion (*Zalophus californianus*)	365	301	662	4	8	666
Stellar sea lion (*Eumetopias jubatus*)	15	19	25	9	0	34
Northern fur seal (*Callorhinus ursinus*)	26	15	38	3	0	41
Northern elephant seal (*Mirounga angustirostris*)	101	72	173	0	1	173
Ribbon seal (*Histriophoca fasciata*)	1	0	1	0	0	1
Hooded seal (*Cystophora cristata*)	3	1	0	4	0	4
Gray seal (*Halichoerus grypus*)	45	18	0	63	3	63
Harp seal (*Phoca groenlandica*)	17	18	0	35	0	35
Harbor seal (*P. vitulina*)	198	191	290	99	11	389
Ringed seal (*P. hispida)*	1	1	2	2	0	2
Spotted seal (*P. largha)*	2	0	2	0	0	2
Guadalupe fur seal (*Arctocephalus gazella*)	2	2	4	0	0	4
Bearded seal (*Erignathus barbatus*)	4	4	0	8	0	8
South American sea lion (*Otaria flavescens)*	0	1	1	0	0	1
Age group						
Pup, ≤1 y of age	725	455	969	211	20	1,180
Adult	55	189	229	14	3	243
Residential status**						
Rescue/rehabilitation	661	626	1,071	216	23	1,287
Resident/other	119	17	127	9	0	136

*A total of 6 and 5 facilities each were located on the Pacific and Atlantic coasts, respectively.
†≈30 animals were reported to have sealpox, but information regarding species type, age group, and sex was available for only 23. Thus, only these 23 were included in the analysis. Of the 23 ill animals, 5 had laboratory-confirmed sealpox virus infection.
‡Fifteen of 780 animals were ill.
§Eight of 643 animals were ill.
¶Fourteen of 1,198 animals were ill.
#Nine of 225 animals were ill.
**Rescue/rehabilitation refers to pinnipeds that were brought in from the wild for rehabilitation; Resident/other refers to animals brought in for purposes other than rehabilitation.

Informants were asked to recall the approximate numbers of animals in which sealpox was clinically diagnosed during the previous year. If informants provided a range of case counts, the lower number was selected. For the 12-month period, an estimated 30 (2%) animals at the 11 facilities had sealpox; 25 diagnoses were presumptive (based on clinical suspicion) and 5 were laboratory confirmed (positive PCR or electron microscopy results). Demographic and health data were not available for 7 animals with presumptive sealpox; thus, these animals were not included in subsequent analyses, leaving a total of 23 infected pinnipeds at 9 centers for analysis of characteristics associated with sealpox virus infection. Individual animals from 4 species were identified as having sealpox: California sea lions (8), harbor seals (11), gray seals (3), and northern elephant seals (1) (Table 2). Sealpox was diagnosed upon arrival at a center for 5 (22%) animals (all wild), <5 weeks after arrival for 11 (48%) animals, and ≥5 weeks after arrival for 2 (9%) animals (Table 2). The timing of illness onset was independent of the animal's geographic location, species, and age. Most (83%) sealpox was diagnosed in pups, but the prevalence of infection was equivalent for adults and pups (1.2% and 1.8%, respectively).

Of the ill animals, 9 (39%) were malnourished, 4 (17%) had concurrent illness, and 10 (44%) had injuries and were malnourished or had concurrent illness (Table 2). Those with concurrent illness died or were euthanized (including all adults in which sealpox was diagnosed), while most animals with malnutrition recovered (Table 2). Informants from 5 marine centers reported transmission of sealpox from infected animals to unhealthy convalescing pinnipeds at their facility, 4 reported transmissions to healthy pinnipeds, and 6 reported transmission among pups. Of the 9 facilities that housed infected animals, 4 reported isolating them to prevent transmission.

To identify factors that might be associated with transmission of sealpox virus in marine mammal facilities, we obtained information about disinfectant use in pens, the number of animals housed per pen, and other housing characteristics (Table 3). Individual facilities had 1 to >16 enclosures and housed ≤83 pinnipeds during the 12 months before completing the survey. All facilities used at least 1 type of disinfectant, and all reported cleaning pens at

Table 2. Outcome of sealpox infection among 23 pinnipeds at 9 marine mammal rehabilitation facilities, North America, 2007–2009

Characteristic	Outcome of infection, no. animals			Total no. animals
	Illness resolved	Animal died or was euthanized	Unknown	
Species				
California sea lion	6	2	0	8
Gray seal	2	1	0	3
Northern elephant seal	0	1	0	1
Harbor seal	8	3	0	11
Male				
Pup, ≤1 y of age	9	3	1	13
Adult	0	1	0	1
Female				
Pup	6	1	0	7
Adult	0	2	0	2
Time of lesion development				
At admission to facility	1	3	0	4
<5 wk after admission	9	2	0	11
≥5 wk after admission	2	0	0	2
Health status				
Diseased	0	4	0	4
Malnourished	8	1	0	9
Injured plus malnourished or diseased	9	1	0	10

least 1× each week (Table 3). To assess whether medical screening and treatment practices might affect detection and prevention of sealpox, we obtained information about facility admission protocols for pinnipeds as well as laboratory and medical resources available at the facility. All facilities had quarantine or isolation enclosures for sick or newly admitted animals, and all facilities included a physical examination as part of their standard protocol for pinnipeds entering the facility. Of the 9 facilities that had animals with sealpox, 9 (100%) reported isolating newly admitted pinnipeds and collecting blood samples for pathogen screening; 4 (44%) also did fecal flotation and culture; and 2 (22%) did other tests, including, including urinalysis and radiography or ultrasound (Table 3). All facilities had veterinarians on site and used third-party diagnostic laboratories; 64% also had a veterinary diagnostic laboratory on site.

Overall, no significant association was found between sealpox infection and species, sex, or age in the study population (Table 2), and no significant associations were found at the facility level. The likelihood of a pinniped having sealpox was greater in facilities on the Atlantic coast (4% of animals) than the Pacific coast (1% of animals) (odds ratio 3.53; $p = 0.002$). However, this observation mainly results from case clusters at 2 facilities on the Atlantic coast. All of the ill animals were characterized as having been rescued or having been in rehabilitation (Table 1). All but 1 facility reported housing adults and pups together and housing different pinniped species together; there was no association with sealpox infection (Table 3). Of the 11 surveyed facilities, 9 (82%) cleaned the enclosures at least daily with bleach-based cleaning products (Table 3). No significant associations were found between type of disinfectant used and odds of sealpox infection. All facilities that housed infected animals reported cleaning enclosures at least daily.

None of the facility informants reported cases of sealpox infections among humans at the facility during the prior 12 months, but informants at 2 facilities reported cases of physician-diagnosed sealpox among workers in the previous 10 years. At both facilities, the affected persons were reported to have handled live animals with skin or oral lesions or both. Use of personal protective equipment (PPE) was reported at all facilities: 100% of respondents reported use of gloves; 91%, rain pants, overalls, or suits; and 9%, goggles and masks.

In addition to the survey of marine mammal rehabilitation facilities, we asked marine mammal workers attending a conference about their knowledge and experience with sealpox. Thirty-one conference attendees completed the survey. Of the 31 respondents, 17 (55%) reported observing pinnipeds affected with sealpox virus in rehabilitation facilities or in the wild, of which 9 were in North America, 5 in northern Europe, and 2 in New Zealand (1 location not specified). (In the United States, human sealpox virus infection is not a nationally reportable condition. However, as a rare infection, it would be considered notifiable in most states.) Of the 17 workers, 13 reported knowledge that sealpox virus can infect humans, and most (15/17) reported that they had learned this information from colleagues or educational material distributed at their workplace. Of the 31 respondents, 7

(23%) reported they had seen or known about a human case of sealpox: 2 reported that a colleague had received a diagnosis of sealpox, and 5 reported that they themselves had received a diagnosis of sealpox at some point during the past 15 years. Two of these 7 persons stated that their physician had prescribed tetracyclines to treat their infections. Tetracyclines are ineffective against sealpox infections; however, antimicrobial drugs are effective against a mycoplasma-associated infection, called seal finger, which is clinically similar to and can be confused with sealpox.

Discussion

For sea lions entering rehabilitation facilities, a history of rehabilitation is a strong indicator of the risk for poxvirus lesions (*14*). In our survey, facility informants reported that 2% of the animals housed in the marine mammal facilities surveyed had laboratory-confirmed or presumptive sealpox during the preceding 12 months. The proportion of infected animals was higher in facilities on the Atlantic than Pacific coast; however, on balance, more animals were housed in rehabilitation facilities on the Pacific coast. California sea lions accounted for the majority of pinnipeds under observation and for the majority of infected animals. Most infected animals were pups and were male, a finding consistent with previous observations (*1*,*14*). Documented infections have typically been reported for young animals brought into rehabilitation environments, which may reflect increased susceptibility among juvenile animals, particularly those under stress (*2*). Malnutrition, a hallmark of rescued pups, seemed not to indicate a poor prognosis, whereas injury, older age, and concurrent illness did.

Table 3. Characteristics of 11 marine mammal rehabilitation facilities that did or did not report sealpox among pinnipeds during the 12 months before survey of marine mammal centers, North America, 2007–2009

Characteristic	Sealpox reported in facility in past 12 mo	
	No	Yes
Animal enclosures		
Shared by adults and pups*	1	2
Shared by different species	2	5
Quarantine space available	2	9
Frequency of pen disinfection		
2×/d	0	3
1×/d	0	6
≤1×/wk	2	0
Disinfectant used†		
Any use	2	9
Bleach	1	8
Virkon	1	3
Chlorhex	2	2
Iodophores	0	2
Standard protocols for newly admitted pinnipeds†		
Isolation/quarantine	1	9
Physical examination	2	9
Blood tests‡	1	9
Vaccination§	1	1
Coast		
Atlantic	1	4
Pacific	1	5
Enclosure material†		
Plastic	1	4
Fiberglass	1	7
Cement	1	5
Epoxy resin	1	1
Wire	0	1

*Adults, >1 y of age; pups, ≤1 y of age.
†Responses were not mutually exclusive.
‡Tests performed included pathogen screening for morbillivirus, *Brucella* spp., rabies virus, *Leptospira* spp., West Nile virus, heartworms, intestinal helminths, and avian influenza virus.
§Vaccinations included those against West Nile virus, rabies virus, and *Leptospira* spp.

Approximately one fifth of infected animals arrived at the rehabilitation facility with symptoms of sealpox infection, and infection was observed within 5 weeks after arrival for half of the animals. We did not attempt to address whether underlying poor health status predisposes an animal to infection with sealpox virus or vice versa. However, 5 facility informants reported sealpox transmission in their facility to both unhealthy and healthy pinnipeds, suggesting that, in captivity, the disease can develop even in apparently healthy animals. The transmission dynamics of sealpox virus have not been well described, but transmission between pinnipeds is likely to occur during suckling; through other forms of skin-to-skin contact; or by fomites, which are of particular concern for captive animals. Further studies are needed to explain transmission dynamics.

Serosurveys suggest that sealpox virus is persistently infecting most wild California sea lions, and other species of pinnepeds may also be persistently infected with other sealpox species (*23*). Thus, for persons who rehabilitate animals, risks for zoonotic transmission of these agents might be appreciable. Vigilant adherence to quarantine and institutional hygiene practices, including the use of PPE by staff, likely serves to diminish risks for virus transmission to humans and may explain why reports of human infection with sealpox virus are rare (*16*,*17*). In our study, all marine mammal workers who knew that sealpox virus is zoonotic reported that they had learned this information from colleagues and educational material provided by their workplace, and all facilities reported that PPE (gloves, boots) were made available to all workers; however, some marine mammal workers indicated that PPE is rarely used when pinnipeds are handled in the wild. Sealpox is only 1 of many potential hazards associated with wildlife rehabilitation activities; workplace education programs should highlight the risk for zoonotic disease transmission from pinnipeds to marine mammal workers and the use of PPE when handling animals in the wild.

Acknowledgments

We thank Hendrick Nollens, Lena Measures, and Tonya Clauss for their advice on survey development.

Dr Roess was an Epidemic Intelligence Service officer at CDC during the conduct of this study and is currently a visiting assistant professor of Global Health at George Washington University. Her areas of research include antimicrobial drug resistance (*Pneumococcus*, *Campylobacter*, and *Salmonella* spp.); emerging, reemerging, and zoonotic diseases; sexually transmitted infections; food safety; pandemic preparedness; and health communications.

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Transmission of Guanarito and Pirital Viruses among Wild Rodents, Venezuela

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Samples from rodents captured on a farm in Venezuela in February 1997 were tested for arenavirus, antibody against Guanarito virus (GTOV), and antibody against Pirital virus (PIRV). Thirty-one (48.4%) of 64 short-tailed cane mice (*Zygodontomys brevicauda*) were infected with GTOV, 1 Alston's cotton rat (*Sigmodon alstoni*) was infected with GTOV, and 36 (64.3%) of 56 other Alston's cotton rats were infected with PIRV. The results of analyses of field and laboratory data suggested that horizontal transmission is the dominant mode of GTOV transmission in *Z. brevicauda* mice and that vertical transmission is an important mode of PIRV transmission in *S. alstoni* rats. The results also suggested that bodily secretions and excretions from most GTOV-infected short-tailed cane mice and most PIRV-infected Alston's cotton rats may transmit the viruses to humans.

The Tacaribe serocomplex viruses (family *Arenaviridae*, genus *Arenavirus*) known to occur in Venezuela are Guanarito virus (GTOV) and Pirital virus (PIRV) (*1*,*2*). GTOV is the etiologic agent of Venezuelan hemorrhagic fever (VHF) (*1*). The human health significance of PIRV has not been rigorously investigated (*3*).

Specific members of the rodent family Cricetidae (*4*) are the principal hosts of the Tacaribe complex viruses for which natural host relationships have been well characterized. It is generally accepted that humans usually become infected with arenaviruses by inhalation of virus in aerosolized droplets of saliva, respiratory secretions, urine, or blood from infected rodents or by inhalation of virus-contaminated dust particles.

The results of published studies (*2*,*5*) indicated that the short-tailed cane mouse (*Zygodontomys brevicauda*) is the principal host of GTOV and that the Alston's cotton rat (*Sigmodon alstoni*) is the principal host of PIRV. The objective of our study was to extend knowledge of the natural host relationships of these arenaviruses, particularly the relative importance of various modes of intraspecies virus transmission and the prevalence of virus shedding among naturally infected rodents.

Materials and Methods

This report is the sixth in a series of publications that include rodents captured in Venezuela in February 1997. The rodents were trapped on Hato Maporal, a farm near the town of Caño Delgadito in the municipality of Guanarito, Portuguesa State (*6*).

Study Sites

Rodents were trapped at 3 sites on Hato Maporal, designated A, B, and C. Traps were set at site A in tall grassy areas on the edge of a grove of trees, site B in tall grassy areas alongside an unpaved road and in a field filled with weeds, and site C in a field filled with crop stubble and in grassy areas alongside the field. Site A was 0.2 km from site B, and site C was 0.7 km from sites A and B.

Capture and Processing of Rodents

The rodents were captured in aluminum live-capture traps (*6*). The traps were placed at 8-m intervals, baited with small pieces of freshly cut pineapple, set 1 h before sunset, and checked at daybreak the following day. Each rodent was assigned a unique identification (FHV, Fiebre

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DOI: http://dx.doi.org/10.3201/eid1712.110393

Hemorrágica Venezolana) number and then killed by exposure to a lethal dose of vaporized chloroform. The identification number, date of capture, trap site and trap number, species identity, sex, total length (tip of nose to tip of tail), length of tail, and other information were recorded on a standardized form. A throat swab; samples of blood, lung, spleen, liver, and kidney; and a sample of urine were collected from each rodent (*6*). These samples were stored in cryo-vials in liquid nitrogen in the field and then shipped on dry ice to the University of Texas Medical Branch (Galveston, TX, USA).

Virus Assay

The samples from the throat swabs; crude 10% wt/vol homogenates of the samples of lung, spleen, and kidney in 0.01 mol/L phosphate-buffered saline; and samples of urine were assayed for arenavirus by cultivation in monolayers of Vero E6 cells (*5*). Cells harvested from the monolayers on day 13 or 14 postinoculation were tested for arenaviral antigen by using an indirect fluorescent antibody test (IFAT) in which the primary antibody was a mixture of a hyperimmune mouse ascitic fluid (HMAF) raised against the GTOV prototype strain INH-95551 (*7*) and an HMAF raised against the PIRV prototype strain VAV-488 (*2*).

Serologic Characterization of Viruses

Strains of GTOV were distinguished from strains of PIRV by an ELISA (*5*). The test antigens were detergent lysates of infected Vero E6 cells. Serial 2-fold dilutions (from 1:800 through 1:204,800 vol/vol) of an anti-GTOV HMAF and anti-PIRV HMAF were tested against each antigen. Antibody (IgG) bound to antigen was detected by using a goat antimouse (*Mus musculus*) IgG peroxidase conjugate in conjunction with the ABTS Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). The reactivity of an HMAF against an antigen was the sum of the optical densities of the 8 reactions in the series of 4-fold dilutions of the HMAF tested against the antigen. The identity of an isolate was determined by direct comparison of the reactivity of the anti-GTOV HMAF versus the reactivity of the anti-PIRV HMAF against the test antigen.

Genetic Characterization of Viruses

The sequences of a 616–619-nt fragment of the nucleocapsid (N) protein genes of the arenaviruses isolated from the spleens of 21 rodents in this study (Table 1) were determined to assess the accuracy of the interpretation of the ELISA data. Total RNA was isolated from monolayers of infected Vero E6 cells by using TRIzol Reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) or Tri Reagent (Sigma Aldrich, St. Louis, MO, USA). First-strand cDNA was synthesized by using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen Life Technologies, Inc.) in conjunction with oligonucleotide 19C-cons (*8*). Amplicons were synthesized from first-strand cDNA by using the MasterTaq Kit (Eppendorf North America, Inc., Westbury, NY, USA) in conjunction with oligonucleotides that flank either a 619-nt fragment of the N protein gene of GTOV strain INH-95551 or the homologous region (a 616-nt fragment) of the N protein gene of PIRV strain VAV-488. Amplicons of the expected size were sequenced directly by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA, USA).

Antibody Assay

Blood samples were rendered noninfectious by irradiation (5×10^6 rads, Co^{60} source), diluted 1:20 vol/vol in phosphate-buffered saline, and then tested for IgG against GTOV strain INH-95551 and PIRV strain VAV-488 by using an IFAT. The cell spots were either a mixture of Vero E6 cells infected with INH-95551 and uninfected Vero E6 cells or a mixture of Vero E6 cells infected with VAV-488 and uninfected Vero E6 cells. Antibody bound to antigen was revealed by using a fluorescein isothiocyanate–conjugated goat antibody raised against mouse (*M. musculus*) IgG (Kirkegaard and Perry). End-point titers against INH-95551 and VAV-488 were measured in the positive samples by using serial 2-fold dilutions beginning at 1:20 and ending at 1:640 vol/vol.

Data Analysis

The male short-tailed cane mice, female short-tailed cane mice, male Alston's cotton rats, and female Alston's cotton rats were assigned on the basis of their nose-to-rump lengths (measured in mm) to 4 size categories. In each instance, the upper boundary of class I was the mean length – 1 SD, the upper boundary of class II was the mean length, the upper boundary of class III was the mean length + 1 SD, and the upper boundary of class IV was the longest nose-to-rump length (Table 2). Animals that were culture-positive or antibody-positive were treated as infected. The acceptable type I error in all statistical tests was $\alpha = 0.05$.

The analyses of the nucleotide sequences included GTOV strain INH-95551 (GenBank accession no. U43686); GTOV strains VAV-623, VAV-952, and AV 97021119 (Table 1); PIRV strain VAV-488 (GenBank accession no. U62561); and PIRV strains VAV-628, VAV-956, and AV 97021016 (Table 1). Strain INH-95551 was isolated from a patient who died of VHF (*7*); VAV-488 was isolated from an Alston's cotton rat captured ≈54 km east-northeast of Hato Maporal (*2*); VAV-623, VAV-628, VAV-952, and VAV-956 were isolated from rodents captured on Hato Maporal in 1994 (*5*); and AV 97021016

Table 1. Strains of Guanarito virus and Pirital virus isolated from the spleens of rodents captured on Hato Maporal and included in the analysis of nucleocapsid protein gene sequences, municipality of Guanarito, Portuguesa State, Venezuela, 1994–1997

Virus and strain*	Rodent no.†	Species	Date captured	Trap site	GenBank accession no.
Guanarito					
VAV-623	FHV-623	*Zygodontomys brevicauda*	1994 Mar 23	–	JF412365
VAV-952	FHV-952	*Z. brevicauda*	1994 Jun 6	–	JF412366
AV 97020997	FHV-4030	*Z. brevicauda*	1997 Feb 4	A	JF412369
AV 97021004	FHV-4037	*Z. brevicauda*	1997 Feb 4	A	JF412370
AV 97021033	FHV-4066	*Z. brevicauda*	1997 Feb 5	A	JF412371
AV 97021034	FHV-4067	*Z. brevicauda*	1997 Feb 5	A	JF412372
AV 97021073	FHV-4106	*Z. brevicauda*	1997 Feb 11	B	JF412373
AV 97021084	FHV-4117	*Z. brevicauda*	1997 Feb 11	B	JF412374
AV 97021092	FHV-4125	*Z. brevicauda*	1997 Feb 11	B	JF412375
AV 97021104	FHV-4137	*Z. brevicauda*	1997 Feb 12	B	JF412376
AV 97021106	FHV-4139	*Z. brevicauda*	1997 Feb 12	B	JF412377
AV 97021113	FHV-4146	*Z. brevicauda*	1997 Feb 12	B	JF412378
AV 97021116	FHV-4149	*Sigmodon alstoni*	1997 Feb 12	C	JF412379
AV 97021117	FHV-4150	*Z. brevicauda*	1997 Feb 12	C	JF412380
AV 97021119	FHV-4152	*Z. brevicauda*	1997 Feb 12	C	AY573922
Pirital					
VAV-628	FHV-628	*S. alstoni*	1994 Mar 23	–	JF412367
VAV-956	FHV-956	*Z. brevicauda*	1994 Jun 19	–	JF412368
AV 97021016	FHV-4049	*S. alstoni*	1997 Feb 4	B	AY573923
AV 97021026	FHV-4059	*S. alstoni*	1997 Feb 4	B	JF412381
AV 97021027	FHV-4060	*S. alstoni*	1997 Feb 4	B	JF412382
AV 97021028	FHV-4061	*S. alstoni*	1997 Feb 4	B	JF412383
AV 97021029	FHV-4062	*S. alstoni*	1997 Feb 4	B	JF412384
AV 97021030	FHV-4063	*S. alstoni*	1997 Feb 5	A	JF412385
AV 97021036	FHV-4069	*S. alstoni*	1997 Feb 5	A	JF412386
AV 97021040	FHV-4073	*S. alstoni*	1997 Feb 5	A	JF412387
AV 97021112	FHV-4145	*S. alstoni*	1997 Feb 12	B	JF412388
AV 97021120	FHV-4153	*S. alstoni*	1997 Feb 12	C	JF412389

*Strains VAV-623, VAV-628, VAV-952, VAV-956, AV 97021016, and AV 97021119 were published previously (*3*,*5*). The nucleotide sequences of the 21 other strains were determined in this study. –,rodents were captured in 1994 before establishment of trap sites.
†FHV, Fiebre Hemorrágica Venezolana.

and AV 97021119 were isolated from rodents captured on Hato Maporal in 1997 and reported previously (*3*). The neighbor-joining analysis of genetic (p) distances was done with MEGA version 4.0 (*9*). Bootstrap support (*10*) for the results of the neighbor-joining analysis was based on 1,000 pseudoreplicate datasets generated from the original multiple nucleotide sequence alignment.

Antibody titers <20 were considered 10 in comparisons of antibody titers to GTOV strain INH-95551 and PIRV strain VAV-488 in individual blood samples. The apparent homologous virus in an antibody-positive sample was the virus associated with the highest titer if the absolute value of the difference between the titers to GTOV and PIRV was ≥4-fold.

Table 2. Prevalence of Guanarito virus infection in short-tailed cane mice (*Zygodontomys brevicauda*) and Pirital virus infection in Alston's cotton rats (*Sigmodon alstoni*) captured on Hato Maporal, municipality of Guanarito, Portuguesa State, Venezuela, February 1997

	Short-tailed cane mice, no. infected/no. tested†			Alston's cotton rats, no. infected/no. tested‡		
Size class*	M	F	Total	M	F	Total
I	1/5	0/2	1/7	5/6	3/3	8/9
II	2/8	5/15	7/23	6/8	3/8	9/16
III	9/13	7/13	16/26	6/11	7/9	13/20
IV	2/3	5/5	7/8	3/7	3/4	6/11
Total	14/29	17/35	31/64	20/32	16/24	36/56

*The boundaries of the size classes were based on analyses of nose-to-rump lengths (measured in mm). Male cane mice: I, 75.0–100.9; II, 101.0–117.1; III, 117.2–133.4; IV, 133.5–147.0. Female cane mice: I, 64.0–99.0; II, 99.1–111.2; III, 111.3–123.4; IV, 123.5–135.0. Male cotton rats: I, 89.0–105.6; II, 105.6–123.6; III, 123.6–141.6; IV, 141.6–150.0. Female cotton rats: I, 83.0–100.8; II, 100.8–119.6; III, 119.6–138.4; IV, 138.4–146.0.
†Guanarito virus (GTOV) was isolated from 29 cane mice. Antibody against GTOV was found in 11 of the 29 culture-positive cane mice and 2 of the 35 culture-negative cane mice.
‡Pirital virus (PIRV) was isolated from all of the infected cotton rats, and antibody against PIRV was found in 1 of the culture-positive cotton rats. The table does not include FHV-4149, the only cotton rat infected with GTOV.

Results

A total of 128 rodents were captured on Hato Maporal in February 1997 in 1,000 trap-nights, with an overall trap success rate of 12.8% (Table 3). Most (121 [94.5%]) of the 128 rodents were short-tailed cane mice or Alston's cotton rats.

Fifty-seven (89.1%) of the 64 short-tailed cane mice and 55 (96.5%) of the 57 Alston's cotton rats were captured in 91 (37.9%) of the 240 traps set on sites A and B. Six Alston's cotton rats from site A were found in traps adjacent to traps in which cane mice were captured, 17 Alston's cotton rats from site B were found in traps adjacent to traps in which cane mice were captured, and a cotton rat and cane mouse were captured on different nights in each of 3 traps on site A and 6 traps on site B. Collectively, these observations suggest that the short-tailed cane mice captured on sites A and B lived in close proximity to Alston's cotton rats and vice versa.

Arenavirus was isolated from the throat swabs; samples of lung, spleen, or kidney; and samples of urine from 29 (45.3%) of the 64 short-tailed cane mice, 37 (64.9%) of the 57 Alston's cotton rats, and none of the 7 pygmy rice rats (Table 4). The analyses of the ELISA data indicated that the arenaviruses isolated from Alston's cotton rat FHV-4149 and the short-tailed cane mice are strains of GTOV and that the arenaviruses isolated from the Alston's cotton rats other than FHV-4149 are strains of PIRV. The results of the neighbor-joining analysis of N protein gene sequence data (Figure) were 100% concordant with the serologic identities of the 21 viruses selected for genetic characterization, GTOV strain AV 97021119, and PIRV strain AV 97021016.

Nucleotide sequence nonidentity between the GTOV strains from 1994 (i.e., VAV-623 and VAV-952) was 6.0%, nucleotide sequence nonidentities among AV 97021119 and the 12 other GTOV strains from 1997 ranged from 0 to 7.3%, and nucleotide sequence nonidentities between the GTOV strains from 1994 and the 13 GTOV strains from 1997 ranged from 0.3% to 6.6%. Similarly, nucleotide sequence nonidentity between the PIRV strains from 1994 (i.e., VAV-628 and VAV-956) was 5.2%, nucleotide sequence nonidentities among AV 97021016 and the 9 other PIRV strains from 1997 ranged from 0 to 6.3%, and nucleotide sequence nonidentities between the PIRV strains from 1994 and the 10 PIRV strains from 1997 ranged from 1.0% to 6.3%.

Antibody (IgG) against GTOV or PIRV was found in 11 (37.9%) of the 29 culture-positive cane mice, 2 (5.7%) of the 35 culture-negative cane mice, 1 (2.7%) of the 37 culture-positive cotton rats, none of the 20 culture-negative cotton rats, and none of the 7 pygmy rice rats (Table 4). The only antibody-positive cotton rat (FHV-4124) was mature (size class III) and antibody positive to GTOV and PIRV. None of the 7 cane mice in size class I was antibody positive to GTOV or PIRV.

The end-point antibody titers to GTOV in the antibody-positive cane mice ranged from 40 to 160, none of the cane mice were antibody positive to PIRV, and the end-point antibody titers to GTOV and PIRV in the antibody-positive Alston's cotton rat were 40 and $\geq$640, respectively. Thus, GTOV was the apparent homologous virus in all of the antibody-positive cane mice, and PIRV was the apparent homologous virus in the antibody-positive cotton rat.

Eight (26.7%) of the 30 short-tailed cane mice in size classes I and II, 23 (67.6%) of the 34 cane mice in size classes III and IV, 11 (68.8%) of the 16 male short-tailed cane mice in size classes III and IV, and 12 (66.7%) of the 18 female short-tailed cane mice in size classes III and IV were infected with GTOV (Table 2). The prevalence of infection in the 34 short-tailed cane mice in size classes III and IV differed significantly from that in the 30 cane mice in size classes I and II (2-tailed Fisher exact test $p<0.01$), but the prevalence of infection in the 16 male short-tailed cane mice in size classes III and IV did not differ significantly from that in the 18 female short-tailed cane mice in size classes III and IV (2-tailed Fisher exact test $p = 0.72$).

Thirteen (86.7%) of the 15 female short-tailed cane mice in size class II, 7 (53.8%) of the 13 female short-tailed cane mice in size class III, and 5 (100%) of the 5 female short-tailed cane mice in size class IV were pregnant. Furthermore, 4 (30.8%) of the 13 pregnant short-tailed cane mice in size class II, 5 (71.4%) of the 7 pregnant short-tailed cane mice in size class III, and all 5 of the pregnant short-tailed cane mice in size class IV were infected with GTOV.

As indicated previously, 12 (66.7%) of the 18 female short-tailed cane mice in size classes III and IV were

Table 3. Prevalence of arenavirus infections in rodents captured on Hato Maporal, municipality of Guanarito, Portuguesa State, Venezuela, February 1997

Site*	No. traps	No. trap-nights	Trap success rate (%)	Prevalence of infection†			Total rodents
				Cotton rat	Cane mouse	Rice rat	
A	80	160	36/160 (22.5)	5/14	12/20	0/2	17/36
B	160	640	82/640 (12.8)	30/41	15/37	0/4	45/82
C	100	200	10/200 (5.0)	2/2	4/7	0/1	6/10
Total	340	1,000	128/1,000 (12.8)	37/57	31/64	0/7	68/128

*Traps were set at site A on February 4 and 5; site B on February 4, 5, 11, and 12; and site C on February 5 and 12.
†Number of infected (culture-positive or antibody-positive) rodents/total number of rodents tested (captured). Cotton rat, Alston's cotton rat (*Sigmodon alstoni*); cane mouse, short-tailed cane mouse (*Zygodontomys brevicauda*); rice rat, delicate pygmy rice rat (*Oligoryzomys delicatus*).

Table 4. Isolation of arenaviruses from 64 short-tailed cane mice (*Zygodontomys brevicauda)* and 57 Alston's cotton rats (*Sigmodon alstoni)* captured on Hato Maporal, municipality of Guanarito, Portuguesa State, Venezuela, February 1997

No.	Sample*					Antibody status
	Throat swab	Lung	Spleen	Kidney	Urine	
Cane mice†						
3	–	+	–	–	–	–
5	–	–	+	–	–	–
6	–	+	+	+	+	–
	–	–	+	+	+	–
1	+	–	+	+	+	–
2	+	+	+	+	+	–
2	+	+	+	+	+	+
2	+	–	+	+	+	+
6	–	–	+	+	+	+
1	–	–	+	+	–	+
2	–	–	–	–	–	+
33	–	–	–	–	–	–
Cotton rats‡						
2	–	–	+	–	–	–
4	–	+	+	+	–	–
21	–	+	+	+	+	–
9	+	+	+	+	+	–
1	–	–	+	–	–	+
20	–	–	–	–	–	–

*–, negative; +, positive.
†The arenaviruses isolated from the cane mice were strains of Guanarito virus.
‡The arenavirus isolated from cotton rat FHV-4149 was a strain of Guanarito virus. The arenaviruses isolated from the 36 other culture-positive cotton rats were strains of Pirital virus.

infected with GTOV. Yet only 1 (14.3%) of the 7 short-tailed cane mice in size class I was infected with GTOV (Table 2). The difference between the prevalence of infection in the female short-tailed cane mice in size classes III and IV and that in the short-tailed cane mice in size class I was significant (2-tailed Fisher exact test p = 0.03).

Seventeen (65.4%) of the 26 Alston's cotton rats in size classes I and II, 19 (61.3%) of the 31 Alston's cotton rats in size classes III and IV, 9 (50.0%) of the 18 male Alston's cotton rats in size classes III and IV, and 10 (76.9%) of the 13 female Alston's cotton rats in size classes III and IV were infected with PIRV (Table 2). The prevalence of PIRV infection in the Alston's cotton rats in size classes III and IV did not differ significantly from that in the Alston's cotton rats in size classes I and II (2-tailed Fisher exact test p = 0.78), and the prevalence of infection in the male Alston's cotton rats in size classes III and IV did not differ significantly from that in the female Alston's cotton rats in size classes III and IV (2-tailed Fisher exact test p = 0.16).

Discussion

The results of this study affirm conclusions drawn from previous studies (*2*,*5*). Specifically, the short-tailed cane mouse is the principal host of GTOV, and Alston's cotton rat is the principal host of PIRV. Examples of GTOV infection in rodents other than the short-tailed cane mouse are limited to the isolation of AV 97021116 from Alston's cotton rat FHV-4149 and the isolation of GTOV from a pygmy rice rat (*Oligoryzomys* sp.) and 4 Alston's cotton rats captured at localities in Venezuela other than Hato Maporal (*11*). Similarly, examples of PIRV infection in rodents other than Alston's cotton rats are limited to the isolation of VAV-956 from a short-tailed cane mouse captured on Hato Maporal in 1994 (*5*) and the isolation of PIRV from 5 short-tailed cane mice and a spiny rat (*Proechimys* sp.) captured at other localities in Venezuela (*12*).

The results of the analysis of the capture data in this study suggest that the short-tailed cane mice captured on sites A and B lived in close physical association with Alton's cotton rats and vice versa. Yet none of the 57 short-tailed cane mice captured on A or B were infected with PIRV, and only 1 of the 55 Alston's cotton rats captured on A or B was infected with GTOV. Collectively, these observations suggest that intimate social interactions between short-tailed cane mice and Alston's cotton rats are infrequent. Alternatively, GTOV-infected short-tailed cane mice are rarely infectious to Alston's cotton rats and PIRV-infected Alston's cotton rats are rarely infectious to short-tailed cane mice.

Chronic infections in individual rodents appear to be critical to the long-term maintenance of arenaviruses in nature. Factors that likely affect the duration of GTOV infection in naturally infected short-tailed cane mice include age at exposure to GTOV, host genetics, virus genetics, inoculum dose, and route of exposure (*13*,*14*).

The positive association between prevalence of infection and size class in the short-tailed cane mice suggests

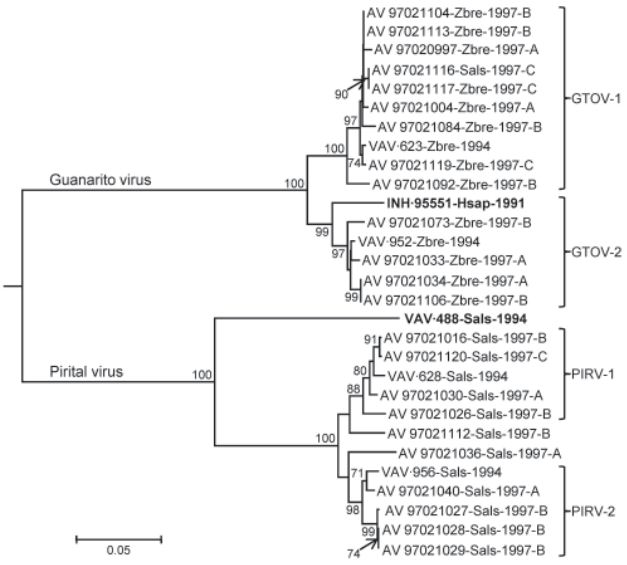

Figure. Phylogenetic relationships among 27 arenaviruses isolated from rodents captured on Hato Maporal, Portuguesa State, Venezuela, 1994 or 1997; Guanarito virus (GTOV) prototype strain INH-95551 (**boldface**); and Pirital virus (PIRV) prototype strain VAV-488 (**boldface**) based on a neighbor-joining analysis of nucleocapsid protein gene sequence data. Branch lengths are proportional to genetic (p) distances; the numbers at the nodes indicate the percentage of 1,000 bootstrap replicates that supported the interior branches; bootstrap support values <70% are not listed; and the analysis was rooted to Oliveros virus strain 3229–1 (GenBank accession no. NC_010248). The branch labels include (in the following order) virus strain, host species, year of isolation, and (viruses from 1997) the study site at which the infected rodent was captured. Hsap, *Homo sapiens*; Sals, *Sigmodon alstoni*; Zbre, *Zygodontomys brevicauda*.

that most GTOV infections in short-tailed cane mice are acquired in an age-dependent manner. Allogrooming, mating, intraspecies aggression, and other activities that entail close physical contact may facilitate horizontal transmission in *Z. brevicauda* mice. The isolation of GTOV from the samples of lung but not the samples of spleen or kidney from 3 antibody-negative short-tailed cane mice (Table 4) suggests that these animals were infected by way of the respiratory tract rather than by wounding or venereal contact. The lack of an association between prevalence of infection and sex in the short-tailed cane mice in size classes III and IV suggests that male animals and female animals contribute equally to the transmission of GTOV in *Z. brevicauda* mice.

Under the assumption that short-tailed cane mice whelp their first offspring after they reach size class III, the high prevalence of infection in the female cane mice in size classes III and IV together with the low prevalence of infection in the cane mice in size class I suggest that vertical (dam-to-progeny) transmission of GTOV among *Z. brevicauda* mice is uncommon. Perhaps GTOV infection in the cane mouse fetus is lethal late in gestation. Alternatively, the survivorship of congenitally infected short-tailed cane mice may be significantly less than the survivorship of their uninfected counterparts during birth through weaning.

Together, the high prevalence of PIRV infection in the Alston's cotton rats in size class 1 and the lack of an association between prevalence of infection and size class in the Alston's cotton rats suggest that most cotton rats become infected with PIRV at an early age, perhaps in utero or immediately postpartum. Hypothetically, vertical (dam-to-progeny) virus transmission is the dominant mode of PIRV transmission in *S. alstoni* rats.

Arenavirus was isolated from the throat swabs and/or samples of urine from 20 (64.5%) of the 31 infected short-tailed cane mice and 30 (83.3%) of the 36 PIRV-infected Alston's cotton rats in this study (Table 4), suggesting that bodily secretions or excretions from most GTOV-infected short-tailed cane mice and most PIRV-infected Alston's cotton rats can transmit the viruses to humans. In a laboratory study (*13*), newborn, juvenile, and some adult short-tailed cane mice inoculated with GTOV strain INH-95551 persistently shed virus in saliva, respiratory secretions, or urine through day 208 postinoculation. Whether the magnitude and duration of virus shedding in PIRV-infected Alston's cotton rats are comparable with that in GTOV-infected short-tailed cane mice has not been investigated.

VHF was first recognized as a distinct clinical entity during an outbreak of hemorrhagic fever that began in 1989 in Guanarito (*1*). From September 1989 through December 2006, the State of Portuguesa recorded 618 VHF cases, with a case-fatality rate of 23.1% (*3*). Most (610 [98.7%]) patients lived or worked in rural areas in Guanarito when they became ill with VHF.

GTOV virus is presumed to be the only agent of VHF; however, the majority of the arenaviruses isolated from VHF patients during September 1989 through December 2006 were identified as strains of GTOV solely on the basis of the results of an IFAT in which extensive cross-reactivity between PIRV and GTOV is possible (*5*). Thus, the arenaviruses isolated from some VHF cases may be strains of PIRV.

PIRV, in association with *S. alstoni* rats, is widely distributed in rural areas in Guanarito and elsewhere in Portuguesa State (*2*,*5*,*12*). Furthermore, Alston's cotton rats (like short-tailed cane mice) are common in grass-dominated habitats, for example, tall grass and hedgerows adjacent to cultivated fields and areas with tall grass alongside human dwellings. Thus, the epidemiology of PIRV infection likely is highly similar to the epidemiology

of GTOV infection in Portuguesa, with most infections in persons who live or work in rural areas in Guanarito.

The neighbor-joining analysis of N protein sequence data separated the viruses from Hato Maporal into 4 groups: GTOV-1, GTOV-2, PIRV-1, and PIRV-2 (Figure). Each group included a strain from 1994 and strains from 1997, suggesting that multiple evolutionary lineages of GTOV and multiple evolutionary lineages of PIRV were maintained on Hato Maporal during mid-June 1994 through early February 1997. Whether GTOV-1 viruses differ from GTOV-2 viruses with regard to pathogenicity in humans or cane mice and whether PIRV-1 viruses differ from PIRV-2 viruses with regard to pathogenicity in humans or cotton rats has not been investigated.

Acknowledgments

We thank Nuris M. C. de Manzione for facilitating the shipment of samples from Portuguesa, Venezuela, to the University of Texas Medical Branch, Galveston.

Support for this research was provided by National Institutes of Health grants AI-39800 and AI-67947.

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Hepatitis E Virus in Rats, Los Angeles, California, USA

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The role of rats in human hepatitis E virus (HEV) infections remains controversial. A genetically distinct HEV was recently isolated from rats in Germany, and its genome was sequenced. We have isolated a genetically similar HEV from urban rats in Los Angeles, California, USA, and characterized its ability to infect laboratory rats and nonhuman primates. Two strains of HEV were isolated from serum samples of 134 wild rats that had a seroprevalence of antibodies against HEV of ≈80%. Virus was transmissible to seronegative Sprague-Dawley rats, but transmission was spotty and magnitude and duration of infection were not robust. Viremia was higher in nude rats. Serologic analysis and reverse transcription PCR were comparably sensitive in detecting infection. The sequence of the Los Angeles virus was virtually identical to that of isolates from Germany. Rat HEV was not transmissible to rhesus monkeys, suggesting that it is not a source of human infection.

Hepatitis E virus (HEV) is a major cause of epidemic waterborne and sporadic hepatitis in developing countries. Hepatitis E is caused principally by HEV genotypes 1 and 2 (*1*). Recently, hepatitis E has been diagnosed with increasing frequency as a cause of sporadic hepatitis in industrialized countries (*2*). Additionally, a large proportion (≤20%) of populations of such countries have antibodies against HEV in the absence of any recognized hepatitis (*3*–*5*), and evidence is increasing that these antibodies might be the result of subclinical infections acquired zoonotically.

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DOI: http://dx.doi.org/10.3201/eid1712.110482

Strains of HEV representing genotypes 3 and 4, which have been isolated from humans with hepatitis E, regularly infect pigs worldwide (*6*), and infection in humans caused by eating undercooked meat from domestic pigs, wild boar, and several species of wild deer has been documented (*6*,*7*). However, many, if not most, persons who have unexplained antibodies against HEV do not eat undercooked pork or venison, raising the possibility that other animals or modes of zoonotic transmission exist. It is noteworthy that swine handlers in the United States have a higher incidence of antibodies against HEV than do healthy blood donors, even though pork is generally thoroughly cooked in the United States. Therefore, eating pork is unlikely to explain the prevalence of antibodies against HEV in this country.

Numerous species, including rodents, have been found to have antibodies reactive with capsid protein of human HEV strains, and HEV closely related to genotypes 3 or 4 has been recently isolated from rabbits (*8*), cattle (*9*), and sheep (*10*). However, an HEV strain recently isolated from rats was unique and only distantly related to known strains (*11*). Thus, it is important to understand how this rat virus is related to human infections. Rats are particularly interesting as a potential source of human infections because although they are not a human food, they have a high seroprevalence of antibodies against HEV (*12*,*13*) and they are ubiquitous and in close contact with humans everywhere.

We have demonstrated that a high proportion of wild-caught *Rattus norvegicus*, *R. rattus*, and *R. exulans* rats trapped in several US cities (Baltimore, Maryland; New Orleans, Louisiana; and the islands of Oahu and Hawaii, Hawaii) were positive for antibodies against HEV (*12*). We studied their seroepidemiology but were unable to obtain

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genomic sequence or to transmit an agent to laboratory rats. Subsequently, in collaborations with the County of Los Angeles Department of Health (Los Angeles, CA, USA) Vector Management Program, we succeeded in transmitting to laboratory rats 2 strains of HEV from rats from Los Angeles but were again unable to obtain genomic sequence (*14*).

Recent cloning of rat HEV obtained from *R. norvegicus* rats in Germany and development of more broadly reactive PCR primers (*11*) prompted us to revisit those experiments. This report describes the partial PCR amplification and characterization of a US strain of rat HEV.

Materials and Methods

Rat Serum

Wild *R. norvegicus* rats were live-trapped by vector-control personnel in urban Los Angeles. The rats were anesthetized, and age and species was determined. Reproductive status and weight were recorded. Blood was obtained by cardiac puncture, and the serum was stored at –70°C.

HEV Strains

We performed transmission studies with genotype 1 strains Sar-55 (*15*), Kashi-87 (*16*), Akluj-90 (*17*); genotype 3 strain Meng swine HEV (*18*); and genotype 2 strain Mex 14 (*19*). All strains were in 10% fecal suspensions, diluted as described in the Results, and all but 1 had been titered for infectivity in nonhuman primates or pigs (Table 1).

Transmission Studies

Because infectivity of HEV in nonhuman primates is ≈10,000-fold less when administered orally than when administered parenterally, commercially acquired, outbred, Sprague-Dawley (*R. norvegicus*) or athymic nude hooded laboratory rats (Harlan, Indianapolis, IN, USA) or rhesus monkeys (*Macaca mulatta*) that were bred and raised in captivity were infected intravenously with serum or homogenized 10% fecal or tissue samples in saline. The animals were housed and maintained at Bioqual, Inc. (Rockville, MD, USA). Housing and care of the animals complied with all relevant guidelines and requirements, and the animals were housed in facilities that are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (Bethesda, MD, USA) and Bioqual, Inc.

Blood samples were obtained weekly and feces were obtained daily to 3×/wk. Serum samples were tested for liver enzyme levels by using standard methods (AniLytics, Inc., Gaithersburg, MD, USA). Postmortem liver tissue was fixed in formalin, embedded, sectioned, and stained with hematoxylin and eosin (American Histo Laboratories, Inc., Gaithersburg, MD, USA) and read under code by one of the authors (S.G.). Samples were scored for liver pathologic changes by the histologic activity index method.

Table 1. Results of testing for transmission of human and swine HEV to laboratory rats, Los Angeles, California, USA*

Inoculum	Genotype	ID_{50}	No. injected	No. with HEV RNA or antibodies against HEV
Sar 55	1	$10^{3.8}$†	2	0
Akluj-90	1	$10^{4.8}$†	2	0
Kashi-87	1	$10^{8.1}$‡	2	0
Mex14	2	$10^{4.3}$†	4	0
Meng	3	$10^{4.3}$§	4	0
Meng	3	$10^{2.3}$¶	4	0

*HEV, hepatitis E virus. The 50% infectious dose (ID_{50}) was administered intravenously.
†In human feces and titered in macaques.
‡Quantitative reverse transcription PCR titer.
§In pig feces and titered in pigs.
¶In macaque feces and titered in macaques.

Serologic Tests

Serum samples were tested for IgG and IgM isotypes against HEV by using a peroxidase-based ELISA as reported (*12*). The antigen used was recombinant open reading frame 2 protein of genotype 1. Serum samples were tested at 10-fold dilutions, and the highest dilution exceeding the cutoff value of optical density was taken as the endpoint titer of the serum.

Nested Reverse Transcription PCR

RNA was extracted from 270 μL of serum by using Trizol LS (Invitrogen, Carlsbad, CA, USA), and purified RNA was resuspended in 20 μL of water. Nested reverse transcription PCR (RT-PCR) was performed with the same primers, enzymes, and thermal profiles as described (*11*). Nested PCR products were separated by electrophoresis on ethidium bromide–stained agarose gels, extracted from the gel, and sequenced to provide the consensus sequence.

Quantitative RT-PCR

RNA was extracted from 50 μL of serum, tissue suspension, or filtered (0.22 μm, UltrafreeMC; Millipore, Billerica, MA, USA) 10% fecal suspension by using the QIAamp Viral RNA Mini Kit, (QIAGEN, Valencia, CA, USA), and total RNA was eluted in a volume of 60 μL. A TaqMan assay was performed by using the 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations. The primers (for a 332-base amplicon) consisted of 900 nmol/L forward (5′-ATG GTG CTT TTA TGG CGA TTG-3′) and 900 nmol/L reverse (5′-CAA ACT CAC TGA

AAT CAT TCT CAA AAA C-3′), and 250 nmol/L probe (5′-6FAM-TAT GTT CAG GAG AAG TTG GAA GCC GCT GT-TAMRA-3′). One-step quantitative RT-PCR (qRT-PCR) cycling conditions were 15 min at 48°C, a 10-min incubation at 95°C, and 50 cycles for 15 s at 95°C and 1 min at 60°C. Rat TaqMan cycle threshold values were indirectly quantified against an in-house HEV genotype 1 quantity standard line that represented a 6-log dynamic range.

Results

Detection of Rats Infected with Human- or Swine-derived HEV Strains

Because isolation of mammalian HEV strains from rats had been reported, we attempted to transmit to laboratory rats 6 well-characterized mammalian HEV strains (genotypes 1, 2, and 3) that can infect primates or pigs (Table 1). Adult Sprague-Dawley rats were injected intravenously with 0.1 mL of inoculum through the tail vein. Rats were bled weekly for 16 weeks and monitored for HEV RNA by real-time PCR with genotype-specific primers and for development of antibodies against HEV by ELISA. None of the animals had any evidence of infection.

Isolation of HEV Strains from Wild Rats

We had reported that wild rats trapped in Baltimore, Maryland, and the Hawaiian Islands had prevalences of antibodies against HEV of 77%–94% (*12*). We tested 134 serum samples from *R. norvegicus* rats trapped in urban Los Angeles. Donor rats were a mixture of male and female animals and adults and juveniles weighing 26–508 g. Of these animals, 105 (78.4%) were positive for IgG against HEV (with or without IgM against HEV), 2 (1.5%) were positive for IgM against HEV only, and 27 (20.1%) were seronegative when tested by ELISA with antigen derived from human HEV (*14*). As we described (*12*), prevalence of antibodies against HEV increased with weight as a measure of age, and ≈50% of the youngest rats were already positive for antibodies against HEV (Figure 1).

Because HEV is neutralized by antibodies against HEV, seronegative serum samples or IgM-positive serum from animals early after infection offer the greatest chance of recovering infectious virus. Therefore, 6 pools of serum were prepared from 27 seronegative rats, and 250 μL of serum from each pool and individual serum samples from 2 IgM HEV-positive rats and 5 IgG/IgM–positive rats were used to infect Sprague-Dawley rats. All 13 rats remained negative for HEV RNA, and only 3 rats (peak ELISA titers 100–400) seroconverted. Two of the seroconverted rats had received pooled seronegative serum, and the other had received IgM-positive serum.

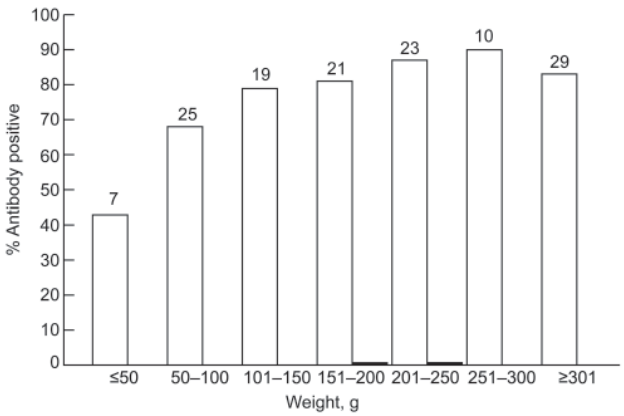

Figure 1. Relationship between prevalence of antibodies against hepatitis E virus (HEV) and weight of *Rattus norvegicus* rats trapped in Los Angeles, California, USA. Rats reach sexual maturity at a weight of ≈150–200 g. White bars indicate IgG, and black bars indicate IgM. Numbers at the top of each bar indicate sample size.

Passage of HEV to Rats

Feces obtained during the first 4 weeks postinfection from the 3 seroconverting rats (B76, B79, and B84) were homogenized and pooled, and 200 μL was used to inject 4 additional rats each. Only 3 of 12 rats injected with feces from rats B76 or B84 seroconverted. Serum was less efficient at transmitting virus, and 0 of 8 rats were infected. To determine the duration of shedding, infectious virus was identified by seroconversion of rats that had been infected intravenously with 200 μL of 10% suspensions of individual serial fecal samples obtained every other day from rats B76 and B84 during the first 4 weeks postinfection; fecal suspensions were also tested by RT-PCR. Feces from rat B76 were positive for ≥11 days and feces from rat B84 were positive for ≥9 days (Table 2). Periods of PCR positivity coincided with intervals of transmissibility on the basis of seroconversions in recipient rats. However, none of the recipient rats became viremic.

Because serum and feces were poor sources of transmissible virus, we tested other clinical materials. Groups of rats injected with fecal pools from rat B76 and rat B84 were exsanguinated on various days, and serum, liver, and intestinal contents were harvested. Serum from these rats was injected into individual rats, which were tested for seroconversion. Only 2 serum samples (from rats B300 and B182) transmitted virus to a new rat. The liver of rat B182 was used for further transmission studies.

To establish a more robust infection, we injected nude rats, which lack a functional adaptive immune system. Nineteen nude rats were injected with 200 μL of a 10% liver homogenate from rat B182 at a dilution of 10^{-1} and 1 rat was killed daily (days 2–20). We then used 200 μL

Table 2. Rat HEV in serial fecal samples of experimentally infected laboratory rats, Los Angeles, California. USA*

	Day postinfection														
Animal	7	9	11	13	15	17	19	21	23	25	27	29	31	33	35
Rat B76															
Infectious†	NT	NT	NT	–	+	–	+	–	–	–	–	–	–	NT	NT
RT-PCR	NT	NT	NT	+	+	+	+	+	+	–	–	–	–	NT	NT
Serum antibody against HEV	NT	NT	NT	–	–	–	–	–	–	–	–	+	+	+	+
Rat B84															
Infectious†	+	–	+	+	+	–	–	–	–	–	NT	NT	NT	NT	NT
RT-PCR	+	+	+	+	+	–	–	–	–	–	NT	NT	NT	NT	NT
Serum antibody against HEV	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+

*HEV, hepatitis E virus; NT, not tested; –, negative; +, positive; RT-PCR, reverse transcription PCR.
†As measured by transmission to another rat.

of a 10^{-2} dilution of serum from the killed rats to infect Sprague-Dawley rats. Only 3 of these rats seroconverted, indicating that only 3 of the nude rats (killed on days 13, 15, and 19) had infectivity titers $\geq 10^2$. One of these 3 nude rats, rat B350, was further studied.

Titer of Rat HEV

To determine the infectivity titer of rat HEV in liver, serum, and feces of selected infected rats, reverse titrations were performed with Sprague-Dawley rats and were monitored for seroconversion (Table 3). In Sprague-Dawley rats, 50% rat infectivity doses (RID_{50}) of 10^4–10^5/g of liver tissue were observed; in nude rat B350, a titer $>10^{6.2}$/g of liver and a titer of $10^{3.7}$ in serum were detected. Titers of virus in feces and intestinal contents of Sprague-Dawley rats were $\leq 10^1$ and $\leq 10^3$, respectively. Feces from nude rats were not tested. These samples were also titered for PCR positivity by qRT-PCR (Table 3). PCR titers of rat HEV paralleled infectivity titers but averaged an ≈10–100-fold higher titer.

Sequence of Rat HEV

A 327-nt product was amplified from the liver of rat B350 by nested RT-PCR and directly sequenced to yield the consensus sequence. The B350 rat virus sequence was as genetically similar to the 2 rat sequences from Germany as they were to each other at the nucleotide and amino acid levels (Table 4).

Effect of Infection on Liver Enzyme Levels

We have shown that some mammalian HEV strains show a dose response: higher doses ($>10^4$ infecting virus) are more likely to be associated with higher serum liver enzyme levels. To determine whether this phenomenon was true also for rat HEV, we infected 6 Sprague-Dawley rats with 200 μL of liver homogenate from rat B350 that contained $10^{4.5}$ RID_{50} of rat HEV. Animals were bled 2×/wk, and levels of alanine aminotransferase, γ-glutamyl transpeptidase, and isocitrate dehydrogenase were measured for 3 months. All 6 animals seroconverted 2.0–3.5 weeks (mean 3.0 weeks) postinfection (Figure 2). As reported, liver enzyme levels varied considerably, but seroconversion and liver enzyme levels were not temporally associated. Thus, these infections were biochemically inapparent infections.

Histologic Evaluation

Two HEV-infected rats (B182 and B300) and 2 uninfected Sprague-Dawley rats were examined under code for histologic evidence of hepatitis. The 2 uninfected rats had essentially normal livers. Rat B182 had parenchymal foci of necrosis and aggregates of lymphocytes and Kupffer cells in hepatic lobules and had mild portal inflammation (Figure 3). Rat B300 had similar but less obvious lesions. This mild hepatitis was consistent with normal liver enzyme levels measured in serum of these animals.

Transmission of Rat HEV to Nonhuman Primates

Because rats and humans often share the environment, especially in inner cities, we tested whether rat HEV was transmissible to nonhuman primates. Seronegative rhesus

Table 3. Titers for HEV in samples from laboratory rats, Los Angeles, California, USA*

Sample	$\log_{10}$ ID_{50}	$\log_{10}$ $RT\text{-}PCR_{50}$
Feces		
76†	≤1	3.4
84†	≤1	3.4
Intestinal contents: 182		
Small intestine	≤3	4.9
Cecum	≤3	5.4
Colon	≤3	4.9
Serum		
182	ND	3.7
300	ND	≤2.2
350	3.7	4.7
Liver		
182	4.7	7.2
300	4.2	5.7
350	6.2	7.7

*Values are per milliliter or per gram. HEV, hepatitis E virus; ID_{50}, 50% infectious dose; $RT\text{-}PCR_{50}$, 50% reverse transcription PCR titer; ND, not determined.
†Serum from wild rats was injected into laboratory rats 76 and 84. Samples from these 2 rats were serially passaged into other laboratory rats (wild rat → rat 76 → rat 300; wild rat → rat 84 → rat 182 → rat 350).

Table 4. Pairwise identity comparisons of a 327-nt fragment amplified from ORF1 of rat HEV, Los Angeles, California, USA*

	% Identity		
Strain	Rat B350	Ger 715	Ger 719
Rat B350		87.5	85.3
Ger 715	96.3		86.2
Ger 719	96.3	95.4	

*Values above the diagonal are nucleotide identities; values below the diagonal are amino acid identities. ORF, open reading frame; HEV, hepatitis E virus. Rat B350, GenBank accession no. JF516246; Ger 715, accession no. GQ504009.1; Ger 719, accession no GQ504010.1.

monkeys, which are surrogates of humans, were injected intravenously with rat liver homogenate containing $10^{3.5}$ RID_{50} of rat HEV from rat B182 or with $10^{5.2}$ RID_{50} of rat HEV from rat B350. The animals were monitored for 15 weeks for seroconversion by ELISA and for genomic RNA by qRT-PCR. The animals remained negative (Table 5). Thus, rat HEV does not appear to be transmissible to rhesus monkeys.

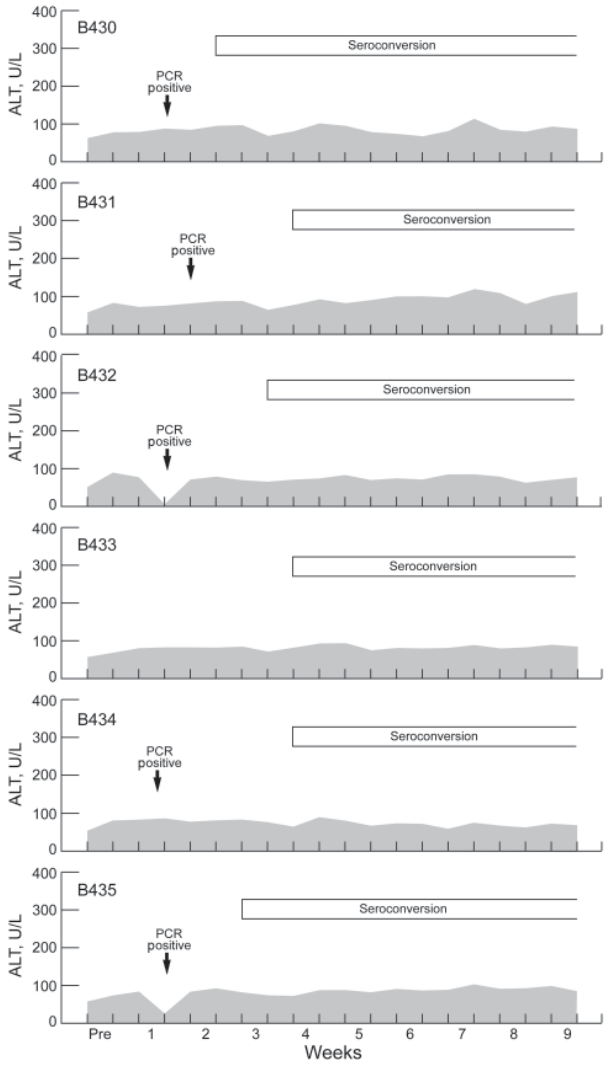

Figure 2. Correlation between virus infection and serum levels of alanine aminotransferase (ALT) (shaded areas) in rats infected with hepatitis E virus, Los Angeles, California, USA. Six Sprague-Dawley rats (B430–5) were infected with a $10^{4.5}$ 50% rat infectious dose of rat HEV and tested 2×/wk for evidence of infection and hepatitis. PCR results were positive for only half a week in 5 of the 6 rats. Pre, preinfection.

Discussion

Previous studies of HEV in rats have been fraught with controversy. The earliest report linked serologic evidence of HEV in rats near a village in the former Soviet Union with an epidemic of hepatitis E in the village (*20*). Later studies reported transmission of HEV in human feces from Nepal (presumably genotype 1) to laboratory rats (*21*) and isolation of genotype 1 HEV sequences from rats trapped in Nepal (*22*). However, the second study was retracted (*23*).

To determine whether rats were susceptible to recognized mammalian strains of HEV, we intravenously injected laboratory rats with human genotype 1 strains of HEV from Sargodha, Pakistan (*15*); Akluj, India (*17*); and Kashi, People's Republic of China (*16*); a human genotype 2 strain from Mexico (*19*); and a swine genotype 3 strain from Illinois, USA (*18*). Infectious titer of virus administered ranged from ≈10^2 to 10^5. None of the animals had evidence of infection, which suggested that rats are not readily susceptible to infection with other mammalian HEVs.

Nevertheless, as reported recently, rats can be infected by HEV strains (*11*). Using published primers, we amplified HEV genomic sequence from 1 of 2 HEV strains isolated in urban Los Angeles. This sequence was similar to sequences isolated from 2 rats in Hamburg, Germany; the virus sequence from Los Angeles was as similar to the 2 sequences from Germany as they were to each other. All 3 strains had only ≈60% identity with other mammalian strains, which suggested that rat HEV comprises a new HEV genotype.

On the basis of our extensive attempts to identify the virus in naturally infected wild caught and experimentally infected laboratory rats, we concluded that rat HEV infections were not robust and that the magnitude and duration of viremia and fecal shedding were less than that usually observed in infections with the other mammalian HEV genotypes. A low titer of rat HEV in rat feces in Germany was also reported (*11*). Rat HEV caused minimal hepatitis in experimentally infected animals; liver enzyme levels seldom increased above baseline levels, and histopathologic lesions during acute infections, although present, were minimal and not associated with clinical disease. Nevertheless, age-specific antibody prevalence in rats suggests that they are easily infected in their natural environment, and most are infected as juveniles and young adults in a pattern similar to that seen for acquisition of antibody against HEV in swine and humans in hepatitis-endemic areas (*24*,*25*).

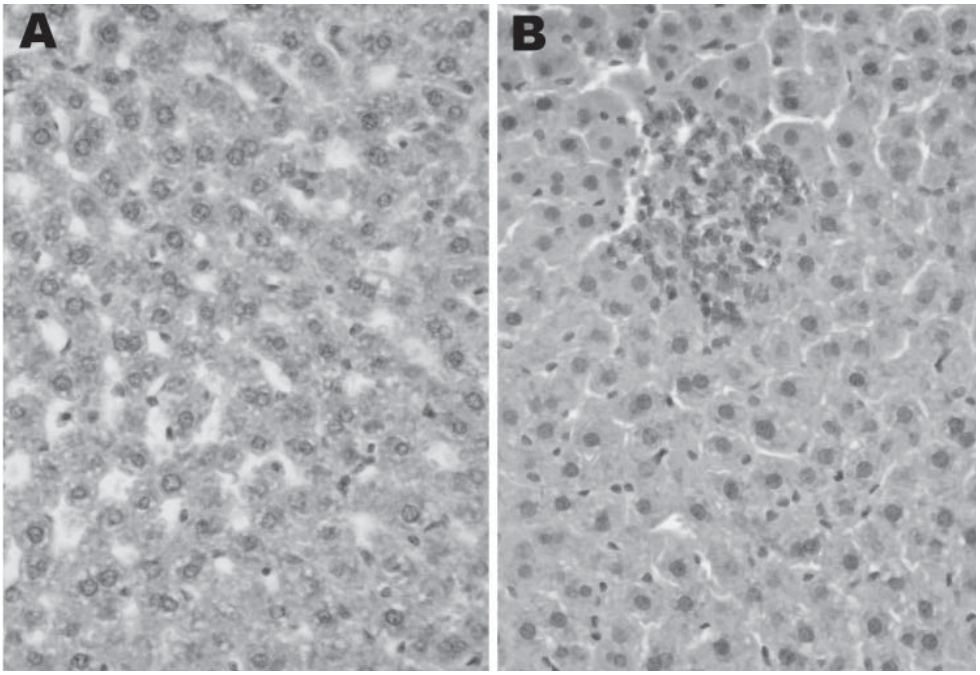

Figure 3. Histologic analysis of infection with rat hepatitis E virus, Los Angeles, California, USA. Hematoxylin and eosin–stained sections of liver from a healthy rat (A) and a rat acutely infected with rat HEV (B). Original magnification ×200.

Antibody against HEV in rats was usually directed against epitopes other than the major neutralization epitope in the carboxy portion of a genotype 1 capsid protein (S.U. Emerson, unpub. data). Seroconversion was relatively sensitive in identifying HEV infection in rats; it was in some cases more sensitive than detecting viremia by PCR. However, PCR was ≈10–100-fold more sensitive than infectivity titrations for quantifying HEV, a difference that is common for many virus infections. Overall, PCR confirmed that the magnitude and duration of viremia and viral shedding are not robust in rats. Whether capsid antigen expressed by rat virus will result in a more specific and sensitive assay for rat HEV antibody and whether it will help to better define the specificity of existing tests for antibodies against HEV in humans should be determined.

The high prevalence of antibodies against HEV in humans living in countries to which HEV is not endemic suggests that HEV infection in such areas might be zoonotic. Nevertheless, a direct association between HEV infection in animals and hepatitis E in humans has been limited, for the most part, to exposure to swine through eating undercooked pork and especially undercooked offal or through environmental exposure to swine feces. However, most persons do not eat undercooked pork or come in contact with swine, and their exposure, especially among those living in inner cities or in cultures without pigs, remains an enigma. In such settings, exposure to rats could be the missing link to HEV infection.

To determine whether this link exists, we attempted to transmit rat HEV to rhesus monkeys, a surrogate of humans that are highly susceptible to mammalian genotypes 1–4, including swine HEVs (*26*–*28*). Although we administered >100,000 infectious doses of virus intravenously to monkeys, they were not infected, as shown by lack of viremia and failure to develop antibodies against HEV. We also demonstrated similar lack of transmissibility of avian HEV to rhesus monkeys in previous collaborative studies (*29*), and we believe that these studies suggest a lack of zoonotic threat to humans from either avian or rat HEV.

Table 5. Results of testing for transmission of rat HEV to rhesus monkeys, Los Angeles, California, USA*

Inoculum	ID_{50}†	No. injected	No. with HEV RNA or antibodies against HEV
Rat B182	$10^{3.5}$	2	0
Rat B350	$10^{5.2}$	2	0

*HEV, hepatitis E virus. The 50% infectious dose (ID_{50}) was administered intravenously.
†In liver homogenate and titered in rats.

This study was supported by the Intramural Research program of the National Institutes of Allergy and Infectious Diseases, National Institutes of Health.

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Enterovirus Co-infections and Onychomadesis after Hand, Foot, and Mouth Disease, Spain, 2008

Maria A. Bracho, Fernando González-Candelas, Ana Valero, Juan Córdoba, and Antonio Salazar

Hand, foot, and mouth disease (HFMD), a common disease caused by enteroviruses (EVs), usually affects children. Clustered and sporadic HFMD cases, followed by onychomadesis (nail shedding), occurred during summer and fall 2008 in Valencia, Spain. Fecal samples from onychomadesis patients, who did or did not have previous HFMD, and from healthy children exposed to onychomadesis patients tested positive for EV. The complete viral protein 1 capsid gene sequence was obtained for typing and phylogenetic analysis. Two EV serotypes, coxsackievirus A10 and coxsackievirus B1 (CVB1), were mainly detected as a monoinfection or co-infection in a childcare center where an onychomadesis outbreak occurred. On the basis of our results, and detection of CVB1 in 2 other contemporary onychomadesis outbreaks in childcare centers in Spain, we propose that mixed infection of an EV serotype that causes HFMD, plus the serotype CVB1, could explain the emergence after HFMD of onychomadesis, a rare and late complication.

Enteroviruses (EVs) are among the most common human viruses, infecting ≈1 billion persons worldwide annually (*1*). On the basis of phylogenetic analysis, the genus *Enterovirus* (family *Picornaviridae*) is divided into 10 species. Members (serotypes) of human enteroviruses (HEVs) are classified into 4 species: HEV-A, HEV-B, HEV-C, and HEV-D (*2*). Although most EV infections are asymptomatic, they can result in a broad range of clinical manifestations, ranging from benign symptoms to notable diseases such as poliomyelitis, severe neonatal systemic disease, encephalitis, meningitis, or myocarditis (*3*).

Hand, foot, and mouth disease (HFMD) typically affects children <10 years of age. The main signs and symptoms are fever; sore throat; general malaise; and, often, vesicular eruptions on the palms of the hands, soles of the feet, oral mucosa, and tongue. Although HFMD is classically a mild disease, outbreaks in Asia have been associated with a high incidence of fatal cardiopulmonary and neurologic complications (*4*). EVs that are most frequently reported as causing HFMD outbreaks include EV71 and coxsackievirus A16 (CVA16) (*5*). Other HEV-A serotypes, such as CVA4, CVA5, CVA6, and CVA10, have also been reported in cases of HFMD and herpangina, a disease that shares clinical symptoms with HFMD (*6*–*9*). HFMD, followed by onychomadesis (nail shedding), was first reported in 2000 in 5 children in Chicago, Illinois, USA (*10*). In 2001, a similar report described it in 4 children in Europe (*11*). Since 2008, several onychomadesis outbreaks (HFMD outbreaks followed by onychomadesis) have been reported in various locations in Spain: Valencia (*12*), Valladolid (*13*), Saragossa (*14*), and A Coruña (*15*). A preliminary case–control study from the 2008 Valencia onychomadesis outbreak established a clear link between HFMD and onychomadesis (odds ratio 5.836, $p<0.001$) (*12*). Finally, onychomadesis cases in the context of a HFMD outbreak have also been reported in Finland in 2008 (*7*,*16*).

Molecular characterization of the etiologic agent involved in onychomadesis after HFMD, either in clustered or sporadic cases, remains controversial. Although serotypes CVA6 and CVA10 co-circulated during the

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DOI: http://dx.doi.org/10.3201/eid1712.110395

2008 HFMD outbreak in Finland (*16*), only CVA6 was explicitly reported in the HFMD cases in which the patients experienced onychomadesis (*7*). In Spain, serotype CVB1 was detected in A Coruña, and both CVB1 and CVB2 were detected in Saragossa. Noticeably, serotypes CVA10 and CVB1 were prevalent in the preliminary reports of the 2008 onychomadesis outbreak in Valencia (*17*). Considering all of these results together, no convincing demonstration has been made to clarify which serotype could account for the HFMD–onychomadesis epidemics.

In this study, to establish a relationship between EV infection and the onychomadesis patients in Valencia, Spain, in 2008, we analyzed fecal specimens from children who experienced onychomadesis after HFMD and from healthy children who had been in contact with onychomadesis case-patients. As a result of identifying EV serotypes and conducting phylogenetic analyses of viral protein (VP) 1 gene sequences, we propose that either co-infection or superinfection with an EV serotype that causes HFMD, along with serotype CVB1, could explain the emergence of recent HFMD–onychomadesis outbreaks. However, further research on future onychomadesis outbreaks that overcome the limitations of this study are necessary to verify this proposal.

Materials and Methods

Patients and Clinical Samples

Fecal samples were obtained from children in clusters of cases or from children with sporadic onychomadesis cases and from their asymptomatic classmates or contacts who were exposed to onychomadesis patients. All study participants were identified during May–December 2008 in Valencia. An onychomadesis case-patient was defined as a person who had lost ≥2 fingernails or toenails unrelated to systemic disease or trauma.

Viral RNA Purification, Reverse Transcription PCR, and Sequencing

Viral RNA was purified from feces by using Nuclisens EasyMag automated extractor (bioMérieux, Durham, NC, USA). Samples that rendered an enterovirus-positive result after real-time amplification (Cepheid's Xpert EV, Sunnyvale, CA, USA) were selected for typing. Sequences corresponding to the gene encoding the entire VP1 protein were obtained by reverse transcription PCR (RT-PCR) followed by direct sequencing. Most of the amplicons corresponding to either HEV-A or HEV-B serotypes were generated by using generic primers 011, 055, 224, and 240 (*18*). cDNA was synthesized in a 20-μL volume reaction containing 10 μL RNA, 500 μmol/L dNTP, 100 U Moloney murine leukemia virus reverse transcriptase (Promega Corp., Madison, WI, USA), 20 U RNasin (Promega) and 1 μmol/L antigenomic primer 240. The RT mixture was incubated at 42°C for 60 min, followed by 4 min at 95°C. A first PCR was performed in 50 μL volume containing 5 μL cDNA, 5 μL 10× PCR buffer, 0.2 mmol/L of each dNTP, 0.4 μmol/L primer 224, 0.4 μmol/L primer 240 and 1.25 U recombinant *Taq* DNA polymerase (TaKaRa Bio Europe SAS, Saint-Germain-en-Laye, France). A nested PCR was subsequently performed in a 50-μL volume with primer pair 011/055. PCR profiles were 94°C for 2 min; 40 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 3 min; and a final extension step at 72°C for 10 min.

The above-described generic RT-PCR strategy was complemented with an additional species-specific strategy. The species-specific protocol was performed for all samples and consisted of using the same RT-PCR conditions but with the specific primers for HEV-A and HEV-B as described (*19*). In all cases, the region encompassing the entire VP1 gene was amplified in 2 overlapping fragments. cDNA was synthesized with either antigenomic primer 489 (HEV-A) or 493 (HEV-B). First-round PCRs included outer pairs 486/489 (HEV-A) or 490/493 (HEV-B); in the second round, 2 heminested PCRs were performed with either pairs 486/488 and 487/489 (HEV-A) or pairs 490/492 and 491/493 (HEV-B). When necessary, for mixed infection, additional PCRs were performed with additional primers (Table 1). Purification of amplicons and sequencing was performed as described (*20*). All primers mentioned were considered potentially useful for PCR or sequencing in both generic and specific strategies. GenBank accession numbers for sequences derived in this study are FR796476–FR796493 and FR797984–FR798004.

Sequence and Phylogenetic Analysis

HEVs were genotyped by sequence comparison by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignments of 5′ and 3′ partial VP1 sequences were obtained with ClustalW (*21*). Maximum-likelihood phylogenetic trees were constructed by using RAxML version 7.2.6 (*22*,*23*) with the general time-reversible model of nucleotide substitution, a gamma-distribution approximation to account for rate heterogeneity and bootstrap support for branches by using 1,000 replicates. Trees were edited with the Tree Explorer tool in MEGA4 (*24*).

Results

Studied Persons

Sixty-five fecal samples, collected from 44 onychomadesis case-patients (28 with HFMD) and 21 children who were exposed to onychomadesis case-patients (3 with HFMD), were tested for EVs. Of these, 38 (59%) samples collected from 29 onychomadesis

Table 1. Primers designed in this study used to amplify and sequence the VP1 gene region*

Name	Sequence, 5′ → 3′	Gene	Virus	Sense	Position	Use
292a	CACCNGTYTCIRCIGC	VP1	EV	A	2582–2597	Seq
7g	TGCTGCARTATATGTATGT	VP1	EV-A	G	2879–2897	Amp, seq
EV71vp1g2	ATGTTTGTACCACCCGGAGCCCC	VP1	EV71	G	2894–2916	Amp, seq
CA10vp1g2	ATGTATGTGCCCCCTGGCGCCCC	VP1	CVA10	G	2894–2916	Amp, seq
CA10_55	GGGACGCATGTGGTGTGGGA	VP3	CVA10	G	2162–2181	Amp, seq
CA10_11	GCGCCGGATTGGTGGCCAAA	A2	CVA10	A	3326–3345	Amp, seq
vp1CA10 g1	TRCAGGCTGCAGAGACGGG	VP1	CVA10	G	2567–2585	Seq
vp1CA10a1	GATGGGTTAGTTGCTGTTTGCCA	VP1	CVA10	A	2945–2967	Seq
vp1CA10a2	GGGGGCACATACATATATTG	VP1	CVA10	A	2888–2907	Seq

*Primer positions are relative to the CVA2-Fleetwood sequence (GenBank accession no. AY421760). VP, viral protein; EV, enterovirus; A, antigenomic; seq, sequencing; G, genomic; amp, PCR amplification; CV, coxsackievirus.

case-patients (23 with HFMD) and 9 exposed persons (1 with HFMD) tested positive. To eliminate likely incidental serotypes not related to onychomadesis, we selected clinical samples from children with sporadic onychomadesis–HFMD by using the following tentative exclusion criteria: time between HFMD symptom onset and specimen collection >90 days and a latency period between the onset for HFMD and the onset for onychomadesis of <2 weeks, which is considerably shorter than the corresponding average in other onychomadesis outbreaks (≈40 days). As a result, 6 EV-positive samples were excluded from the study.

All 32 children studied (19 boys) were <5 years of age (mean 2.1, range 1.3–4.2 years) (Table 2). They attended 9 different childcare centers, except for 5 children, 3 of whom were siblings. A cluster of onychomadesis cases

Table 2. Clinical and epidemiologic data for symptomatic and asymptomatic children and genotyping results of 32 fecal samples collected in the HFMD–onychomadesis outbreak, Valencia, Spain, 2008*

Childcare center	Isolate no.	Age, y/sex	HFMD	Date of onset for HFMD	Onych	Date of onset for onych	Date of sampling	Serotypes	Days from HFMD onset to sampling	Days from onych onset to sampling
None	54574	1.5/F	Yes	Apr 27	Yes	May 27	Jun 25	CVA10	58	28
	54696	1.7/F	Yes	May 24	Yes	Jun 13	Jul 1	CVA10	37	18
	54697	1.7/F	Yes	May 24	Yes	Jun 13	Jul 1	CVA10	37	18
	54698	1.7/M	Yes	Jun 24	Yes	Jun 13	Jul 7	CVA10/CVA6	37	18
	54682	1.4/F	Yes	Jun 9	Yes	Jun 23	Jun 30	CVA16	21	7
1	54624	2.4/M	Yes	May 9	Yes	Jun15	Jun 26	CVA10	47	41
	54628	2.3/F	Yes	May 3	Yes	May 18	Jun 26	CVA10/E9	53	38
	54629	1.7/M	Yes	Apr 18	Yes	Jun 28	Jun 26	CVB1	68	28
	56643	1.8/M	Yes	May 1	Yes	Jun 19	Jun 26	CVA10	55	37
	54582	1.5/M	Yes	Apr 20	Yes	Jun 20	Jun 25	CVA10/CVB1	65	5
	54636	1.3/M	Yes	May 12	Yes	Jul 7	Jun 25	CVA10/CVB1	43	–12
	54602	2.2/F	Yes	Apr 15	Yes	Jun 9	Jun 25	CVA10/CVA5	70	16
	54573	1.4/M	No		Yes	May 2	Jun 25	CVB1		50
	54576	2.0/M	No		Yes	May 30	Jun 25	CVA10		25
	54622	2.4/M	No		Yes	Jun 3	Jun 26	CVA10		23
	54667	1.4/M	No		No		Jun 29	CVA10/CVB1		
	54572	2.4/M	No		No		Jun 25	CVA10		
	54575	3.2/F	No		No		Jun 25	CVA10		
	54579	1.4/M	No		No		Jun 25	CVB1		
	54601	2.0/M	No		No		Jun 25	CVA10		
	54599	2.9/M	No		No		Jun 25	CVA10		
	54632	3.4/M	No		No		Jun 27	CVB1		
2	54693	2.8/M	Yes	May 21	Yes	Jun 25	Jul 1	CVB1	40	6
3	54657	2.1/F	Yes	May 13	Yes	Jun 21	Jun 26	CVA6/CVB1	43	5
4	54753	4.2/M	No		No		Jul 2	CVA10		
5	54694	1.6/F	Yes	May 20	Yes	Jun 5	Jul 1	CVA6	41	26
6	54633	2.8/M	Yes	NA	Yes	May 15	Jun 27	CVA5		
7	54678	1.7/F	Yes	Apr 1	Yes	May 14	Jul 1	EV71/CVA5	90	47
8	54720	1.7/F	No		Yes	May 1	Jul 2	EV71		61
9	1023	1.9/F	Yes	Oct 18	Yes	Nov 28	Nov 4	CVA6	16	–31
	1215	2.0/F	Yes	Oct 21	Yes	Dec 1	Oct 31	CVA6	9	–24
	1031	2.0/M	Yes	Oct 23	No		Nov 4	CVA6	11	

*HFMD, hand, foot, and mouth disease; onych, onychomadesis; none, no childcare center attendance; CV, coxsackievirus; E, echovirus; NA, not available; EV, enterovirus.

during May–July 2008 was first reported in childcare center 1 from which 17 samples from onychomadesis patients and exposed children were studied. By the time the epidemiologic study was conducted in childcare center 1, some sporadic onychomadesis cases were reported in other childcare centers, and EV-positive samples from corresponding case-patients and 1 exposed person were analyzed (childcare centers 2–8). In October 2008, another cluster of HFMD cases was identified in childcare center 9, from which fecal samples were taken even before onychomadesis symptoms developed in HFMD case-patients. In our study, nail shedding appeared an average of 32 days after HFMD onset (95% confidence interval [CI] 24–39 days), and fecal samples were collected an average of 44 days after HFMD onset (95% CI 35–54 days) and 25 days after onychomadesis onset (95% CI 18–32 days), excluding data from samples collected before onychomadesis onset (negative days in Table 2).

EV Typing

All EV-positive samples could be typed, and 7 different serotypes were found, 5 belonging to HEV-A species (CVA5, CVA6, CVA10, CVA16, and EV71) and 2 to HEV-B (CVB1 and echovirus [E] 9). In 4 samples, CVB1 was found in dual infections with either CVA10 or CVA6. The most prevalent serotypes were CVA10 (45%) and CVB1 (22.5%), which were mainly detected in childcare center 1 and in children not attending any childcare center, followed by CVA6 (15%), the only serotype detected in the 3 children from childcare center 9. Other serotypes were found with more marginal frequencies: CVA5 (7.5%), EV71 (5%), CVA16 (2.5%), and E9 (2.5%). Sporadic onychomadesis cases that matched exclusion criteria for likely incidental infections were analyzed and presented HEV-B serotypes CVB3 (n = 2), E3 (n = 1), E9 (n = 2), and E3/E9 co-infection (n = 1). A substantial number of mixed infections (25%) was detected. All 3 mixed infections CVB1/CVA10 occurred in childcare center 1 and were found in 2 symptomatic children (with both HFMD and onychomadesis) and 1 healthy child. Thus, mixed infection of both serotypes was found in children who stayed in the childcare center where the first onychomadesis case were identified. Other combinations of mixed infections were detected only once.

Additionally, viral extracts from 16 EV-positive samples were inoculated into cell culture (human cervical carcinoma, human rhabdomyosarcoma, and human embryo fibroblasts), followed by EV detection with immunofluorescence (data not shown). Eight samples typed as HEV-A (mixed or monoinfection) produced negative results, whereas all 5 samples typed as CVB1 and all 3 samples with the mixed infection CVA10/CVB1 showed positive results.

Phylogenetic Analyses

Given their relevance in this study, only phylogenies for serotypes CVB1, CVA10, and CVA6 are shown (Figures 1–3). The PCR strategy amplified a region that ranged from 1,084 nt (CVB1) to 1,174 nt (CVA6). Notably, most currently available sequences from these serotypes cover only a fragment of the VP1 coding region (either the 3′ or the 5′ part), with typical lengths of ≈300 nt and 400 nt, respectively. In consequence, to attain a global view of the relationships between our sequences and representative isolates circulating worldwide, we performed 2 parallel phylogenetic reconstructions using each part of the coding region.

CVB1

Nine serotype CVB1 sequences were obtained from 6 onychomadesis case-patients (5 with previous HFMD) and 3 healthy classmates. Eight of the 9 CVB1 sequences were virtually identical (99.9%) and shared 94% nucleotide identity with the relatively divergent 54693 isolate. These CVB1 isolates from Valencia clustered together and were phylogenetically close to isolates circulating in the United States during 2007–2008 (nucleotide identities 96.6%–99.0%) (Figure 1, panel A) and to isolates circulating in Spain in 2008–2009, including those isolates detected in the onychomadesis outbreak reported in A Coruña in 2009 (*15*) (97.7%–99.5%) (Figure 1, panel B).

CVA10

Serotype CVA10 was detected in 18 children: 12 onychomadesis case-patients (10 with HFMD) and 6 healthy children. Phylogenetic analyses with representative CVA10 sequences are shown in Figure 2. Both 5′ and 3′ partial VP1 analyses (panels A and B, respectively) showed that all but 1 of the CVA10 sequences from the current outbreak clustered with a nucleotide identity of 97%–100%. This CVA10 group showed a close relationship with Slovakian sequences collected in 2007, with a clade that comprises many strains circulating in the People's Republic of China during 2008–2010 and showed pairwise identities of ≈80% compared with the main cluster of 2008 isolates from Finland that contains the divergent isolate 54602, collected in childcare center 1. Nucleotide identities between isolate 54602 and sequences in the 2008 Finland cluster were 94%–100%.

CVA6

CVA6 serotype was found in 6 children: 3 from the onychomadesis outbreak reported in fall 2008 (childcare center 9) and 3 from patients with sporadic onychomadesis cases reported in summer 2008. In contrast to the collection of other isolates (except 54636), the samples from the HFMD cluster in childcare center 9 were collected

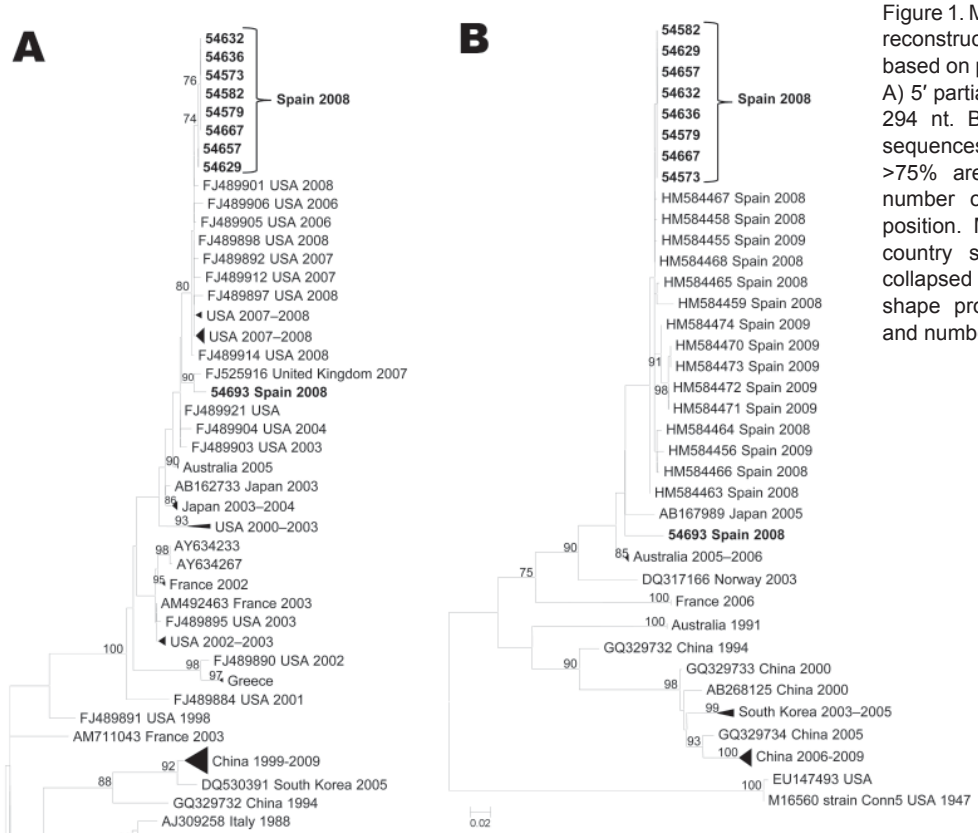

Figure 1. Maximum-likelihood phylogenetic reconstructions for coxsackievirus B1 based on partial viral protein 1 sequences. A) 5′ partial coding region (93 sequences, 294 nt. B) 3′ partial coding region (49 sequences, 390 nt). Bootstrap values >75% are shown. Scale bars indicate number of substitutions per nucleotide position. Multiple strains from the same country sharing the same node were collapsed and shown as triangles with shape proportional to branch distances and number of sequences.

≈1 month before onychomadesis onset. Phylogenetic relationships of CVA6 isolates (Figure 3) showed that 4 of our isolates formed a cluster with an isolate collected in Great Britain in 2008. These highly similar isolates (identities ≈100%) formed a relatively distant sister cluster to a group that included most CVA6 isolates circulating in the 2008 HFMD outbreak in Finland. In contrast to the results shown above for the CVA10 serotype, these CVA6 isolates from Spain and Finland showed higher nucleotide identities (94%–98%). On the other hand, the divergent CVA6 sequence from isolate 54698 tended to cluster with isolates circulating in Japan, Iceland, and Greece during 2007 and with a divergent isolate that circulated in the 2008 Finland outbreak. Isolate 54657 (a short CVA6 sequence not included in the phylogenetic analyses) was found in a mixed infection with CVB1 in a child with HFMD–onychomadesis (childcare center 3). This partial sequence was identical to sequence 54694 and could likely group within the cluster of Spanish sequences.

Other Serotypes

Three CVA5 isolates were detected in different children with onychomadesis after HFMD. Isolates 54602 and 54633 were identical and similar to isolate 54678 and to an isolate from China that was circulating in 2008 (data not shown).

Two EV71 sequences, which shared a 99.6% nt identity, were detected in samples from patients with sporadic onychomadesis cases, with and without previous

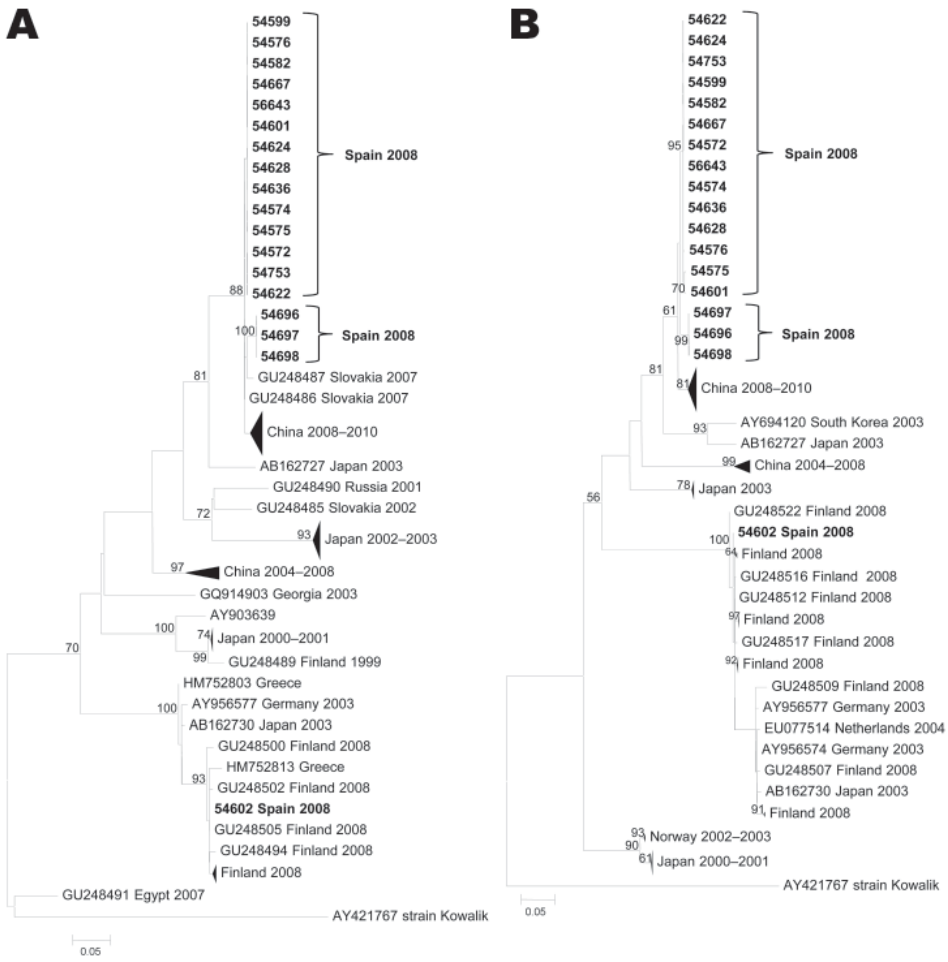

Figure 2. Maximum-likelihood phylogenetic reconstructions for coxsackievirus A10 based on partial viral protein 1 sequences. A) 5′ partial coding region (89 sequences, 246 nt); B) 3′ partial coding region (87 sequences, 397 nt). Bootstrap values >75% are shown. Scale bars indicate number of substitutions per nucleotide position. Multiple strains from the same country sharing the same node were collapsed and shown as triangles with shape proportional to branch distances and number of sequences.

HFMD. Phylogenetic analysis indicated (data not shown) that both EV71 isolates clustered with sequences circulating Europe during 2006–2009.

One CVA16 isolate was detected in a child with sporadic HFMD followed by onychomadesis (isolate 54682); the child was not attending any childcare center. Phylogenetic analysis indicated the isolate grouped with endemic strains that were circulating in China during 2000–2008 (data not shown).

Serotype echovirus 9 (E9) was detected in a dual infection with CVA10 in isolate 54629. Phylogenetic analysis grouped this strain within a cluster of numerous isolates circulating in Spain (2003–2008), Australia (2005–2006), and United Kingdom (2007–2008) (data not shown).

Discussion

The main EVs detected in the HFMD–onychomadesis outbreak in Valencia in 2008 included HEV-A serotypes that caused HFMD (CVA6 and CVA10) and an HEV-B serotype (CVB1), currently associated with meningitis and myocarditis and detected recently in clusters of severe systemic neonatal illness (*25*) and onychomadesis outbreaks (*14*,*15*).

The other EVs detected in our survey (CVA5, EV71, CVA16, and E9) could be incidental to the outbreak because they were found rarely and, except for CVA16, were identified from fecal samples collected long after HFMD onset (*26*). In fact, if more stringent exclusion criteria had been followed, they would have been excluded from the analysis. For instance, the time from onset of HFMD and onychomadesis symptoms to specimen collection for isolate 54678 (90 and 47 days, respectively) and from onychomadesis onset to specimen collection in isolate 54720 (61 days), although both contained EV71 serotype, was considerably longer than the average from symptom onset to fecal sample collection (44 and 25 days for HFMD and onychomadesis, respectively). This delay could also be the case for E9, which was detected only once in a mixed infection (but detected in 3 excluded samples collected >90 days after HFMD onset).

Phylogenetic analyses found that divergent strains within serotypes CVA6 and CVA10 were isolated in

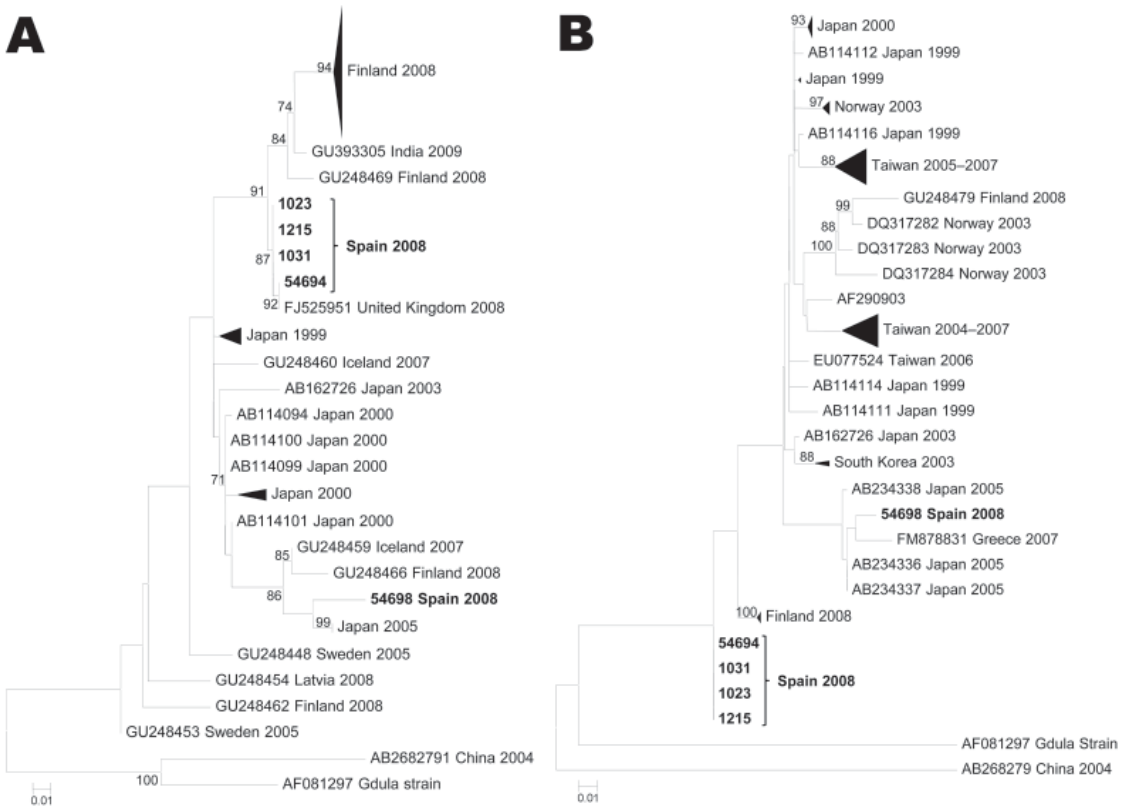

Figure 3. Maximum likelihood phylogenetic reconstructions for coxsackievirus A6 based on partial viral protein 1 sequences. A) 5′ partial coding region (81 strains, 293 nt). B) 3′ partial coding region (68 sequences, 377 nt). Bootstrap values >75% are shown. Scale bars indicate number of substitutions per nucleotide position. Multiple strains from the same country sharing the same node were collapsed and shown as triangles with shape proportional to branch distances and number of sequences.

the contemporary HFMD–onychomadesis outbreaks in Valencia and Finland. Phylogenies of CVB1 showed that a virtually identical CVB1 strain was detected in both onychomadesis outbreaks in Valencia and A Coruña (*15*), along with a relatively divergent strain from Valencia. Consequently, no single serotype or strain within serotype can account exclusively for onychomadesis. The same conclusion arises after considering serotyping results from previous HFMD–onychomadesis studies in which CVA6 (*16*), CV6 and CV10 (*7*), and CVB1 (*15*) or CVB1 and CVB2 (*14*) were detected as single infections. On the contrary, we found, although in a low proportion, dual infections of CVA10/CVB1 and CVA6/CVB1 in onychomadesis cases that led us to suggest that a mixed infection of serotypes from 2 different EV species might account for this unexpected and late complication. Two different serotypes could have infected patients either simultaneously (co-infection) or sequentially (superinfection). Constituents of this mixed infection that possibly causes HFMD–onychomadesis would be 1 HEV-A serotype that causes HFMD, CVA10 or CVA6, and 1 HEV-B serotype, CVB1, never found before in clusters of only HFMD. These 3 serotypes co-circulated during spring and fall 2008 in Valencia and accounted for a cumulative 85.5% of all detected infections in our study.

Identifying serotype CVB1 as a cofactor that contributes to onychomadesis in a HFMD context would solve satisfactorily the question of which serotype is responsible for the onychomadesis feature but also would generate new concerns. First, is CVB1 an incident serotype detected in the 3 onychomadesis outbreaks in Spain or a true cofactor? Second, why do typing results among HFMD–onychomadesis studies not agree?

Prevalence of CVB1 in Spain before 2008 was low. Eighteen CVB1 isolates (0.6%) were detected in Spain from 2,814 typed EV isolates, mainly collected from children during 1998–2007 (*27*). The detection of 17 CVB1 isolates collected in 3 distant onychomadesis outbreaks ≈1,000 km from A Coruña to Valencia) during a year (May 2008–April 2009) seems too high to be considered just a chance event.

Differences in specimens and methods may explain the discordant typing results among HFMD–onychomadesis

studies, especially in the use of viral culture (*28*,*29*) or different sets of RT-PCR primers (*30*,*31*). In the Saragossa and A Coruña outbreaks, typing was performed after viral culture (*15*). By using the same cell lines, we obtained EV-positive cultures when they were injected with either CVB1 or HEV-A/CVB1 viral extracts, but cultures were EV-negative when they were inoculated with HEV-A strains alone. Similarly, 8 CVA6 RT-PCR–positive specimens could not be cultured in the outbreak in Finland (*16*). Therefore, the method applied in these 2 outbreaks in Spain is highly likely to have missed HEV-A strains, even if present, and selected for, and indeed found, only CVB1 isolates.

A noteworthy inconsistency, if our hypothesis holds, is that CVB1 was not detected in the HFMD–onychomadesis outbreak in Finland. Specimens studied in the 3 Spanish surveys were sampled a long time after HFMD diagnosis, whereas in the 2 surveys from Finland, acute-phase specimens were obtained (*7*,*16*). Typing methods in Finland (*16*) were based on primers specific for CVA6-VP1 or melting point comparison in the 5′ noncoding region, which are not advised for typing at the serotype level. Not surprisingly, only the CVA6 serotype was found. In fact, the cited study could not detect co-circulating CV10. The second publication (*7*) about the same outbreak, in which a different method was used, proved co-circulation of CVA6 and CVA10. In this report, protocols seem suitable for detecting any serotype, but the researchers did not specify the number of fecal samples (and their collection data) that were typed directly from onychomadesis case-patients. Curiously, only supernatants from consecutive cultures that showed 100% cytopathic effect underwent typing. However, in the surveys from A Coruña (*15*) and Valencia, all viral cultures were tested, irrespective of cytopathic effect, and CVB1 was detected in A Coruña (viral cultures were not typed in our survey). The highly stringent conditions used in the Finland survey could have seriously compromised the ability to detect CVB1, even if it was initially present.

Our study combined the use of fecal samples along with 3 distinct strategies for molecular amplification of isolates, thus improving previous strategies used in onychomadesis outbreaks. That all EV-positive samples could be typed and that the variability detected was high clearly supported the robustness of our approach.

Our study has some limitations, however. First, virions in feces correspond to viral shedding from the whole patient and add an eventual possibility of detecting incidental EVs. Moreover, the possibility of detecting incidental serotypes increases with time between HFMD onset and specimen collection as reflected in typing results from excluded samples. Therefore, we recommend limiting sample collection to ≤2 months after HFMD onset. Second, our hypothesis has no statistical support because a Fisher exact test (applied to a 2-way contingency table) performed with our data failed to detect a significant association between the presence of CVA6/CVB1 or CVA10/CVB1 mixed infections and onychomadesis. However, this lack of significant association does not necessarily invalidate our hypothesis because the quality of the specimens may affect detection of some serotypes and, consequently, the result of the test. For instance, distant serotypes may differ in their persistence pattern in feces (*26*), which could have led to the poor association between mixed infections detected and expected according to our hypothesis. Finally, our studied population was temporally and geographically restricted.

Further research of HFMD–onychomadesis outbreaks will be needed to confirm or negate our hypothesis. Adequate specimens to test the hypothesis would comprise, ideally, nail sampling and serial fecal sampling from time of HFMD diagnosis to ≈2 months after HFMD onset.

The standardization of protocols and techniques in typing is essential for EV surveillance and worldwide comparisons. In this context, we strongly recommend that the complete VP1 gene be sequenced.

Acknowledgments

We thank Ignacio G. Bravo and David Martinez-Torres for critical reading of the manuscript. We also thank Beatriz Acosta for her work on viral cultures.

This study was funded by Instituto de Salud Carlos III–Fondo de Investigación Sanitaria project CP07/00078 (M.A.B.) from Ministerio de Sanidad y Consumo and project BFU2008-03000 (F.G.-C., M.A.B.) from Ministerio de Ciencia e Innovación.

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Experimental Infection of Horses with Hendra Virus/Australia/Horse/2008/Redlands

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Hendra virus (HeV) is a highly pathogenic zoonotic paramyxovirus harbored by Australian flying foxes with sporadic spillovers directly to horses. Although the mode and critical control points of HeV spillover to horses from flying foxes, and the risk for transmission from infected horses to other horses and humans, are poorly understood, we successfully established systemic HeV disease in 3 horses exposed to Hendra virus/Australia/Horse/2008/Redlands by the oronasal route, a plausible route for natural infection. In 2 of the 3 animals, HeV RNA was detected continually in nasal swabs from as early as 2 days postexposure, indicating that systemic spread of the virus may be preceded by local viral replication in the nasal cavity or nasopharynx. Our data suggest that a critical factor for reducing HeV exposure risk to humans includes early consideration of HeV in the differential diagnosis and institution of appropriate infection control procedures.

Hendra virus (HeV) is a zoonotic paramyxovirus harbored by Australian mainland flying foxes, from which it is believed to be transmitted directly to horses. In horses, HeV causes a severe, often fatal, febrile illness associated with respiratory and neurologic signs (*1*). Since its emergence in Queensland, Australia, in 1994, HeV infection of horses has regularly recurred. Of the 32 equine outbreaks, 5 have extended to involve infection of humans; of the 7 known human case-patients, 4 have died. Human infection has typically occurred after close contact with infected horses, usually horses in the terminal stages of disease or at postmortem examination, except for 1 person for whom epidemiologic findings suggested the most likely exposure to an infected horse occurred during incubation (*2*). Currently, HeV is an unmanaged emerging infectious disease.

Since the serious zoonotic potential of HeV was confirmed, clinical and laboratory evaluation of disease horses from outbreaks has been limited. In particular, the relationship between the onset of clinical signs and duration of viral shedding has not been determined, and the understandably few equine experimental infection studies conducted in the mid-1990s (*3*) yielded limited data that could guide effective management of the exposure risk to humans.

Further concern arose after an HeV outbreak in the Brisbane suburb of Thornlands (Redlands Shire), Queensland, in 2008, in which the major clinical signs in horses were attributable to disease of the central nervous system (*4*). Although nervous system signs have been associated with previous outbreaks, HeV is more commonly considered to induce a respiratory syndrome in horses. In the Redlands 2008 outbreak, credible alternate provisional diagnoses and thus delay in definitive diagnosis likely contributed to an increased HeV exposure risk to attending staff and to in-contact horses; 2 staff members became infected, 1 fatally (*4*).

The objectives of this study were to monitor potential routes of shedding for evidence of HeV replication in horses experimentally exposed to Hendra virus/Australia/

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DOI: http://dx.doi.org/10.3201/eid1712.111162

Horse/2008/Redlands and to compare the associated clinical syndrome with that observed after infection with the HeV isolate from the first outbreak in 1994. These data would provide a framework for assessing the relative transmission risk posed by horses at various times during acute HeV infection and permit incorporation of recommendations for reducing the transmission risk to humans and other horses into advisory and outbreak management strategies. Following the observation in the Redlands outbreak of a predominantly neurologic disease, an experimental challenge was carried out under BioSafety Level 4 conditions at the Australian Animal Health Laboratory, Geelong, Victoria, Australia, in late 2008.

Methods

Animals

Three mares were housed in individual pens within 1 room at BioSafety Level 4. Room temperature was maintained at 22°C with 15 air changes per hour; humidity varied from 40% to 60%. Animal husbandry methods and experimental design were endorsed by the Commonwealth Scientific and Industrial Research Organization, Australian Animal Health Laboratory and Animal Ethics Committee, and aligned with the Code of Practice for the Welfare of Horses (Bureau of Animal Welfare, Victoria, Australia).

Horses were acclimated to the facility for 1 week before exposure to HeV. Immediately before viral challenge, each mare was fitted with an intrauterine (transcervical) temperature transponder to continuously record core body temperature (*5*). An indwelling catheter was placed in the jugular vein of each animal and sutured in position.

Horses were clinically assessed at least 2×/d, and when a predetermined humane endpoint was reached, they were euthanized. The humane endpoint was defined as fever accompanied by depression or other signs consistent with localization to respiratory or neurologic systems. This clinical assessment permitted documentation of the incubation period, duration and route of virus shedding from the time of virus exposure to readily detectable disease, and time for localization of virus to various body tissues.

Virus

HeV (Hendra virus/Australia/Horse/2008/Redlands) was isolated on Vero cells from the spleen from a horse with naturally acquired disease in the Redlands outbreak. Genetic analysis of this isolate showed 99.6% similarity at the amino acid level to the original HeV isolate (Hendra virus/Australia/Horse/1994/Hendra) (*6*). Because the median infectious dose of HeV in horses is not known, the virus challenge dose was selected to mimic previous experimental studies (*3*); virus (2×10^6 50% tissue culture infectious dose) was administered oronasally to each horse.

Clinical Data and Biological Sample Collection and Analysis

Clinical observations were recorded 2×/d beginning 3 days before HeV exposure and included rectal temperature (to augment records from the implanted transponders) and heart rate measured with an electronic monitor. The character of the respiratory pattern and effort was also noted on each occasion (resting respiratory rate had previously been assessed as highly variable from time to time in each animal and so was not routinely recorded).

Biological samples (nasal, oral, and rectal swab specimens and blood) were collected every day. Urine and feces were collected from the pen floor and mid-stream urine was collected on an opportunistic basis. All biological samples, except blood for serologic tests, were placed immediately on wet ice and then stored at –80°C until processed. Serum samples were stored at –20°C until tested for antibody against HeV.

Nasal, oral, and rectal swab specimens; urine; feces; and blood were analyzed for HeV nucleic acid by quantitative real-time PCR directed against the N and P genes (*7*) and for live HeV by virus isolation in Vero cells. After being euthanized, each horse underwent postmortem examination. Diverse samples were retained for virus isolation, real-time PCR, histopathologic examination, and immunohistochemical testing by using rabbit α-Hendra N-protein antiserum.

Results

Clinical Observations

Horse 1

The horse remained well until day 5 postchallenge, when its temperature began to rise (Figure 1) in parallel with a steady rise in resting heart rate (Figure 2). At this time the horse was eating well and otherwise appeared clinically normal.

On day 6, the horse was clinically depressed with reduced appetite. Temperature and heart rate had continued to rise, and over several hours, the horse exhibited continuous restlessness, with constant shifting of weight between all 4 limbs, especially the hind limbs. By afternoon, the horse was disinterested in its surroundings and had begun to stand with its head facing the side of the pen. The horse was euthanized, and postmortem examination was conducted.

Horse 2

A slight bilateral serous nasal discharge was observed in this horse on day 2 postchallenge. The horse remained otherwise well until day 7, when a temperature above baseline developed in parallel with a rise in resting heart rate (Figure 2). At this time, the horse did not exhibit

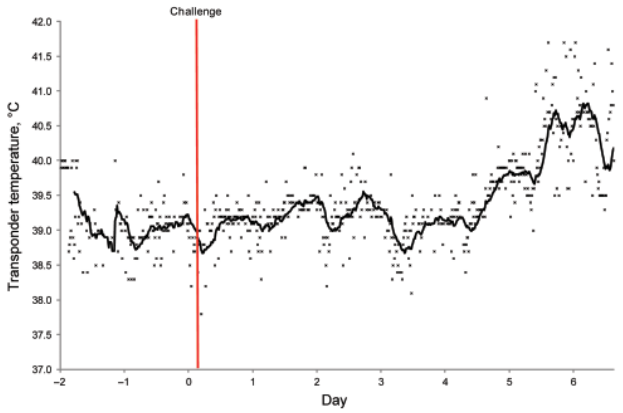

Figure 1. Temperature transponder data for horse 1 during experimental infection with Hendra virus, Australia. Before viral challenge, each mare was fitted with an intrauterine (transcervical) temperature transponder to allow continuous recording of core body temperature. Temperature was measured every 15 minutes in each horse. Solid line represents the moving average based on 20 temperature readings.

any other abnormal clinical signs. The following day, the horse was slightly depressed, and the elevated heart rate and temperature appeared to have stabilized. However, on day 9, the heart rate continued to rise, and the horse exhibited mild dyspnea with a prolonged expiratory phase. This normally quiet mare also became agitated when approached. The horse was euthanized on the afternoon of day 9, and postmortem examination was conducted.

Horse 3

A slight bilateral serous nasal discharge was observed in this horse on day 2 postchallenge. The horse remained otherwise well until day 6 when its temperature began to rise above baseline. Fever was established by day 7, and a concomitant rise in heart rate was also noted (Figure 2). The serous nasal discharged had resumed, but the horse was otherwise well and eating normally. On day 8, temperature and heart rate were continuing to rise, and small amounts of blood coated in mucus were seen in the feces. The mare exhibited a rigid forelimb stance, alternating with general restlessness and constant shifting of weight from limb to limb, difficulty eating, frequent head shaking, and irritability with attempts to bite her handlers. A panting type of respiration was noted. The horse was euthanized on the afternoon of day 8.

Postmortem and Histopathologic Findings

Horse 1

Significant gross abnormalities comprised enlarged and edematous submandibular lymph nodes, and several 2 × 0.5–cm subpleural hemorrhages were noted on the left lung. A small (6 × 6 cm) area of brownish pink consolidation was present on the ventral border of the left lung posterior to the cardiac notch. A 2.5-cm follicle was noted in the left ovary.

On histologic examination, systemic vasculitis was observed that affected meninges, nasal mucosa, trachea, lung, diverse lymph nodes, spleen, kidney, heart, uterus, ovary, and intestine. Edema, syncytial cells, viral inclusion bodies, and alveolitis were seen in lung sections. Focal necrosis of the adrenal gland was identified, together with glomerulitis and syncytial cell formation in the kidney. HeV antigen was detected in tissues and organs, including meninges, alveolar walls, lymph nodes, renal glomeruli, and adrenal glands and in blood vessels supplying each of these. In addition, the nasal mucosa, trachea, spleen, heart, uterus, ovary, and intestine showed HeV antigen.

Horse 2

Significant gross abnormalities comprised enlarged and edematous submandibular and bronchial lymph nodes and heavy lungs that oozed fluid from the cut surface. Numerous petechial hemorrhages were found over the surface of the diaphragmatic regions of the lung. The liver was small with an irregular finely nodular surface.

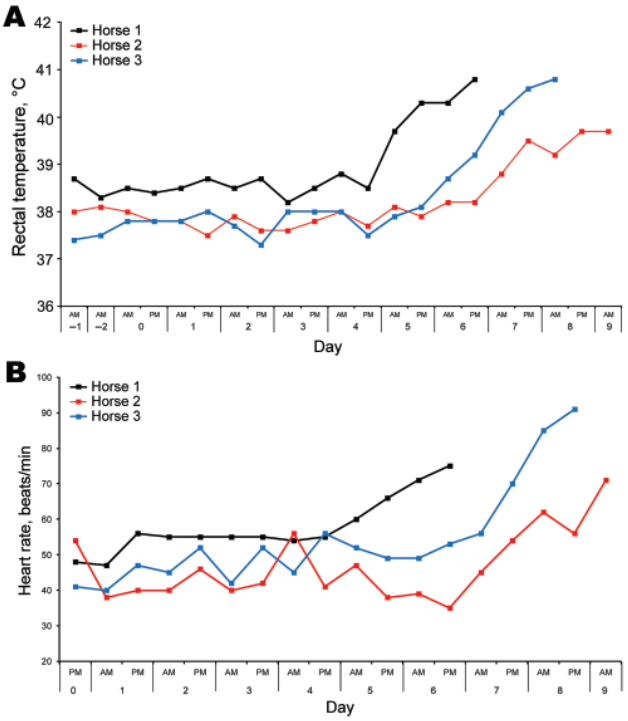

Figure 2. Rectal temperatures (A) and heart rates (B) of each horse after experimental infection with Hendra virus, Australia. Data were collected by using an electronic monitor 2×/d, along with comments on general demeanor. Data were used to determine a humane endpoint for each animal.

On histologic examination, systemic vasculitis was observed that affected meninges, brain (Figure 3, panel A), nasal mucosa, trachea, lung, diverse lymph nodes, spleen, liver, kidney, heart, uterus, ovary (Figure 3, panel B), and intestine. Edema, syncytial cells, viral inclusion bodies, and alveolitis were seen in lung sections. Focal necrosis and syncytial formation within lymph nodes were identified, together with glomerulitis and syncytial cell formation in the kidney. Acute myocarditis and focal necrosis of corpus luteum tissue were also identified. HeV antigen was detected in tissues and organs, including meninges, alveolar walls, lymph nodes (Figure 4), renal glomeruli, myocardium, and ovary and in blood vessels supplying each of these. Again, HeV antigen was detected in nasal mucosa, liver, spleen, adrenal gland, uterus, and intestine. Hepatic amyloidosis was also noted but was considered to be an incidental finding.

Horse 3

At postmortem examination, we identified swollen and edematous submandibular, sternal, and bronchial lymph nodes and dilation of lymphatic vessels at ventral lung lobe margins (Figure 5). We also found endometrial edema with purplish discoloration of the serosal surface of the uterus.

On histologic examination, systemic vasculitis was observed affecting meninges, nasal mucosa, lung, diverse lymph nodes, tonsil, spleen, liver, kidney, heart, uterus, ovary, and intestine. Edema, syncytial cells, viral inclusion bodies, and alveolitis were seen in lung sections. Focal necrosis and syncytial formation within lymph nodes was identified, together with glomerulitis and syncytial cell formation in the kidney. Acute myocarditis and focal necrosis of adrenal and corpus luteum tissue was detected. HeV antigen was also detected in tissues and organs including alveolar walls, lymph nodes, tonsil, renal glomeruli, myocardium, and ovary and in blood vessels supplying each of these, as well as in nasal mucosa, liver, spleen, adrenal gland, uterus, and intestine.

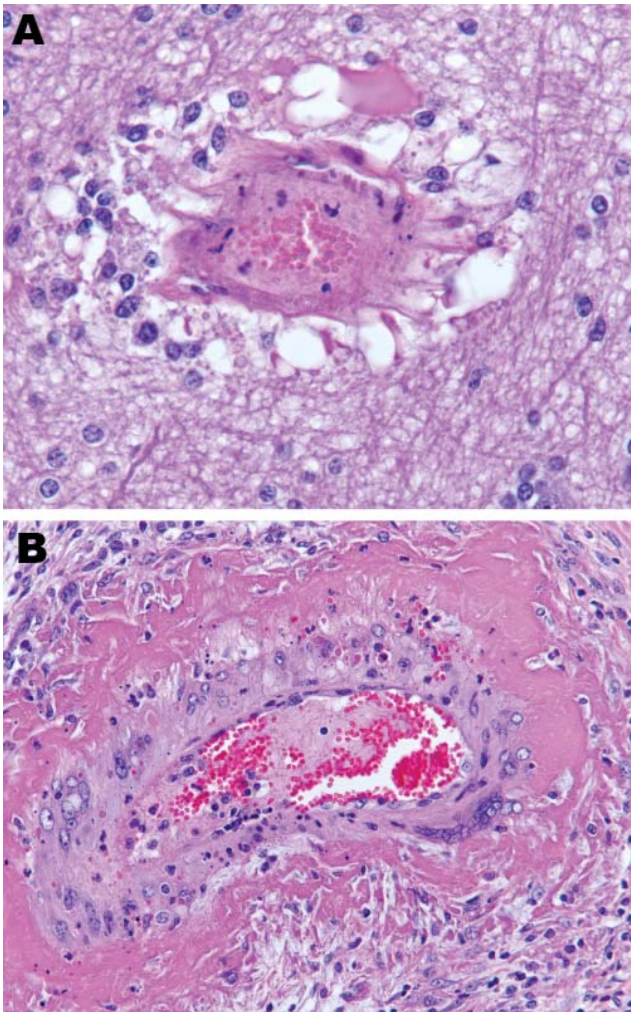

Figure 3. Brain vasculitis in horse experimentally infected with Hendra virus, Australia. A) Parenchyma and B) ovary of horse 2. Original magnification ×200.

Virus Loads in Clinical Samples

Viral genetic material was detected in nasal swabs from 2 days postchallenge (Table, only P gene data shown), consistently in 2 of the animals and intermittently in the third. The steady increase in relative copy numbers over time is consistent with viral replication in the upper respiratory tract and shedding into the nasal cavity in nasal secretions. Viral RNA was first found in the blood of each horse at least 1 day before onset of fever. After onset of fever, but before development of other clinical signs of illness, HeV genome was detected in the oral swabs, urine, and feces of each horse; the rectal swab only of horse 2 was positive. Fecal material on the floor of the pen could have been contaminated by urine containing viral genetic material. In addition, the smaller amount of material collected on the rectal swab could have influenced sensitivity of the test. Once clinical disease was established, all samples had detectable levels of HeV genome, except the rectal swabs of horses 1 and 3. All samples in which viral RNA was found were examined for live virus by passage in Vero cells. Virus was not reisolated from any sample collected before postmortem examination. Blood samples collected during acute disease, as well as samples of urine and feces, were highly toxic to tissue cultures, and virus might have been present at low titer in some of these samples.

Virus Loads in Tissue Samples

Viral RNA (N and P genes) was detected in all tissues sampled at postmortem examination (Figure 6, only P gene data displayed). Reisolation of virus was attempted

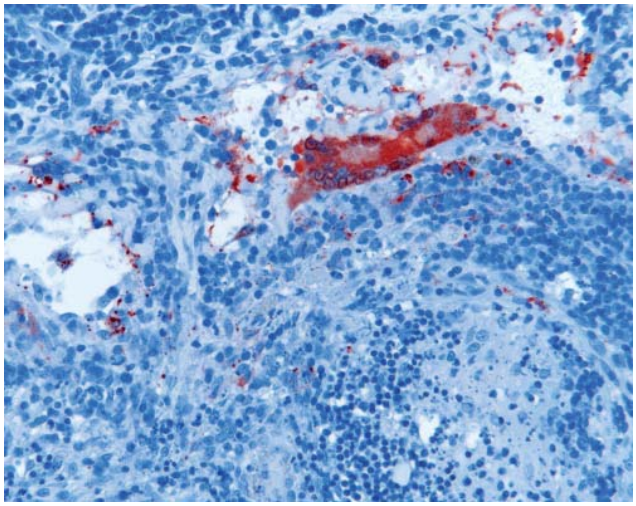

Figure 4. Lymphadenitis with syncytial cell formation in horse 2 experimentally infected with Hendra virus (HeV), Australia. Immunohistochemical staining of HeV N protein showing presence of antigen in red. Original magnification ×200.

for all tissues; tissues from which virus was recovered generally were those with the highest levels of target genes. From horse 1, these tissues were kidney; lung; and submandibular, inguinal, and renal lymph nodes. From horse 2, virus was recovered from the guttural pouch; pharynx; submandibular, inguinal, bronchial, and renal lymph nodes; lung; spleen; kidney; heart; large intestine; spinal cord; brain; and intrathoracic sympathetic chain. From horse 3, virus was recovered from the guttural pouch; submandibular, inguinal, bronchial, and renal lymph nodes; lung; kidney; heart; adrenal gland; spinal cord; brain; cerebrospinal fluid; and meninges.

Discussion

The mode and critical control points of HeV spillover from flying foxes to horses, along with the risk for transmission of virus from infected horses to other horses and to humans, is poorly understood. In this study, we successfully established systemic HeV in 3 horses exposed to 2×10^6 50% tissue culture infectious dose HeV (Hendra virus/Australia/Horse/2008/Redlands) oronasally. In 2 of the 3 animals, HeV RNA was continually detected in nasal swabs over the course of the incubation period, strongly suggesting that systemic spread of virus may be preceded by local viral replication in the nasal cavity or nasopharynx.

These data indicate that nasal secretions of asymptomatic horses may pose a transmission risk during the early phase of disease that precedes viremia, fever, or other discernable clinical signs of HeV infection. However, the increasing gene copy number recovered over time also suggests that the risk provided by these animals is relatively low, compared with animals in the immediate presymptomatic and symptomatic stages of infection. Duration of exposure also contributes to infection risk because longer contact time increases the potential for acquisition of an infectious dose of virus. Additionally, certain types of contact or procedures may contribute to infection, such as nasal intubation or routine dental procedures, where operator risk is increased even in the preclinical stage of infection.

The febrile, and then symptomatic, horse likely poses a greater transmission risk not only from virus shed in its nasal secretions but also from excretions, such as urine, and blood. However, the activity likely to pose the highest transmission risk is postmortem examination of a horse that has died of acute HeV infection. The potentially high virus load in the animal at this time provides a scenario for gross contamination of operator and assistants with infective material and the associated additional risk inherent in the handling of sharp instruments. Of the 7 known human HeV infections, 2 have been associated with postmortem examination of affected horses (*8*,*9*) and the remainder with contact with clinically ill horses in the late incubation period (*2*,*10*).

Clinical signs observed in our study, including fever, tachycardia, inappetence, depression, dyspnea, and restlessness, were generally consistent with signs recorded in the first HeV outbreak (*10*–*12*) as well as earlier experimental studies that used the original HEV isolate (Hendra virus/Australia/Horse/1994/Hendra) (*13*). The early field observations also mention ataxia (*10*,*14*) and myoclonus (*14*), which suggest that neurologic presentations are regularly associated with HeV disease in horses. Similarly, nonsuppurative meningoencephalitis is commonly found in experimentally induced infection (*15*), and the florid neurologic signs noted in field cases in

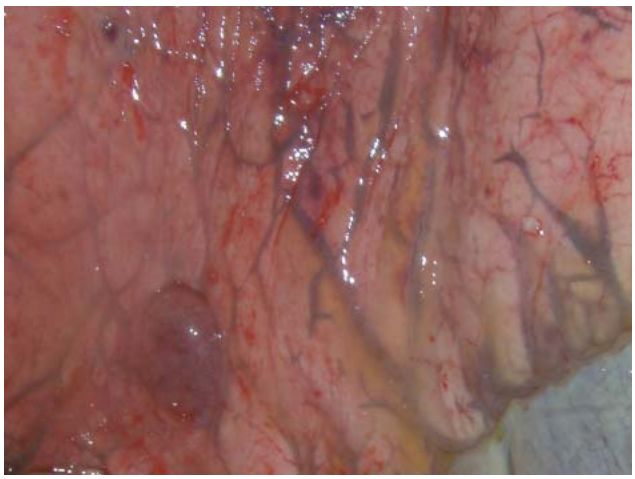

Figure 5. Dilation of lymphatic vessels and ventral lung lobe margins of horse 3 experimentally infected with Hendra virus, Australia. Original magnification ×10.

Table. Real-time PCR detection of HeV in daily shedding samples in experimental infection of horses, Australia, 2008*

	Cycle threshold values from HeV P gene real-time RT-PCR†									
	Days postinfection									
Animal/sample	0	1	2	3	4	5	6	7	8	9
Horse 1										
Blood	−	−	−	−	39.4	33.0	31.2			
Urine	−	−	−	41.9	−	41.6	36.2			
Feces	−	−	−	−	−	40.7	36.1			
Rectal swab	−	−	−	−	−	−	42.0			
Nasal swab	−	−	37.5	34.7	35.9	29.5	32.8			
Oral swab	−	−	−	−	−	41.2	38.5			
Horse 2										
Blood	−	−	−	−	−	−	37.3	32.2	31.4	29.9
Urine	−	−	−	−	−	−	−	36.2	36.3	33.5
Feces	−	−	−	−	−	−	40.7	37.0	35.0	35.2
Rectal swab	−	−	−	−	−	−	−	43.6	36.4	36.5
Nasal swab	−	−	36.3	32.4	38.9	34.3	31.1	28.1	29.2	35.2
Oral swab	−	−	−	−	−	−	−	36.6	35.8	34.5
Horse 3										
Blood	−	−	−	−	−	39.1	36.3	32.8	31.6	
Urine	−	−	−	−	−	−	40.7	39.0	34.3	
Feces	−	−	−	−	42.2	−	39.2	35.3	34.5	
Rectal swab	−	−	−	−	−	−	−	42.9	42.8	
Nasal swab	−	−	42.0	NA	41.4	43.0	−	37.9	32.9	
Oral swab	−	−	−	−	41.4	41.8	40.3	39.5	38.5	

*HeV, Hendra virus; RT-PCR, reverse transcription PCR; −, negative by real-time PCR testing; NA, sample not available for testing. Shading indicates that horse was euthanized.
†Individual samples were taken, RNA extracted, and samples tested by real-time PCR in duplicate.

the Redlands 2008 outbreak (*4*) might merely reflect the normal spectrum of HeV in horses. Although the route of natural infection of horses by HeV is not known, primary exposure likely occurs through the upper respiratory tract or the oropharynx. In previous experimental studies using HeV, either route has been used to successfully establish infection and therefore was selected for the current study. Notably, 3 of 5 horses at Redlands had medical problems involving the head (corneal lesion, nasal granuloma, and mandibular fracture) (*4*), which created the potential for an exposure route that bypassed mucosal protective mechanisms. If so, these routes of infection may also have influenced the course of infection.

Our data suggest that a critical part of reducing HeV exposure risk to veterinarians and animal attendants includes early consideration of HeV in the differential diagnosis of the febrile horse, with institution of appropriate infection control procedures, at least until a definitive diagnosis is obtained. Accordingly, published guidelines on handling potential HeV cases have been revised to account for this improved understanding of the dynamics of HeV infection in the horse (*1*). Most febrile horses are not infected with HeV but will often require ongoing veterinary interventions for underlying disease. We acknowledge that case management under these circumstances is not straightforward, particularly with respect to suitable personal protective equipment, because neither the human infectious dose nor the virus load in the air are known.

In spite of attendant risk, postmortem examination of affected animals remains particularly valuable when atypical disease is observed or in other situations where

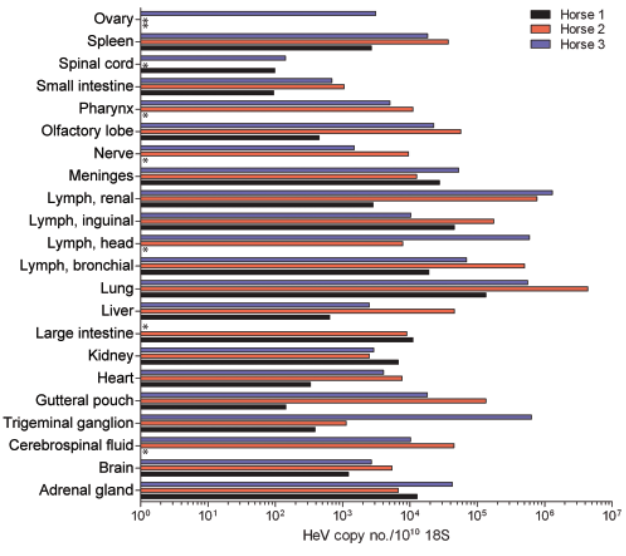

Figure 6. Relative abundance of Hendra virus (HeV) P RNA in different horse tissues at postmortem examination after experimental infection with HeV, Australia. Values are expressed relative to ribosomal 18S copies. Tissue origins are indicated along the y-axis. *Sample not available for testing.

a diagnosis is essential, such as when human exposure is suspected or transmission to other animals might have occurred. Necropsy can be conducted safely by suitably experienced and equipped operators with predetermined strategies and infrastructure for personnel and environmental decontamination. A level of risk reduction should be adopted for inexperienced operators or those ill prepared to safely manage such a procedure, especially where diagnostic confirmation of a typical HeV case is sought. In such cases limited collection of tissues, such as superficial lymph nodes, may offer a tolerable balance between the value of diagnostic confirmation and the infection risk associated with achieving it.

Acknowledgments

We thank the histopathology staff at the Australian Animal Health Laboratory for processing tissue samples and Biosecurity Queensland field and laboratory staff for providing samples from Hendra virus–infected horses. We also thank Ina Smith and Cameron Stewart for critical review of this manuscript.

This work was supported by funds supplied by the Australian Biosecurity Cooperative Research Center for Emerging Infectious Disease.

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Lineage and Virulence of *Streptococcus suis* Serotype 2 Isolates from North America

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We performed multilocus sequence typing of 64 North American *Streptococcus suis* serotype 2 porcine isolates. Strains were sequence type (ST) 28 (51%), ST25 (44%), and ST1 (5%). We identified nonrandom associations between STs and expression of the virulence markers suilysin (SLY), muramidase-relased protein (MRP), and extracellular factor (EF). Expression of pili encoded by the *srtF* and *srtG* pilus clusters was also nonrandomly associated with STs. ST1 strains were SLY+ EF+ MRP+ *srtF* pilus+ *srtG* pilus–. ST25 strains were SLY– EF– MRP– *srtF* pilus– *srtG* pilus+, and most ST28 strains were SLY– MRP+ EF– *srtF* pilus+ *srtG* pilus+. ST28 isolates proved essentially nonvirulent in a mouse infection model; ST25 strains showed moderate virulence and ST1 isolates were highly virulent. ST1 is responsible for a high proportion of *S. suis* disease in humans worldwide. Its presence in North America indicates that potential zoonotic *S. suis* outbreaks in this continent cannot be disregarded.

Streptococcus suis causes meningitis and septicemia in pigs and is a zoonotic agent (*1*). In the Western hemisphere, human *S. suis* disease is infrequent and usually affects workers in the swine industry. However, *S. suis* is the most commonly reported cause of streptococcal meningitis in adults in Vietnam and the second in Thailand (*2*,*3*). Two outbreaks of human *S. suis* disease have occurred in People's Republic of China, affecting hundreds of persons and causing 39 deaths (*4*). Most cases of animal and human *S. suis* infection have been caused by serotype 2 strains (*5*). The percentage of *S. suis* serotype 2 strains recovered from diseased pigs and the number of cases of human disease is lower in North America than in other parts of the world (*6*,*7*).

Multilocus sequence typing (MLST) has shown that *S. suis* serotype 2 strains can be divided into at least 16 sequence types (STs). Closely related STs are grouped in the so-called ST complexes. Although ST complexes 1, 27, and 87 dominate the *S. suis* population, most invasive isolates belong to the ST1 complex (*8*). For example, most strains isolated from human patients in Japan were ST1 (*9*), whereas those causing the human outbreaks in People's Republic of China were ST7, included in the ST1 complex (*10*,*11*). However, Takamatsu et al. showed that 80% of the isolates recovered from blood or cerebrospinal fluid of humans in Thailand belonged to STs grouped in the ST27 complex (*12*).

Most of the *S. suis* serotype 2 strains genotyped so far by MLST originated in Europe and Asia (*8*–*12*). Isolates from Canada and the United States have received less attention. In this study, we used MLST to genotype a relatively large collection of US and Canadian *S. suis* serotype 2 strains.

Materials and Methods

S. suis Field Strains

Sixty-four strains of *S. suis* serotype 2 isolated from pigs with clinical disease in different and nonrelated farms in major swine production areas of Canada and the United States were used. For comparison purposes, 19 porcine and 1 human *S. suis* serotype 2 strains isolated in Japan and

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DOI: http://dx.doi.org/10.3201/eid1712.110609

12 human *S. suis* serotype 2 strains isolated in Thailand were included (*12*,*13*). All strains are listed in the online Appendix Table (wwwnc.cdc.gov/EID/article/17/12/11-0609-TA1.htm).

MLST and Phylogenetic Analysis

S. suis genomic DNA was prepared from overnight cultures by using the QIAamp DNA Minikit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. MLST was performed by PCR amplification and DNA sequencing of the *cpn*60, *dpr*, *recA*, *aroA*, *thrA*, *gki*, and *mutS* genes as described (*8*). For each isolate, the alleles at each of the 7 loci defined the ST. MLST information in the *S. sui*s database (http://ssuis.mlst.net) identified the phylogenetic position of strains. eBURST software (*14*) was used to identify *S. suis* clonal complexes and to display the overall structure of the population.

PCRs for Virulence Markers and Pili Cluster Genes

Amplification of *sly*, *mrp*, and *epf* genes was performed by PCR as described (*6*). Genes in the *srtF* and *srtG* pilus clusters were amplified by PCR by using the primers and conditions described by Takamatsu et al. (*13*).

MRP, EF, and Pili Expression and Hemolysis Assays

S. suis strains were grown in Todd-Hewitt broth at 37°C (at 28°C for detection of the *srtG* pilus). Bacteria were harvested by centrifugation during the late exponential phase of growth, and supernatants were concentrated 10-fold by using Ultrafree-MC centrifugal filters (Millipore Corp., Bedford, MA, USA). Expression of extracellular factor (EF) and muramidase-released protein (MRP) was determined by Western blotting of the concentrated supernatant fraction by using monoclonal antibodies against MRP or EF, as described (*15*). Mutanolysin extracts were prepared from pelleted bacteria as described (*16*,*17*) and used to detect pili encoded by the *srtF* and *srtG* pilus clusters by Western blotting with antibodies directed against the major subunit of each pilus (*16*,*17*). The ability of strains to lyse horse erythrocytes (an indication of the production by the strains of the hemolysin known as suilysin, SLY) was determined as described (*6*).

Experimental Infection of Mice

All animal experiments followed the guidelines of the Canadian Council on Animal Care and were approved by the Ethics Committee, Université de Montréal. We used a validated CD1 mouse infection model (*18*). In a first experiment, 60 female 6-week-old mice (Charles River Laboratories, Wilmington, MA, USA) were divided in 4 groups. Group 1 was inoculated with ST1 strain P1/7; groups 2 and 3 received ST25 strains 89-1591 and 1085543, respectively. Group 4 received ST28 strain 1088563. The inocula (5×10^7 CFU/animal) were delivered intraperitoneally. Mice were monitored 3×/d for 10 days for clinical signs and assigned clinical scores as described (*18*). Blood was collected daily from the tail vein (5 μL) and at necropsy by cardiac puncture and used to evaluate bacterial load by plating onto sheep blood agar plates and enumeration. Colonization of the liver and spleen of infected animals was evaluated at necropsy as described (*18*). A second experiment was performed essentially as described above, but the mice received a 10-fold higher dose of ST28 strains 1088563, 1054471, and 1097205. In this second experiment, groups contained 5 mice.

Results

Most of the 64 strains from North America were ST28 (n = 33) or ST25 (n = 28). Together, these 2 STs accounted for 95% of all *S. suis* serotype 2 strains from North America that were investigated (Table 1). However, a higher ST28 prevalence was true only for the United States; most strains from Canada were ST25. The remaining 3 strains belonged to ST1, which is commonly found in Europe and Southeast Asia.

Nonrandom Association between STs and Expression of Virulence Markers

SLY (encoded by the *sly* gene), MRP (*mrp* gene), and EF (*epf* gene) are virulence markers that have been used in elaborated genotypic and phenotypic schemes to try to predict the virulence of a given *S. suis* strain (*1*,*19*). For example, Silva et al. designed a multiplex PCR test that can discriminate between at least 6 naturally occurring genetic variants of *mrp*, named *mrp*^{s}, *mrp*, *mrp**, *mrp***, *mrp****, and *mrp***** (*20*). We investigated possible associations between STs and these widely used markers in our collection of *S. suis* serotype 2 strains from North America. To assess whether associations found are independent of the geographic origin of the strains, we included 32 described (*12*,*13*) *S. suis* serotype 2 strains of STs 28, 25, and 1 isolated in Japan and Thailand (online Appendix Table).

Independently of geographic origin, we found clear, nonrandom associations between STs and expression of virulence markers. All but 2 ST1 strains had the phenotype SLY+MRP+ EF+. All ST25 strains were SLY−MRP−EF− and all ST28 strains were SLY−MRP (or its variants)+ EF− (Table 2). Most ST1 strains had an *sly*+*mrp*+*epf*+

Table 1. STs identified among the *Streptococcus suis* serotype 2 isolates from North America*

Country	No. strains	ST1	ST25	ST28
Canada	44	0	26	18
United States	20	3	2	15
Total	64	3	28	33

*ST, sequence type.

Table 2. Association of *Streptococcus suis* serotype 2 STs and commonly used virulence markers in isolates from North America†

		Presence of factor-encoding gene										
			mrp variant‡							Phenotype		
ST	No. strains	*sly*	*mrp*	*mrp*^{S}	*mrp**	*mrp***	*mrp****	ND	*epf*	Hemolysis§	MRP¶	EF
1	11	11	9	0	0	0	0	2	11	11	9	11
25	36	0	0	1	1	8	23	3	0	0	0	0
28	49	0	42	6	1	0	0	0	3	0	49	0

†ST, sequence type; SLY, suilysin; MRP, muraminidase-released protein; ND, no amplification of the *mrp* gene was detected by PCR under the conditions used; EF, extracellular factor.
‡Variants of the *mrp* gene are those described by Silva et al. (*20*).
§Hemolysis of horse erythrocytes by the strains was considered to be an indication of the expression of SLY.
¶Molecular mass MRP variants identified by Western blotting were in agreement with those expected on the basis of the *mrp* gene variant identified by PCR.

genotype, in agreement with results of previous reports (*11*–*13*). ST25 and ST28 strains had an *sly*– genotype and, with the exception of 3 ST28 strains, an *epf*– genotype. No clear relationships were found between ST25 strains and a particular *mrp* gene variant genotype. All but 3 ST25 strains were positive by PCR for 1 *mrp* gene variant, yet none of these strains expressed the protein (Table 2). In a recent report, all *mrp*+/MRP– strains that were investigated (of various *S. suis* serotypes) had truncations or point mutations in the *mrp* gene that prevented expression of MRP (*6*). Although we have not sequenced the *mrp* gene in our collection of strains, we hypothesize that similar genetic rearrangements are likely to explain the *mrp*+/MRP– results we observed in ST25 strains in this study. Three *mrp* gene variants were associated with ST28, although variant *mrp* was the most prevalent (85%) among this ST.

Nonrandom Association between STs and Expression of Pili

Takamatsu et al. reported associations between particular STs and the presence or absence of putative pilus gene clusters, designated *srtBCD*, *srtE*, *srtF*, and *srtG* clusters (*13*). All ST25 and ST28 strains investigated by these authors were positive by PCR for all genes in the *srtF* and *srtG* pilus clusters (*13*). Consistently, we found that all ST25 and ST28 strains in our collection were positive for all genes in these 2 pilus clusters (Table 3). Furthermore, by using specific antibodies directed against the major pilin subunits (*16*,*17*), we identified a clear, nonrandom association between ST28 strains and expression of both pili (Table 3). However, although all ST25 strains expressed the *srtG* pilus, none produced the *srtF* pilus (Table 3).

It has been shown that one ST25 isolate from Canada, which does not have a discrete *srtF* pilus cluster and is unable to express the *srtF* pilus, is nonetheless PCR positive for each of the individual *srtF* genes because PCR amplicons can be generated from homologs of these genes found at various genome locations (*13*,*16*). We hypothesized that the ST25 strains analyzed in our study have a genetic organization similar to that ST25 isolate. Consistent with our hypothesis, our attempts to amplify the *srtF* pilus cluster in ST25 strains by using a primer pair annealing to the first and last gene of the *srtF* cluster were unsuccessful (data not shown). All the ST1 strains had the *srtF* cluster genes but, with the exception of 3 strains, not the *srtG* cluster genes. When we assessed the pilus phenotype by Western blotting, all ST1 strains expressed the *srtF* pilus but none expressed the *srtG* pilus (Table 3). The reason(s) the 3 ST1 strains that have the *srtG* cluster genes do not express the corresponding pilus are currently under investigation.

Mouse Infection Model

Inasmuch as the MLST data showed that more than half of the strains from North America analyzed were ST28 and the second most represented ST was ST25, we performed a comparison of the virulence of representative ST25 and ST28 strains by using a standardized mouse infection model (*18*). For comparison, we included the well-characterized and highly virulent ST1 strain P1/7. Most mice in the ST1 group showed severe clinical signs

Table 3. Association of *Streptococcus suis* serotype 2 STs and *srtF* and *srtG* pilus clusters in isolates from North America*

		srtF pilus cluster					*srtG* pilus cluster			
		Gene†					Gene†			
ST	No. strains	*srtF*	*sfp1*	*sfp2*	*sipF*	Pili expression‡, Sfp1	*srtG*	*sgp1*	*sgp2*	Pili expression, Sgp1‡
1	11	11	11	11	11	11	3	3	3	0
25	36	36	36	36	36	0	36	36	36	36
28	49	49	49	49	49	46	49	49	49	47

*ST, sequence type.
†The presence of the genes was detected by PCR by using primers and conditions described by Takamatsu et al. (*13*).
‡Expression of pili encoded by the *srtF* and *srtG* pilus clusters was performed by Western blotting by using described antibodies directed against the major subunits of these structures (*16*,*17*).

of septicemia, such as depression, swollen eyes, weakness, and prostration during the first 24 hours postinoculation. Several mice died of septicemia during the first 2 days of the trial, and the remaining animals were humanely killed for ethical reasons at day 3 postinoculation (Figure 1). *S. suis* was isolated in pure cultures at high titers (>1 × 10^7 CFU/mL) from blood samples and organs, such as the liver and spleen, of septicemic animals in the ST1 group (>1 × 10^7 CFU/0.5 g of tissue in most animals).

The virulence of ST25 strains was intermediate. They caused moderate clinical signs and relatively low mortality among inoculated mice (Figure 1). Statistical analysis demonstrated that ST25 strains were significantly less virulent than ST1 strains. However, ST25 strains were significantly more virulent than ST28 strains. In fact, no mice in the ST28 group died (Figure 1) or showed clinical signs associated with *S. suis* infection, with the exception of slight depression immediately after inoculation, which subsided after 24 hours postinoculation. Bacteria could not be isolated from the blood of most mice in this group >48 postinoculation, and we could not isolate *S. suis* from different organs at necropsy (results not shown). Given this surprising absence of clinical signs, we repeated the experiment by inoculating 3 additional groups of 5 mice each with the previously used and 2 other ST28 strains by using an infective dose that was 10-fold higher than the one previously used. Despite this increased infective dose, similar low virulence was observed for ST28 strains (Figure 2).

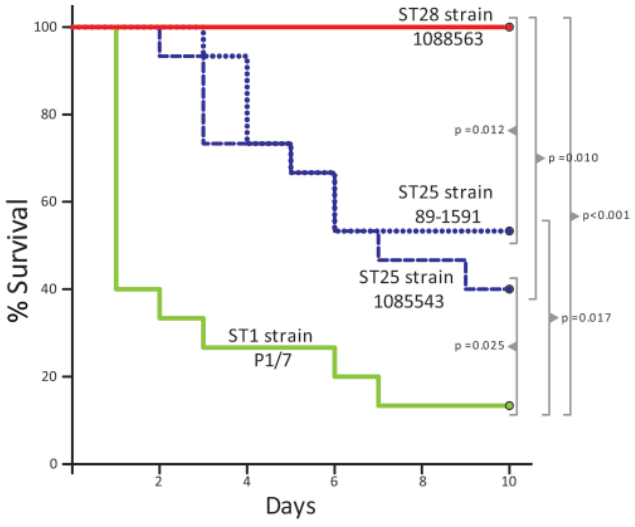

Figure 1. Survival of CD1 mice inoculated with *Streptococcus suis* strains of different sequence types (STs). Most animals that received the ST1 strain P1/7 died from septicemia during the first 3 days of the trial. Several animals in this group died from meningitis from day 6 postinfection. Two groups of mice received ST25 strains 89–1591 and 1085543, respectively. Survival of mice in these 2 groups was higher than in the group that received the ST1 strain. However, >40% of the animals in the 89–1591 group and 60% of the animals in the 1085543 group died or were killed for ethical reasons before the end of the trial. In strong contrast, all 15 mice in the ST28 strain group survived the trial. Significant differences in survival were noted between groups (log-rank test, p values indicated in the figure body).

Discussion

In this article, we show that most *S. suis* isolates from North America belong to ST28 and ST25 and that strains of these STs are significantly less virulent than ST1 strains. Although ST28 strains were essentially nonvirulent for mice, ST25 strains were of intermediate virulence and able to induce severe disease.

With a population of ≈115 million pigs, Canada and the United States combined are second only to the People's Republic of China in terms of swine production. Although *S. suis* infections are a main cause of postweaned piglet deaths in North America, the prevalence of *S. suis* serotype 2 strains is much lower on this continent than in other regions of the world (*6*,*7*). We show here that in North America the most common STs among *S. suis* serotype 2 strains are ST28 and ST25. By using a mouse infection model, we also show that *S. suis* serotype 2 ST28 and ST25 strains are of lower virulence than ST1 strains. In contrast to Europe and Asia, where >60% of virulent serotype 2 isolates are ST1 (*21*–*23*), in North America only a small percentage (5%) of strains belonged to this more virulent ST.

Only 3 cases of *S. suis* serotype 2 in locally infected humans have been reported in North America (*5*). Our results suggest that this low prevalence of human infections might be connected to the lower virulence of the circulating serotype 2 strains among the swine population in North America. In addition to a low prevalence of ST1 strains, we did not find any strains in our collection from North America belonging to STs 101, 102, 103, and 104, which are agents of human disease in Thailand (*12*). On the basis of its low frequency of isolation, we speculate that the ST1 strains we identified were introduced in North America by importation of animals. Human travel might also contribute to dissemination of ST1 strains, as exemplified by a reported case of human *S. suis* meningitis caused by an ST1 strain involving a patient who contracted *S. suis* in the Philippines but in whom clinical signs appeared only after he returned to the United States (*24*). The deadly human outbreaks in Asia caused by ST1 complex strains (*2*–*5*) and the fact that ST1 strains are replacing at a fast pace STs of lower virulence and causing human disease in countries such as Vietnam and Thailand (*21*,*25*,*26*) highlight that maintaining a low prevalence of ST1 strains among the swine population in North America is crucial for animal and human health. Of note, the only locally acquired human infection in the United States described so far (*27*) was caused by an ST1 strain (M. Gottschalk, unpub. data).

Another concern for the swine industry and for public health authorities is the presence in North America of *S.*

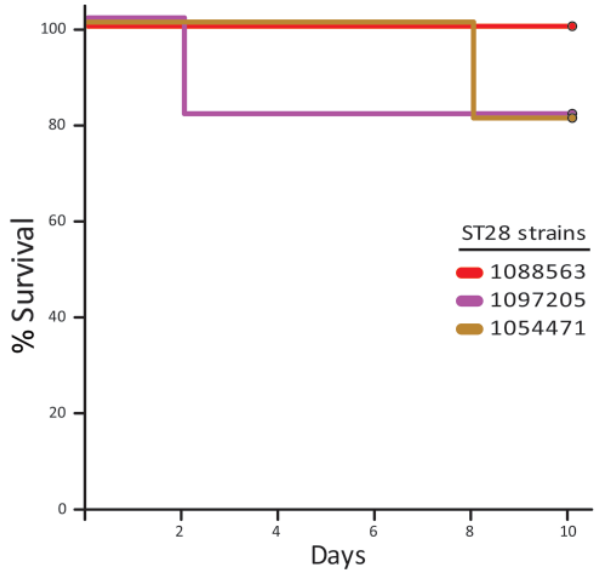

Figure 2. Survival of CD1 mice inoculated with the different *Streptococcus suis* sequence type 28 strains from North America. In this experiment, the infectious dose was 1 × 10^8 CFU/animal, 10-fold higher than in the previous experimental inoculation. Doses were intraperitoneally injected into the animals. No significant differences were found between groups.

suis ST25 strains. Many human cases reported in Thailand and 2 cases in Canada of *S. suis* serotype 2 disease were caused by ST25 strains (*5*,*12*). On the other hand, we found that strains of the most prevalent ST28 are of low virulence. Two strains shown here to be ST28 (1330 and 0891; see online Appendix Table) had been reported as nonvirulent *S. suis* serotype 2 (*8*). No human *S. suis* cases attributable to ST28 strains have been reported in North America. However, all ST28 strains included in this study were isolated from diseased pigs, and 1 human case in Japan and 1 human case in Thailand were caused by ST28 strains (*9*,*12*). Nonvirulent *S. suis* strains have been hypothesized to cause disease in immunocompromised animals or humans who have a concurrent infection with another bacterial or viral pathogen(s) (*5*). Porcine *S. suis* infections in North America are usually associated with a concomitant infection with the porcine respiratory and reproductive virus (*1*). We do not know the immunologic status of the animals from which the ST28 strains were isolated to test the aforementioned hypothesis. Toward this goal, however, we are developing a co-infection model of *S. suis* and porcine respiratory and reproductive virus.

Our results provide evidence that genotyping schemes based on *sly*, *mrp*, *epf*, and pilus cluster genes, although useful in discriminating highly virulent ST1 strains from other groups (*8*,*13*,*20*), are of limited use in differentiating between ST25 and ST28 strains. Although not ideal because protein expression levels may be affected by many factors, typing methods based on protein expression of these markers might a priori differentiate these STs of different virulence. The fact that ST25 strains do not express MRP or the *srtF* pili, yet they are more virulent than ST28 strains, further demonstrates the dispensability of these factors for the full virulence of *S. suis* (*16*,*28*). Our results also highlight that subunit vaccines based on purified MRP or *srtF* pilus subunits might be of little use to counter *S. suis* infections caused by ST25 strains.

Our work provides more support to the longstanding hypothesis that *S. suis* serotype 2 strains in North America are of lower virulence than strains from Eurasia. However, we do not yet understand the reasons for this lower virulence. The genome sequences of several *S. suis* serotype 2 ST1 and an ST25 strains have been published or made available (*25*,*29*,*30*). Genome sequencing of a larger number of additional *S. suis* strains of these and other STs could help elucidate the genetic basis of virulence differences among strains of this swine pathogen and zoonotic agent.

This study was supported by grants from the Natural Sciences and Engineering Research Council of Canada to M.G. (no. 154280 and Discovery Accelerator Supplement 380299) and Minister of Economic Development, Innovation and Export Trade, China-Quebec Collaboration grants to M.G and J.X. (no. 2008FA31830 and 2008DFA31830, respectively). N.F. is partially supported by the Canadian Institutes of Health Research.

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West Nile Virus Infection of Birds, Mexico

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West Nile virus (WNV) has caused disease in humans, equids, and birds at lower frequency in Mexico than in the United States. We hypothesized that the seemingly reduced virulence in Mexico was caused by attenuation of the Tabasco strain from southeastern Mexico, resulting in lower viremia than that caused by the Tecate strain from the more northern location of Baja California. During 2006–2008, we tested this hypothesis in candidate avian amplifying hosts: domestic chickens, rock pigeons, house sparrows, great-tailed grackles, and clay-colored thrushes. Only great-tailed grackles and house sparrows were competent amplifying hosts for both strains, and deaths occurred in each species. Tecate strain viremia levels were higher for thrushes. Both strains produced low-level viremia in pigeons and chickens. Our results suggest that certain avian hosts within Mexico are competent for efficient amplification of both northern and southern WNV strains and that both strains likely contribute to bird deaths.

In Mexico, West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus*) was first isolated in 2003 from a common raven (*Corvus corax*) carcass in Tabasco (southeast Mexico) (*1*). According to findings of WNV-neutralizing antibodies in horses from the coastal states of eastern Mexico and in resident birds in the Yucatan Peninsula, the virus had spread to Mexico at least 1 year earlier (*1*–*3*). In the United States and Canada, morbidity and mortality rates for WNV infection are high among humans, horses, and birds; but in Mexico and other regions of Latin America, the health effects of this virus remain unknown (*4*). Low numbers of cases in humans, equids, and birds in Mexico have been reported, primarily from the northern border with the United States, where isolated WNV strains (e.g., Tecate) were genetically related to the North American 2002 strain circulating in the southwestern United States (*5*).

The paucity of reported WNV cases in Mexico might be the result of multiple factors involved in local virus ecology. The interactions of amplifying hosts, vectors, and virus strains in Mexico, combined with external factors such as climate, habitat, and circulation of interfering flaviviruses, may result in relatively low levels of transmission and disease. Virus–host interactions in Mexico, including susceptibility and competence of candidate amplifying hosts, remain unknown. Assessment of the response of various avian species to WNV infection could elucidate aspects of the transmission ecology in tropical ecosystems and provide insight for potential surveillance strategies.

To address knowledge gaps regarding transmission and to investigate whether the apparently low prevalence of WNV disease in Mexico could result from reduced virulence of WNV strains from Mexico, during 2006–2008 we experimentally infected birds. We selected birds of several common species as potential WNV-amplifying hosts, including domestic chickens (*Gallus gallus*), rock pigeons (*Columba livia*), house sparrows (*Passer domesticus*), great-tailed grackles (*Quiscalus mexicanus*),

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DOI: http://dx.doi.org/10.3201/eid1712.110294

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and clay-colored thrushes (*Turdus grayi*). We measured viremia, virus shedding, survival rates, and tissue tropism and calculated reservoir competence index values in birds infected with WNV strains from southern Mexico (Tabasco) or northern Mexico (Tecate).

Materials and Methods

Experimental Birds

All birds in the study were adults and originated in Mexico. They were either acquired commercially (chickens) or trapped by using mist nets (house sparrows and clay-colored thrushes) or walk-in traps (rock pigeons and great-tailed grackles). The birds were moved to indoor housing, where blood samples were collected and serum was tested for neutralizing antibodies to WNV as determined by plaque-reduction neutralization test (*6*). All birds were cared for in animal holding facilities at the National Institute of Forestry, Agriculture and Livestock, Palo Alto, Mexico City.

Experimental Inoculation and Sampling Protocol

Low-passage WNV strains originally isolated from tissues harvested from common ravens from southern Mexico (Tabasco; GenBank accession no. AY660002, 7 Vero passages) and northern Mexico (Tecate; GenBank accession no. DQ080060, 2 Vero passages) were used to inoculate birds. Because of the additional passages of the Tabasco strain, we sequenced the viral protein coding region to determine the presence or absence of potential vertebrate virulence determinants, such as the glycosylation motif at positions 154–156 of the envelope (E) protein. Birds seronegative for WNV and St. Louis encephalitis virus (*Flaviviridae*) were subcutaneously inoculated in the pectoral region at concentrations of ≈100,000 Vero PFU/0.1 mL in sterile phosphate-buffered saline. Sample sizes of 4–6 birds were inoculated for each species–virus strain combination, and 1–2 additional birds per group were sham inoculated as negative controls. Blood was collected from all birds (sparrows 0.1 mL; all others 0.2 mL), and oral and cloacal swab samples were collected at ≈24-hour intervals for 6 or 7 days postinoculation (dpi). Coagulated blood was centrifuged to separate serum, which was placed in cryovials. Serum and swab samples were stored at −80°C until tested. A postinfection 0.6-mL blood sample was collected from survivors at 14–28 dpi. All surviving birds were euthanized, and necropsies were performed. The following tissues were collected: heart, kidney, liver, spleen, skin, and brain from all species except pigeons; intestine from thrushes, grackles, and sparrows; and pancreas and lung from grackles and sparrows. Tissues were frozen at −80°C. Some blood samples from chickens and pigeons had been destroyed before viremia could be determined. All animal studies were approved by the US Centers for Disease Control and Prevention Institutional Animal Care and Use Committee 05-26-005-MSA and by the National Institute of Forestry, Agriculture and Livestock Animal Bioethics Committee.

Laboratory Assays

To determine viral loads in tissue homogenates, swab samples, and serum samples, we used plaque assay for end-point titration in Vero cell culture (*6*). Tissue homogenates were prepared by placing ≈0.5 cm^3 of each tissue into 2-mL polypropylene tubes containing 1 mL BA-1 medium (medium 199 with Hank balanced salt solution; 0.05 mol/L Tris buffer, pH 7.6; 1% bovine serum albumin, 0.35 g/L of $NaHCO_3$, 100 mg/L streptomycin, 100 U/mL penicillin G, 1 μg/mL amphotericin B) supplemented with 20% fetal bovine serum and a 4.5-mm–diameter copper-coated steel bead. Samples were macerated in a mixer mill (Retsch GmbH, Haan, Germany) for 5 min at 25 cycles/s and clarified by centrifugation. Swab samples were soaked in 1 mL BA-1 supplemented with 20% fetal bovine serum and vortexed for 5–10 s. Serum samples were diluted 1:10 in BA-1.

Antibodies were detected in serum samples by using the plaque-reduction neutralization test in Vero cell monolayers prepared in 6-well polystyrene culture plates (*6*). Samples were heat inactivated at 56°C for 30 min and tested for neutralizing antibodies at a 1:10 dilution against WNV strain NY99–4132, originally isolated from the brain of a dead crow in New York, and St. Louis encephalitis virus strain TBH-28, originally isolated from a person in Florida, USA.

Mathematical and Statistical Analyses

Viremia titers were log transformed for statistical tests. Mean log viremia titers were compared by the Student *t* test, and the Bonferroni adjustment was applied for multiple comparisons. Using the vertebrate reservoir competence index, we analyzed viremia data to determine the potential of each species to infect vector mosquitoes (*7*). Species-specific reservoir competence index values, C_i, were calculated according to the equation $C_i = S \times I \times D$, where S is susceptibility to infection (0.0–1.0), I is mean daily infectiousness (0.0–1.0) with units representing the average proportion of *Culex quinquefasciatus* mosquitoes that are expected to become infectious after feeding on an infectious bird, and D is duration, the number of days that viremia remained infectious with titers $\geq 10^{4.7}$ PFU/mL serum for any given bird. Viremia titers below this threshold were considered zero (i.e., not infectious). Infectiousness, I, was inferred from viremia measurements according to the formula derived by Kilpatrick et al. (*8*): $I = 0.1349 \times \log_{10}(\text{viremia}) - 0.6235$.

Confidence intervals of means were calculated by using the following standard equation:

$$95\%\ \text{confidence interval} = \text{mean} \pm 1.96 \left(\frac{StdDev}{\sqrt{N}} \right)$$

C_i for an arbovirus represents the relative number of vectors that a bird is inherently able to infect during its viremic phase. Overlapping confidence intervals around calculated means indicated lack of significant differences.

Results

Viremia and Reservoir Competence Index Values

Observed viremia titers for mature chickens and pigeons did not reach infectious levels for mosquitoes, making these birds, at least when adults, incompetent hosts for the 2 strains of WNV from Mexico used in this study. Conversely, the 3 passerine species examined were competent hosts. Log-transformed mean peak viremia titers did not statistically differ between the 2 virus strains for any of the species tested (Table 1). The viremia profiles for each strain did not dramatically differ within a species, except for thrushes, because 1 thrush infected with the Tecate strain maintained a high level of viremia while the others experienced declines (Figures 1–3). Among the 3 passerine species, moderately infectious viremia for each of the 2 strains developed in the thrushes, whereas infectiousness for each of the 2 strains was higher for the sparrows and grackles. Also among the 3 passerine species, peak viremia titers differed significantly for the Tabasco strain ($p<0.005$, $\alpha = 0.05$ with Bonferroni adjustment for 3 comparisons) but not for the Tecate strain. To evaluate the potential of these passerines to infect vector mosquitoes, we compared reservoir competence index values, which predict the relative number of infectious vectors, i.e., those that will transmit virus after feeding on a bird of each species. An individual sparrow and a thrush were each predicted to generate ≈2- and 20-fold more infectious vectors when infected with Tecate than with Tabasco strain viruses, respectively (Table 1). A grackle infected with the Tabasco strain, however, would generate ≈1.5-fold more infectious mosquitoes. However, none of these quantitative differences were significant. Regardless, a thrush was predicted to infect fewer mosquitoes with either strain than would a grackle or sparrow (confidence intervals around C_i values did not overlap). Thus, among the passerine species tested, the clay-colored thrush seemed to be less of an amplifying host for the 2 WNV strains from Mexico than were house sparrows and great-tailed grackles.

Shedding

Shedding was evaluated in the same 3 passerine species: great-tailed grackles, house sparrows, and clay-colored thrushes. Thrushes were sampled on 6 and 7 dpi only and were negative (n = 4 for each strain). Most grackles and sparrows orally shed infectious virus of the Tabasco and Tecate strains. Swab sample collection from birds inoculated with the Tabasco strain was not adequate for assessing cloacal shedding, although it was noted for at least 1 grackle and 1 sparrow; cloacal shedding was noted for most birds inoculated with the Tecate strain (Table 2). Oral swab samples generally contained more virus particles than cloacal swab samples, and the Tabasco strain was shed in higher concentrations than the Tecate strain. No shedding was observed in negative control birds.

Table 1. Viremia parameters and reservoir competence index values for birds from Mexico infected with West Nile virus*

	Tecate strain viremia					Tabasco strain viremia				
Species	No.	Duration (95% CI)†	Infect (95% CI)‡	Mean peak (range)§	Comp (95% CI)¶	No.	Duration (95% CI)†	Infect (95% CI)‡	Mean peak (range)§	Comp (95% CI)¶
House sparrow	6	3.3 (2.8–3.9)	0.34 (0.23–0.44)	9.4 (5.9–10.1)	1.12 (0.43–1.81)	6	2.3 (0.9–3.7)	0.27 (0.20–0.34)	7.7 (5.7–8.4)	0.62 (0.11–1.14)
Great-tailed grackle	4	4.5 (3.8–5.2)	0.28 (0.19–0.38)	9.7 (6.5–10.3)	1.28 (0.49–2.07)	4	4.2 (2.7–5.8)	0.47 (0.36–0.58)	9.8 (8.2–10.2)	2.01 (1.14–2.88)
Clay-colored thrush	4	1.5 (0.3–2.7)	0.12 (0.03–0.21)	6.3 (3.7–6.9)	0.18 (0.00–0.50)	4	0.5 (0.0–1.2)	0.03 (0.03–0.03)	4.3 (2.5–4.8)	0.01 (0.00–0.04)
Domestic chicken	9	0	0	2.9 (0–3.3)#	0.00	11	0	0	3.3 (0–4.1)#	0
Domestic pigeon	11	0	0	2.0 (1.8–2.2)#	0.00	9	0	0	3.4 (1.3–4.0)#	0

*Infect, infectiousness; comp, competence; CI, confidence interval.
†No. days that viremia titers were $\geq 10^{4.7}$ PFU/mL serum for any given bird.
‡Average proportion of *Culex quinquefasciatus* mosquitoes that are expected to become infectious vectors after feeding on an infectious bird.
§Expressed as log PFU/mL.
¶Species-specific reservoir competence index. Values are calculated as the product of duration, infectiousness, and susceptibility to infection, which was 1.0 for all species in the table.
#Mean peak viremia values presented for chickens and pigeons may be underestimated because of intermittent sample collection.

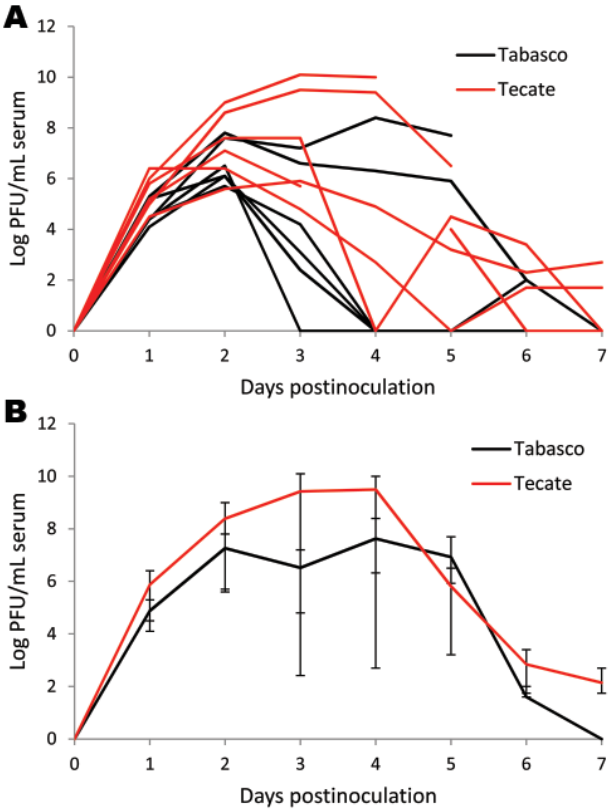

Figure 1. Viremia profile for house sparrows experimentally inoculated with Tabasco or Tecate strains of West Nile virus. Virus titers are plotted on a logarithmic scale. A) Individual birds; B) group means. Error bars represent ranges of observed titers.

Illness and Death

During the 7-dpi period, lethargy and fluffed feathers were observed among some grackles and sparrows but not among thrushes, pigeons, or chickens. Birds died within 24 hours after onset of clinical signs. Among sparrows, of the 6 inoculated with the Tabasco strain, 4 (≈67%) died; and of the 6 inoculated with the Tecate strain, 4 (≈67%) also died. The sham-inoculated sparrow showed no signs of illness. Among grackles, of the 4 inoculated with the Tabasco strain, 3 (75%) died; and of the 4 inoculated with the Tabasco strain, 1 (25%) died. Of the 2 sham-inoculated grackles, 1 (50%) died. A high viremia titer developed in 1 thrush at 5 dpi, and the bird was found dead at 8 dpi.

Tissue Tropism

Viral loads were determined in brain, viscera, and skin from each of 5 sparrows and 4 grackles that died acutely. Sample numbers were too low to detect significant differences in tissue tropisms or viral loads among strains or species. However, high-titered viral loads (>10^7 PFU/0.5 cm^3) were found for both WNV strains in brain, heart, spleen, kidney, lung, and skin of sparrows and grackles (Table 3).

Antibody Responses

All inoculated animals tested for seroconversion at 14–28 dpi had a strong detectable neutralizing antibody response to WNV (100% neutralization by 1:10 serum); there was no noticeable effect of strain used for inoculation (n = 6 sparrows, 3 grackles, 7 thrushes). Sham-inoculated controls showed no evidence of antibodies against WNV or viremia.

Virus Sequencing

Approximately 11 kb of the Tabasco strain was sequenced, encompassing almost all the genome except the 5′ untranslated region (UTR) and the 20 3′ terminal nucleotides of the 3′ UTR. Comparing the sequence from the consensus TM-171–03 isolate previously published (*5*), the following amino acid differences were identified: prM-T141I, E-P156S, E-V159A, NS3-D401Y, NS4B-V245I, NS5-I898T. The most notable difference was that of a glycosylation motif between E154 and E156. Of the 6 aa differences between the TM-171–03 sequence and the Tabasco virus stock used in this study, 4 of these differences in the stock described herein were synonymous with that of the prototype NY99 strain. The strain used to

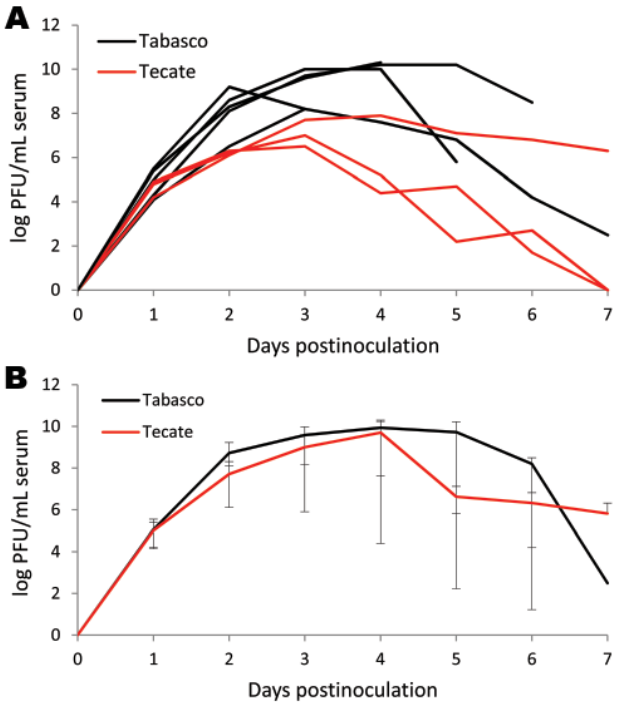

Figure 2. Viremia profile for great-tailed grackles experimentally inoculated with Tabasco or Tecate strains of West Nile virus. Virus titers are plotted on a logarithmic scale. A) Individual birds; B) group means. Error bars represent ranges of observed titers.

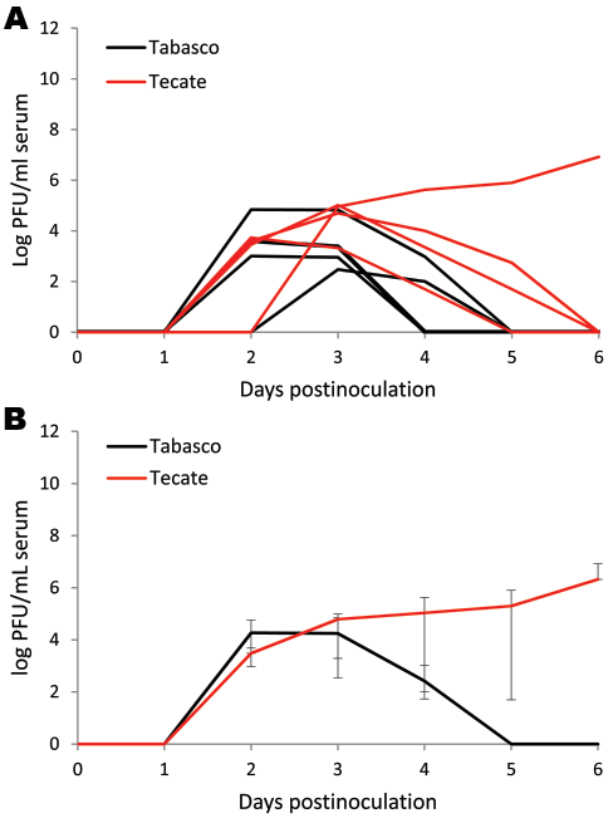

Figure 3. Viremia profile for clay-colored thrush experimentally inoculated with Tabasco or Tecate strains of West Nile virus. Virus titers are plotted on a logarithmic scale. A) Individuals birds; B) group means. Error bars represent ranges of observed titers.

inoculate birds differed from NY99 at E-V159A (present in all WN02 genotypes) and NS3-D401Y. Several 3′ UTR nucleotide mutations were also noted between the published TM-171–03 and the stock used in this study at c10,772t, g10,828t, and a10,989g. Only the c10,772t transition was unique to this stock; the other mutations were synonymous with the prototype NY99 strain.

Discussion

The results of inoculation of birds with WNV strains of Mexican origin are difficult to compare with results of published WNV infection studies because of variation in bird species, virus strains, and inoculation method (e.g., needle vs. mosquito bite). House sparrows from Mexico that were needle inoculated with the Tabasco strain were almost as infectious as house sparrows originating in the United States that were needle inoculated with the WNV NY99 strain but 3-fold less infectious than mosquito-inoculated sparrows from the United States (*9*). Responses of adult chickens to needle inoculation with strains from Mexico and the NY99 strain were similar (*10*). Chickens and pigeons consistently failed to become infectious regardless of infection method or virus strain (*11*,*12*). Common grackles (*Quiscalus quiscula*) inoculated by mosquito bite with NY99 have been found to be more competent amplifying hosts than American robins (*Turdus migratorius*) (*12*) in concordance with the findings of our studies that used the 2 strains from Mexico in species of these same genera. However, the American robin was a moderately competent host for NY99, whereas the clay-colored thrush was minimally competent for the Tabasco strain. The 20-fold greater competence of the clay-colored thrush for the Tecate strain was still relatively less than competence for its counterpart from the United States for the NY99 strain and is somewhat contrived because the clay-colored thrush is absent from northwest Mexico where the Tecate strain was isolated. The observed competence of the great-tailed grackle for the Tabasco strain is about as high as or higher than that observed for any species regardless of strain or inoculation method (*8*). This species is likely a major reservoir throughout its range from the central plains of the United States south throughout Mexico and Central America and into Colombia.

Observed differences in quantitative viremia measurements and calculated competence index values between the 2 virus strains from Mexico could not be substantiated by using statistical tests on direct comparisons of like measurements. However, binomial statistics applied across measurement categories did indicate a significant difference. The Tecate strain generally seemed to be more virulent than the Tabasco strain for the avian species examined, except for grackles. One basis for these differing outcomes could be the presence of virulence factors in the viral genome of the Tecate strain that may be absent from the Tabasco strain.

Table 2. Virus shedding by 2 bird species experimentally inoculated with West Nile virus from Mexico*

			Oral shedding			Cloacal shedding		
Species	No.	WNV strain	Peak titer, log PFU/swab†	When detected, dpi	Proportion positive	Peak titer, log PFU/swab†	When detected, dpi	Proportion positive
House sparrow	6	Tecate	5.0	2–7	1.00	1.4	2–3	0.83
	6	Tabasco	7.2	2–6	0.67	4.9	4	NR
Great-tailed grackle	4	Tecate	3.7	2–7	0.75	2.4	3	0.75
	4	Tabasco	6.2	5–6	0.75	4.6	3–7	NR

*dpi, days postinoculation; NR, not reported because of insufficient sample collection.
†Peak titers and end point of detection might be underestimated because of intermittent sample collection.

Table 3. Tissue tropism and viral loads in birds that died after experimental inoculation with WNV, Mexico*

Species†	WNV strain	dpi	Viral load by tissue type, PFU/0.5 cm^3								
			Brain	Heart	Spleen	Kidney	Lung	Liver	Pancreas	Intestine	Skin
House sparrow	Tecate	4	8.0	7.6	NR	NR	9.3	8.1	4.9	<0.7	7.6
	Tecate	5	8.2	7.7	7.6	7.2	9.2	8.0	5.2	3.1	8.0
	Tabasco	6	2.3	2.9	NR	3.6	4.5	5.7	<0.7	<0.7	4.9
	Tabasco	7	<0.7	<0.7	<0.7	1.3	1.9	<0.7	<0.7	<0.7	<0.7
	Tabasco	7	3.1	7.7	4.8	5.9	5.2	5.2	<0.7	NR	NR
Great-tailed grackle	Tecate	4	7.4	8.2	7.5	8.8	9.3	7.6	6.7	<0.7	8.0
	Tecate	12	3.4	5.1	5.5	5.9	5.2	5.8	6.0	3.0	4.9
	Tabasco	3	NR	8.1	3.8	8.6	8.5	7.9	<0.7	NR	NR
	Tabasco	4	8.1	7.7	6.6	7.9	NR	7.4	4.7	2.7	7.8

*WNV, West Nile virus; dpi, days postinoculation; NR, not reported because of insufficient sample collection.
†Deaths occurred in sparrows and grackles but not thrushes and chickens; thrushes and chickens were euthanized on 21 and 7 dpi, respectively, and tissues from these birds were negative for infectious WNV with the exception of 1 chicken-derived skin sample ($10^{2.0}$ PFU/0.5 cm^3).

Genetic determinants associated with high titers of viremia in the American crow (*Corvus brachyrhynchos*) include a glycosylation site on the E protein (*5*,*13*) and an amino acid substitution in the NS3 helicase (*14*). The original, unpassaged Tabasco strain was shown to contain at least 2 genotypes, 1 of which was determined to have reduced virulence and lacks the glycosylation site on the E protein (*5*). However, the passaged Tabasco strain we used to inoculate birds from Mexico retained the E protein glycosylation site. Previous data have indicated the association of glycosylation of the E protein for virulence in mice and hatchling chickens (*13*,*15*,*16*). The absence of this reported virulence factor, as well as the presence of additional potential attenuating mutations in the original unpassaged Tabasco strain, could explain the lack of observed bird deaths in Mexico and Latin America, assuming a widespread circulation of this genotype throughout the region. This attenuated phenotype could be explained by selection for lower virulence in migrating birds (*4*). Migrating birds with WNV infections have been shown to maintain their migratory behavior during the viremic phase of infection (*17*). Therefore, viremic migrating birds that survive long-distance (i.e., trans-Gulf) migration to the Tabasco region may have contributed to selection for mutant, low-virulence genomes. However, after serial passage in vertebrate cells, the Tabasco strain used in this study could have reverted to a more virulent phenotype. As evidence of this potential, previous studies have demonstrated that repeated passage of WNV (Kunjin) has resulted in attainment of a glycosylated phenotype after as few as 2 passages in Vero cells (*18*). The 3 additional amino acid differences (prM-T141I, NS4BV245I, or NS5-I898T) in this Tabasco stock that were identical at these positions to the NY99 avian virulent strain could individually or in combination also impart the enhanced avian virulent phenotype observed in these studies.

Both WNV strains from Mexico were pathogenic, leading to death in birds of 2 species (house sparrows and great-tailed grackles), although the stress of captivity and handling may have exacerbated illness among these birds (as indicated by the death of the 1 sham-inoculated grackle). This result signifies that birds in the tropics are probably dying of WNV infection. Therefore, surveillance of bird deaths from WNV may be useful for early warnings of outbreaks in Mexico, as it has been in the United States (*19*), although challenges include lack of public involvement and rapid disappearance of carcasses. The link between surveillance of bird illness and deaths and emerging zoonotic pathogens such as highly pathogenic avian influenza virus (Asian strain subtype H5N1) (*20*) suggests that a large effort should be made to investigate bird deaths.

From our data, thrushes do not appear to be amplifying hosts of the WNV Tabasco strain. However, house sparrows and great-tailed grackles are highly competent hosts and susceptible to infection and some associated deaths, suggesting that high rates of WNV transmission in the American tropics is being overlooked. Alternatively, infection rates are not high among sensitive species such as house sparrows and great-tailed grackles, or these species are fed upon by vectors at lower rates than expected. Blood meal identification studies of *Culex* spp. mosquitoes have demonstrated that these mosquitoes feed on house sparrows and common grackles (*Q. quiscula*) at a frequency lower than expected from the relative abundance of these avian species (*21*,*22*). The same studies report a strong preference of *Culex* spp. mosquitoes for blood meals from American robins. Because clay-colored thrushes were not highly competent hosts for strains of WNV from Mexico, preference of infected *Culex* spp. mosquito vectors for blood meals from this congener could lead to zooprophylaxis. Relatively high viral loads in tissues of birds infected with Tabasco or Tecate WNV strains (i.e., loads greater than or equal to those of birds infected with WNV NY99) (*12*) could result in higher rates of oral transmission to predatory or carrion-eating vertebrates, even if mosquito-borne transmission is less supported in tropical than in temperate regions. For example, feral dogs and cats, raptors, corvids, and other animals may become orally infected by eating

WNV-infected birds and carcasses (*23*–*25*). In addition, birds from Mexico that are inoculated with Tabasco and Tecate strains shed infectious WNV from oral and cloacal cavities, as did birds infected with the WNV NY99 strain (*12*). Shedding of WNV could serve as an additional source of non–vector-borne transmission.

This study indicates that WNV is probably contributing to deaths of some species of birds in the tropics, where numerous unique bird populations are often geographically isolated because of islands of fragmented habitats. In contrast, high levels of biodiversity, such as those found in the Neotropics, lend themselves to reduced WNV transmission (*26*,*27*). Urban locations are less biodiverse yet colonized with numerous species of birds competent for amplifying WNV, like house sparrows and, even more so, great-tailed grackles. The lack of peridomestic corvid populations in the Neotropics would seemingly contribute to reduced human risk for WNV infection in the region (*28*). However, there could be an alternative, less susceptible, super spreader, as with the American robin in the United States.

Aside from ecological explanations of reduced WNV transmission in the tropics, human factors may ultimately explain the lack of an obvious public health problem. Three major human factors stand out in this regard: 1) the high incidence of secondary flavivirus infections, mainly caused by dengue virus holoendemicity, which may cause high levels of cross-reactive flavivirus-reactive antibodies; 2) the low investment in surveillance and diagnostic services because of the lack of confirmed human cases of West Nile neurologic disease, and finally, 3) the inability of arbovirus reference laboratories to use serologic methods to diagnose WNV-induced neurologic illness in persons with circulating heterologous antibodies against flavivirus. This last possibility raises concerns that WNV might indeed cause a substantial amount of disease in Mexico, as it does in the United States, but it might be difficult to detect.

Acknowledgments

We thank Pedro Paz-Salazar, Carmen Castro, Arturo Campomanes-Cortes, Mario Solis-Hernandez, and Roberto Navarro-Lopez for providing WNV strains from Mexico. We also thank Graciela Velasco Santiago and personnel from Zoológico de Zacango, especially J. Frieventh, for help with bird collection, and G. Young and N. Panella for laboratory assistance. B. Biggerstaff, M. DeLorey, and K. Horiuchi provided statistical advice.

J.G.E.-F. was partially supported by US National Institutes of Health grant NO1-AI25489. S.G.-S. was supported by the Mexican National Research Council of Science and Technology (CONACYT) grant CHIS 2005-C03-075), S.C.-R. by CONACYT-SAGARPA 2003.025, and M.T.T.-O. by Fondo Mixto Chiapas-CONACYT CHIS030755.

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Isolation of Prion with BSE Properties from Farmed Goat

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Transmissible spongiform encephalopathies are fatal neurodegenerative diseases that include variant Creutzfeldt-Jakob disease in humans, scrapie in small ruminants, and bovine spongiform encephalopathy (BSE) in cattle. Scrapie is not considered a public health risk, but BSE has been linked to variant Creutzfeldt-Jakob disease. Small ruminants are susceptible to BSE, and in 2005 BSE was identified in a farmed goat in France. We confirm another BSE case in a goat in which scrapie was originally diagnosed and retrospectively identified as suspected BSE. The prion strain in this case was further characterized by mouse bioassay after extraction from formaldehyde-fixed brain tissue embedded in paraffin blocks. Our data show that BSE can infect small ruminants under natural conditions and could be misdiagnosed as scrapie. Surveillance should continue so that another outbreak of this zoonotic transmissible spongiform encephalopathy can be prevented and public health safeguarded.

Transmissible spongiform encephalopathies (TSEs) are fatal diseases characterized by neurodegenerative changes in the central nervous system that include vacuolation, gliosis, and accumulation of an abnormal isoform (PrP^{Sc}) of a naturally occurring host-encoded protein (PrP^{C}) (*1*). According to the prion hypothesis, PrP^{Sc} is the major or the sole infectious agent (*1*). Although this hypothesis has not received universal acceptance, PrP^{Sc} is ubiquitous in all known naturally occurring TSEs, and its detection is widely used for their diagnosis.

Bovine spongiform encephalopathy (BSE), a TSE of cattle, was first detected in 1986 (*2*) and has since been linked with emerging TSEs in other species (*3*,*4*) including humans (*5*,*6*). Because of its ability to cross species barriers and particularly its zoonotic potential, BSE is considered a public health risk, and extensive measures have been established to detect and eliminate the disease.

Scrapie, a naturally occurring TSE affecting small ruminants, has been known for centuries (*7*) and is not considered to pose a public health risk (*8*). Under experimental conditions, however, small ruminants are susceptible to BSE, with pathogenesis and clinical signs that are not readily distinguishable from scrapie (*9*–*12*). Additionally, the fact that small ruminants were exposed to BSE-contaminated food before the exclusion of meat and bone meal from ruminant feedstuffs led to the possibility that sheep and goats on commercial farms could be affected by BSE that could be misdiagnosed as scrapie (*13*,*14*). The response to this potential risk was the implementation of extensive statutory active surveillance, elimination, and breeding for resistance programs in the European Union (EU).

In 2005, as part of a review of historical TSE-positive cases of sheep and goats in France, a specimen from a goat slaughtered for human consumption in 2002 was reported to be "indistinguishable from a BSE isolate on the basis of all identification criteria available." (*15*). In response to this report, 2 retrospective studies were initiated in the United Kingdom to analyze archived samples from goat cases that were initially diagnosed as scrapie (*16*,*17*). Because only fixed material was available, both studies had to use differential immunohistochemical analysis (D-IHC), a technique that can discriminate scrapie from experimentally induced BSE in sheep (*18*). These studies identified a single case, originally diagnosed in 1990 as scrapie, that had a D-IHC signature indistinguishable from BSE (*16*).

Given the wide phenotypic variance of scrapie in sheep and our limited knowledge of this variance in goats, the

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DOI: http://dx.doi.org/10.3201/eid1712.110333

D-IHC result on its own was insufficient for an unequivocal diagnosis. In accordance with EU regulation 36/2005 (*19*), the case was referred to the EU Reference Laboratory Strain Typing Expert Group, which recommended further investigation by bioassay.

Bioassay is conventionally undertaken by using unfixed tissues to prepare inocula. Much historical tissue is available only as formalin fixed or formalin fixed and paraffin wax embedded. TSE infectivity persists in such material but with a lower infectious titer than with unfixed frozen tissue (*20*). However, the potential effects on biological activity, and therefore strain characterization, of fixation and processing are unknown. Thus, further investigation of this case required an extensive panel of controls. We report the results of the bioassay analysis and confirm the diagnosis of BSE in a goat in the United Kingdom.

Materials and Methods

Sample Preparation

Whenever fixed tissue was used, it had been processed and embedded in paraffin wax. To recover the fixed, paraffin-embedded tissue from the wax blocks, samples were processed in reverse. Specifically, the wax was liquefied by immersing the tissue blocks in a wax bath preheated to 55°C. The samples were then placed in an ether bath and subsequently were immersed in 100% ethanol to remove the ether. This process was followed by sequential washes in alcohol solutions of decreasing concentrations to gradually remove the alcohol and rehydrate the sample. Finally, the samples were suspended in normal saline (10% wt/vol) before homogenization. Unfixed samples were kept frozen at –80°C. After thawing, they were suspended in normal saline (10% wt/vol) before homogenization. All homogenates were examined microbiologically and treated with antimicrobial drugs as required. Only microbiologically cleared inocula were used to challenge animals.

Experimental Design

The only tissue available from the 1990 suspected UK case (*16*) was paraffin wax–embedded brain (supplied by Martin Jeffrey, Veterinary Laboratories Agency, Lasswade, UK). Several sources were used to control for TSE strain, host species, and tissue condition (i.e., frozen vs. fixed and wax-embedded) to ensure unequivocal interpretation of the results (Table). Paraffin wax–embedded material from 2 field cases that were contemporary with the suspected case but gave a scrapie profile on D-IHC (*16*,*17*) and an experimental caprine BSE case (supplied by Nora Hunter, Roslin Institute, Edinburgh, UK) were used to control for strain variation in material that had been handled and stored in the same way and for a similar time as material from the suspected case (*21*).

Additional controls of fixed and frozen brain tissues from the same source were used to assess the effect of fixation, processing, and retrieval on the biological properties of the TSE agents present. All samples included in this study were from animals showing clinical signs of TSE. These came from animals with confirmed TSE sourced through passive surveillance schemes, with the exception of an ovine BSE case that was produced experimentally (*11*). Because the sampling site of the brain may also affect the infectious titer, in addition to the above parameters we identified a bovine BSE case for which whole frozen brain stem was available. Given the left-right symmetry of PrP^{Sc} distribution, which was verified by IHC analysis of the adjacent rostral and caudal coronal levels of the selected sample, we assumed that titer did not vary substantially on either side of the midline. Therefore, the obex was cut sagitally in half. Half was processed histologically and was subsequently recovered and rehydrated to replicate the process applied in the fixed samples; the other half was kept frozen. Each half was homogenized and inoculated into mice.

Each source was administered to 3 panels of wild-type inbred mice (C57/BL6, RIII, and VM) and a transgenic mouse line (tg388 line was provided by Hubert Laude, Institut National de la Recherche Agronomique, Jouy-en-Josas, France). C57/BL6 and RIII mice share the same PrP sequence (PrP-a), but it is believed that RIII alone could be used to discriminate BSE from other TSEs on the basis of lesion profile (LP) data on first passage (*5*), although this belief has been challenged (*22*). VM mice have a different PrP sequence (PrP-b) and have been used to identify BSE after 2 serial passages on the basis of incubation period (IP) and LP data (*23*). The tg338 mouse line overexpresses an ovine VRQ transgene and has been proved to be susceptible to scrapie (*24*,*25*) and relatively resistant to BSE (*26*,*27*).

Serial passage from the suspected case was initiated only in the VM mouse line because subpassage of BSE in this line gives rise to the mouse-adapted BSE strain 301V, which has a characteristically short IP that can be used to discriminate BSE from scrapie (*23*). The acquired data were compared with an experimental ovine BSE case and with a 301V reference strain that were serially passaged in VM mice in different studies.

The number of mice inoculated with each source varied from 5 to 20 for each mouse line depending on availability of material (Table). Serial passages used 10 mice. Where tissue availability was limited, the mouse lines of choice were RIII and tg338.

Animal Procedures

Because of the small amount of available material, only intracerebral inoculations (20 μL of 10% of brain homogenate in normal saline) were performed (*22*,*28*). For secondary passage, VM mice were challenged

intracerebrally with 20 μL of 1% brain homogenate. Mice were monitored for signs of clinical disease and euthanized either at specified clinical endpoints (*29*) or on the basis of animal welfare justification (intercurrent losses). The brains were removed under sterile conditions by using disposable equipment. Each brain was cut parasagittally, and the smaller fraction was frozen for biochemical analysis or serial bioassay; the larger fraction, which included the midline, was fixed in formal saline and processed for histopathologic and IHC analysis. All animal procedures were performed in compliance with the Animal (Experimental Procedures) Act 1986 under license from the UK Home Office and were approved by the local ethics committee.

Histopathologic and IHC Analyses

Sections (3 μm thick) of 4 different coronal levels (frontal, thalamic, midbrain, and medulla) were stained

Table. Bioassay results of first passage (ruminant to mouse) to determine presence of BSE*

Sample source and treatment†	Tissue‡	Mouse line	No. PrP^{Sc}-positive mice/total no. mice inoculated§	Clinically positive and H&E-positive mice	Mean (SD) incubation period postinoculation, d
Caprine BSE suspected (V459/90) (*16*)					
Fixed and embedded	Brainstem and cerebellum	C57/BL6	5/20	0	NA
		RIII	5/20	3	525 (93)
		VM	9/20	6	620 (32)
		tg338	8/10	0	NA
Natural caprine scrapie 1 (E90/89) (*16*)					
Fixed and embedded	Brainstem	C57/BL6	0/20	0	NA
		RIII	0/20	0	NA
		VM	0/20	0	NA
		tg338	6/10	0	NA
Natural caprine scrapie 2 (84/1549) (*17*)					
Fixed and embedded	Brainstem	C57/BL6	18/20	18	506 (26)
		RIII	8/20	2	413 (98)
		VM	15/20	6	586 (87)
		tg338	10/10	5	196 (13)
Experimental caprine BSE (45x48) (*21*)					
Fixed and embedded	Brainstem	C57/BL6	2/20	1	588 (NA)
		RIII	3/20	2	535 (5)
		VM	6/20	4	592 (51)
		tg338	10/10	0	NA
Experimental ovine BSE (PG0341/00) (*11*)					
Fixed and embedded	Medulla	RIII	7/20	6	388 (23)
		tg338	5/5	0	NA
Frozen	Medulla	RIII	6/20	0	NA
		tg338	NA	NA	>785¶
Natural bovine BSE (PG0475/05, UK passive surveillance)					
Fixed and embedded	Medulla	C57/BL6	2/20	1	713 (NA)
		RIII	6/20	5	477 (49)
		VM	13/20	2	578 (35)
		tg338	7/10	0	NA
Frozen	Medulla	C57/BL6	7/20	3	535 (76)
		RIII	10/20	9	431 (29)
		VM	16/20	3	568 (31)
		tg338	6/10	0	NA
Natural ovine scrapie (PG2413/98, UK passive surveillance)					
Fixed and embedded	Medulla	RIII	0/20	0	NA
		tg338	5/5	3	97 (2)
Frozen	Medulla	RIII	9/20	8	490 (31)
		tg338	10/10	10	64 (2)

*BSE, bovine spongiform encephalopathy; PrP, host-encoded prion protein; H&E, hematoxylin and eosin; NA, not applicable; UK, United Kingdom.
†Animal identification numbers are indicated in parentheses and, where available, references are supplied.
‡Brainstem inocula were prepared by pooling separate samples of brainstem (including medulla, midbrain, and thalamus) retrieved from paraffin wax blocks.
§Positive for PrP^{Sc} by using immunohistochemical analysis.
¶Postinoculation time, after which 2 of 10 inoculated mice were still alive at the time of writing; 5 of the 8 mice that died were PrP^{Sc} positive when examined by using immunohistochemical analysis.

with hematoxylin and eosin according to standard methods (*22*,*30*). Positive histologic diagnosis was based on the identification of TSE-related vacuolation. The intensity of vacuolation in 9 gray matter areas was assessed semiquantitatively, and the resultant scores were plotted against the respective brain areas as described (*22*,*30*,*31*).

For IHC evaluation, each section was labeled with a rabbit polyclonal antibody against PrP (Rb486) according to established methods (*22*,*28*). The distribution of different PrP^{Sc} types in the rodent brain at primary passage can provide a means of identifying different strains in wild type (*22*,*28*) and transgenic mice (*32*). By examination of immunolabeled sections, different PrP^{Sc} deposits were identified and their distribution in different neuronatomic brain areas recorded as described (*22*,*28*).

Western Blotting Analysis

Western blotting (WB) was applied only for PrP-a mice (C57/BL6 and RIII) because PrP-b mice inoculated with either scrapie or BSE produce similar banding profiles and cannot be distinguished by this approach (*33*). Brain homogenates (10% wt/vol for murine samples and 20% wt/vol for ruminant controls) were prepared by using ribosylation tubes (Bio-Rad Laboratories, Hercules, CA, USA). Dilutions of TSE-positive brain homogenates were prepared in known TSE-negative brain homogenates of the same mouse strain. Each brain homogenate was subjected to proteinase K digestion as directed by the manufacturer (Bio-Rad Laboratories) and subsequently prepared for analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and WB according to the TeSeE WB protocol (Bio-Rad Laboratories) with detection of PrP^{Sc} by SHA31, epitope ^{148}YEDRYYRE^{155} (proprietary kit reagent) and12B2 (provided by Jan Langeveld, Central Veterinary Institute, Lelystad, the Netherlands), epitope ^{93}WGQGG^{97} (0.2 μg/mL) and 12% BisS/Tris (Criterion) acrylamide gels (Bio-Rad Laboratories) in 3-(N-morpholino)propanesulfonic acid buffer. Relative band mass was measured by using Quantity One software (Bio-Rad Laboratories).

Results

Attack Rate and IP Analysis

As anticipated (*20*), the use of fixed tissue had a negative effect on attack rate (AR) and IP irrespective of TSE source or mouse line (Table). The most reliable data, suggesting that fixation causes a decrease in titer, are those relating to the bovine BSE, where not only the same source but also the same neuroanatomic region was sampled because of the symmetric distribution of PrP^{Sc} with respect to the midline of the brainstem.

The IP of RIII and VM mice inoculated with material from the goat with suspected BSE were similar to that of the experimental caprine BSE control, but the value of this comparison and that of the other controls was limited because of the generally low ARs observed and the proportion of positive mice that did not progress to show clinical disease. However, because none of the C57/BL6 or tg338 mice inoculated with fixed brain from the goat with suspected BSE showed development of clinical signs of TSE, comparisons of IP in these recipients could not be made.

LP and Histopathologic Analysis

It is generally accepted that, during first passage, LPs from RIII mice can be used to discriminate BSE from scrapie (*5*), though this principle has been challenged (*22*). An LP is considered to be reliable when ≥5 clinically and pathologically positive mice contribute to the evaluation (*22*,*28*). Although the ovine and bovine BSE controls fulfilled this requirement, few of the goat-derived samples complied (Table). Therefore, LPs could not be used with any confidence to classify the suspected case.

However, the histopathologic lesions observed individually in all clinically positive mice that were inoculated with material from the caprine BSE-suspected case were consistent with those observed in the known BSE controls, irrespective of whether they came from fixed or frozen tissue, and with previous studies of BSE isolates (*22*,*30*). In addition, they were distinct from the lesions observed in the scrapie controls. These BSE-specific lesions included minimal vacuolation in the ventral midbrain and the cerebellum, characteristic vacuoles in the trigeminal nerve nucleus, and confluent vacuolation in the dorsal cochlear nuclei in PrP-a mice (Figure 1) as described elsewhere (*5*,*22*,*30*).

IHC Analysis

Samples from the goat with suspected BSE and samples from the experimentally BSE-infected goat and experimentally infected sheep generated equivalent PrP^{Sc} distribution patterns in PrP-a mice (Figure 2, panels A–F), which were clearly distinct from the PrP^{Sc} patterns generated by goat scrapie in the same mice (Figure 2, panels G and H). In PrP-b mice, the suspected goat was also indistinguishable from BSE and distinct from scrapie (Figure 3). The BSE-associated PrP^{Sc} distribution pattern was identified in all mice that were inoculated with either frozen or fixed BSE tissues from various sources (Table), suggesting that the histologic processing and suboptimal storage conditions of the archived samples do not alter the biologic properties of the agent. These BSE-related patterns were distinct from the classical scrapie samples that were analyzed here or reported (*22*,*25*).

Clinical stage TSE (Table) did not develop in any of the BSE-challenged tg338 mice. Therefore, the distribution

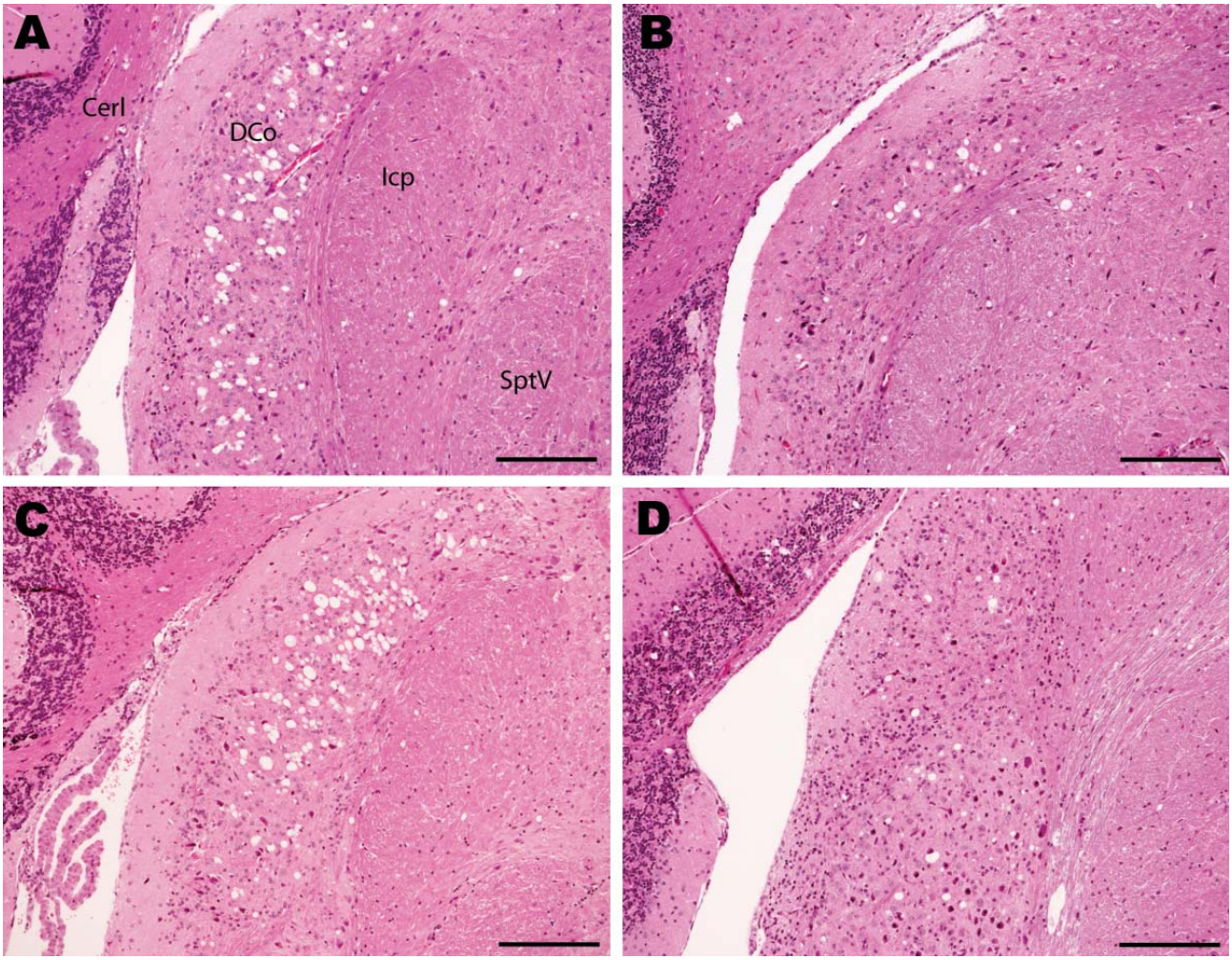

Figure 1. Histopathologic analysis of cochlear nuclei from host-encoded prion protein (PrP)-a mice (C57/BL6) inoculated with (A) fixed material from the suspected case, (B) fixed material from experimental goat bovine spongiform encephalopathy (BSE), (C) unfixed material from experimental sheep BSE, and (D) fixed material from experimental goat scrapie. The BSE-challenged mice (A–C) show confluent vacuolation in the dorsal cochlear nucleus that extends ventrally with increasing lesion severity. Even in mild cases (B) this lesion can be distinguished from the low-frequency randomly dispersed vacuoles observed in scrapie (D). Note the unaffected nature of the lesion between fixed (A and B) and unfixed (C) samples. Cerl, cerebellum; DCo, dorsal cochlear nucleus; Icp, inferior cerebellar peduncle; SptV, spinal tract of the trigeminal nerve. Scale bars = 200 μm.

of spongiform lesions and PrP^{Sc} deposits in tg338 mice in which BSE was diagnosed was limited, and intensity of the labeling was weak. Despite this finding, where PrP^{Sc} distribution was widespread, individual mice challenged with BSE differed qualitatively from those challenged with scrapie (data not shown).

WB Analysis

When examined by WB, brain tissues from PrP-a mice that were inoculated with the sample from the goat with suspected BSE showed PrP^{Sc} bands that were indistinguishable from those of mice inoculated with the various BSE sources (Figure 4). The lower unglycosylated band had a molecular mass of ≈18.8 kDa, and the samples demonstrated lower binding with the 12B2 antibody, confirming that the proteinase K cleavage site was indistinguishable from that of ovine BSE. In contrast, mice inoculated with various scrapie sources demonstrated a 20.1-kDa unglycosylated band and increased reactivity with 12B2 (Figure 4).

Secondary Passage Data in PrP-b Mice

After 1 serial passage in PrP-b (VM) mice, the sample from the goat with suspected BSE generated IP of 128 ± 4 (mean ± SD) days postinoculation similar to serially passaged ovine BSE (109 ± 4) and the 301V mouse-adapted BSE strain (107 ± 6). The comparatively longer IP generated by that goat sample relative to these mouse-

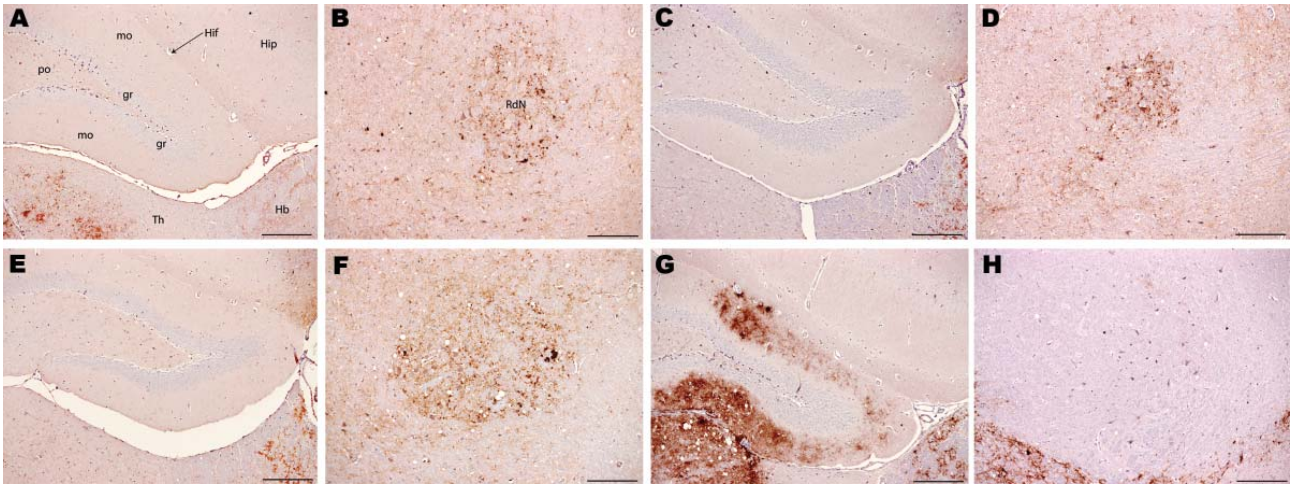

Figure 2. Immunohistochemical analysis of brains of host-encoded prion protein (PrP)-a mice (RIII) inoculated with (A and B) fixed material from the goat with suspected bovine spongiform encephalopathy (BSE), (C and D) fixed material from experimental goat BSE, (E and F) unfixed material from experimental sheep BSE, and (G and H) fixed material from experimental goat scrapie. No PrP^{Sc} was detected in the molecular layer of the dentate gyrus in the suspected case (A) and the BSE controls (C and E); in the scrapie control (G) the same area is heavily affected. In the red nucleus, small PrP^{Sc} aggregates were observed in the suspected case (B) and in the BSE controls (D and F), whereas the same nucleus seem to be unaffected in the scrapie control despite evident PrP^{Sc} deposits in the surrounding area. Hip, hippocampus; Hif, hippocampal fissure; Hb, habenular nuclei; RdN, red nucleus; Th, thalamus; gr, mo, and po, granular, molecular, and polymorph layers, respectively, of the dentate gyrus. Scale bars = 200 μm.

adapted BSE isolates is a common observation at second passage; for example, the IP of the serially passaged ovine BSE at second passage was 148 ± 3 days postinoculation. In these mice, the LPs were indistinguishable from those produced by serially passaged experimental ovine BSE and similar to the 301V strain (Figure 5). After serial passage of material from the goat suspected to have BSE in VM mice, the PrP^{Sc} patterns observed were indistinguishable

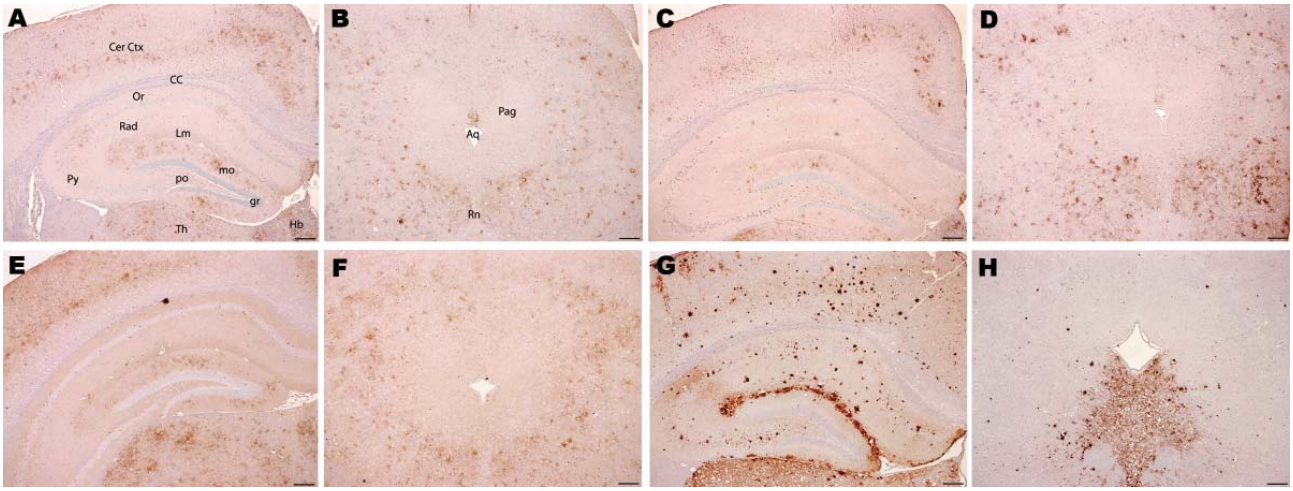

Figure 3. Immunohistochemical analysis of brains of host-encoded prion protein (PrP)-b mice (VM) inoculated with (A and B) fixed material from the goat with suspected case, (C and D) fixed material from experimental goat bovine spongiform encephalopathy (BSE), (E and F) unfixed material from experimental sheep BSE, and (G and H) fixed material from experimental goat scrapie. In thalamus, cerebral cortex, and hippocampus the suspected case (A) and the BSE controls (C and E) showed mainly granular PrP^{Sc} deposits with comparable distribution. In the scrapie control (G), the predominant PrP^{Sc} type was large aggregates and plaques. In the periaqueductal gray matter, mice challenged with the suspected case (B) and with BSE controls (D and F) showed a manifold lower staining intensity of PrP^{Sc} labeling compared with the surrounding area, but the scrapie control (H) showed an intense labeling in the ventral periaqueductal region associated with substantial reduction of labeling in all neighboring areas. Aq, aqueduct; CC, corpus callosum; Cer Ctx, cerebral cortex; Hb, habenular nuclei; Rn, raphe nucleus; Pag, periaqueductal gray; Th, thalamus; Or, Py, Rad, and Lm, oriens, pyramidal cell, radiatum, and lacunosum-moleculare layers, respectively, of the hippocampus; gr, mo, and po, granular, molecular, and polymorph layers, respectively, of the dentate gyrus. Scale bars = 200 μm.

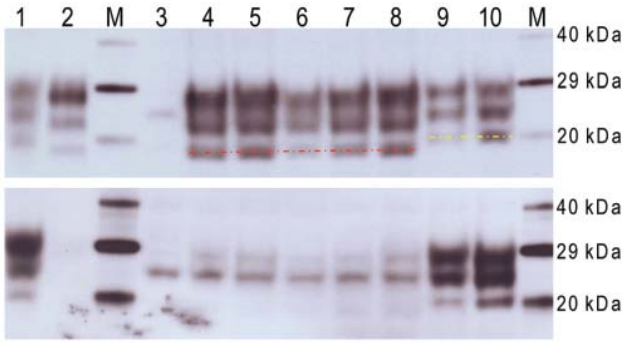

Figure 4. Western blot analysis of a range of murine transmissible spongiform encephalopathy–affected brain homogenates in host-encoded prion protein (PrP)–a (RIII) mice. A) Western blot probed with SHA31, 15-s exposure time. B) Western blot probed with 12B2, 5-min exposure time. M, biotinylated marker; lane 1, ovine scrapie field case; lane 2, bovine spongiform encephalopathy (BSE) field case; lane 3, unchallenged mouse; lane 4, bovine BSE-challenged mouse; lane 5, ovine BSE-challenged mouse; lane 6, caprine BSE-challenged mouse; lanes 7 and 8, mice challenged with suspected case; lane 9, caprine scrapie-challenged mouse; lane 10, ovine scrapie-challenged mouse. Molecular weights are indicated kDa. Red line indicates 19 kDa unglycosylated band; yellow line indicates 20 kDa unglycosylated band. Identical results were also obtained with C57/BL6 mice.

from those induced by other mouse-adapted BSE isolates (data not shown).

Discussion

We confirmed that the agent responsible for TSE in a UK goat, which was initially reported as scrapie in 1990 and subsequently as suspected BSE in 2006 (*16*), was a BSE agent. This conclusion was based on bioassay of nervous tissue in mice demonstrating similarities of histopathologic lesions, PrP^{Sc} mapping in the brain, and WB of PrP^{Sc} with those of mice inoculated with BSE from various ovine, caprine, and bovine sources.

From a method perspective, the data suggest that AR, IP, and LP are not optimal bioassay parameters for differentiating TSE sources during first passage because they represent mean values derived from a group of animals that have been inoculated with a specific source. Therefore, a substantial number of animals must die of clinical TSE for these parameters to be meaningful. This finding is a limiting factor in instances in which TSE is diagnosed in only a few animals because of low titer, restricted permissiveness of specific TSE strains in certain laboratory animals, or both. These limitations can be overcome by application of IHC and WB to differentiate BSE from scrapie confidently in individual mice on first passage. Use of IHC has shown that different PrP^{Sc} deposits can be identified, and the distribution of each deposit in the brain can be mapped (*22*,*28*,*32*). This approach generates high-resolution data that appear to be specific to individual TSE strains.

The data show that the TSE agents in this study were not altered by the adverse conditions applied to them during histologic procedures. However, titer may decrease, suggesting that the effect of histologic processing is quantitative not qualitative. Therefore, bioassay is a valid approach for identifying BSE in archived histologic material when other techniques are not applicable, as in the current study. Regarding the suitability of different mouse lines for confirming BSE, our data show that any mouse line in which the agent can propagate sufficiently is suitable. An additional requirement at a practical level is the ability to characterize the agent on first passage. In this respect, use of PrP-a mice is preferable because in addition to AR, IP, histopathologic analysis, and PrP^{Sc} patterning, WB can also be applied to diagnose BSE. In contrast, its application in PrP-b mice is less informative (*33*).

These methods can also be applied to analyze bioassay data derived from validated transgenic mouse lines that offer the advantage of higher AR and decreased IP, provided that appropriate transgenic lines are selected and the TSE source and the donor species under investigation are taken into consideration. In this particular instance, our first choices would have been the use of a mouse line overexpressing a bovine transgene in combination with 1 that overexpresses a caprine transgene. At initiation of the

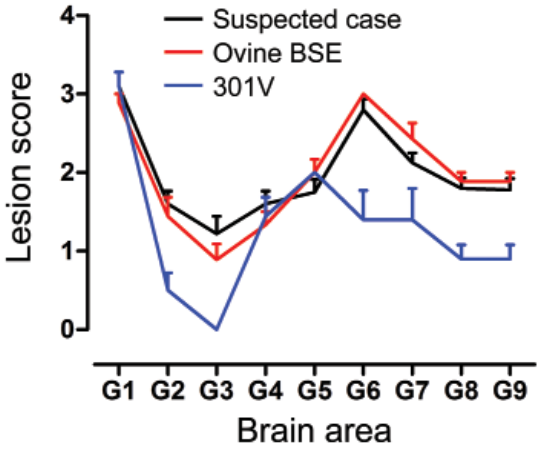

Figure 5. Lesion profiles from VM mice after second passage of the suspected case, serial passage of an ovine bovine spongiform encephalopathy (BSE) source, and a 301V control. Profiles were made on the basis of the lesion score, which is the quantification of transmissible spongiform encephalopathy–specific vacuolation in 9 neuroanatomical gray matter areas: G1, dorsal medulla nuclei; G2, cerebellar cortex of the folia including the granular layer, adjacent to the fourth ventricle; G3, cortex of the superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septal nuclei of the paraterminal body; G8, cerebral cortex (at the level of G4 and G5); G9, cerebral cortex (at the level of G7). At least 9 clinically and histopathologically positive mice contributed to each profile. Error bars indicate SEM.

study, an established bovinised line was not available to us, and the data generated from the wild-type mice were considered sufficient to identify unequivocally the agent strain. Caprine transgenic mouse lines are still under development and not characterized or widely available. Instead, we used tg338 mice although they show <100% AR and extended IP when inoculated with BSE (*26*,*27*). Our data show that this ovinized line offers a feasible alternative for detecting and differentiating caprine TSEs.

The 2 cases of naturally occurring BSE in small ruminants—the 1 reported here and the 1 identified in France (*15*)—occurred in different countries, during different time periods, and before strict BSE control measures were fully implemented. Therefore, the most likely origin of these 2 cases would be exposure to BSE-contaminated food supplements. Although in France goats constitute 14.3% of the small ruminant population, in the United Kingdom they account for only 0.3% of small ruminants. It is intriguing, therefore, that the only naturally occurring BSE cases in small ruminants in France and particularly in the United Kingdom were detected in goats and not in sheep, although they have also been exposed to contaminated food supplements. A possible explanation could be that goats are generally managed more intensively than sheep and thus might have been exposed to higher doses of the infectious agent because of the more frequent use of concentrates in intensive dairy farming. Similar observations have been reported in cattle, in which the incidence of BSE was significantly higher in dairy herds and in which management is much more intensive than in beef herds (*34*). In the United Kingdom, most of the commercial goat herds are kept for milk production in a typically intensive production system, similar to dairy cattle.

The BSE case we have confirmed was 1 of 26 historic goat samples examined in the United Kingdom collected during 1984–2002 (*16*,*17*). Since 1993, scrapie in goats has been a notifiable disease in the United Kingdom, and since 2005, samples from all suspected cases of TSE in small ruminants are required to be tested for BSE-like features by using WB (*19*). No BSE cases have been identified, although an intermediate case in a goat was reported and is under investigation by bioassay for final resolution (*35*,*36*). This screening of brain samples from all small ruminant cases offers reassurance that BSE is not present in the contemporary small ruminant population. However, application of WB to sheep experimentally co-infected with BSE and scrapie detected only the scrapie agent (*37*). Also, in contrast to cattle, where infectivity is mainly confined to the nervous system, in small ruminants the BSE agent is widely distributed in peripheral tissues and can be transmitted horizontally (*11*,*38*). Therefore, feed ban measures alone would be inadequate to control a BSE outbreak in small ruminants. Also, it would be impossible to prevent BSE from entering the human food chain through consumption of food products derived from small ruminants.

Because TSEs in goats are still a problem, particularly in Mediterranean countries, our data suggest that extensive surveillance and breeding schemes must remain in place to prevent a BSE outbreak in small ruminants and to safeguard public health. This report also highlights several issues regarding the use of mouse bioassay to identify TSE strains. As governing bodies seek confirmation of equivocal cases that are identified worldwide, they must be aware of the limitations, cost, and timescale demands of confirming such cases.

Acknowledgments

We thank John Sheehan for tissue retrieval from wax-impregnated tissue blocks; Angel Ortiz-Pelaez for epidemiologic assistance; histopathology employees at Veterinary Laboratories Agency for expert technical support in histopathology and immunohistochemistry; and Animal Services Unit employees at Veterinary Laboratories Agency for expert support with animal procedures and care.

This work was supported by a Department of Environment, Food and Rural Affairs grant (project SE1849).

Dr Spiropoulos is a veterinary researcher at Veterinary Laboratories Agency with a particular interest in animal pathology. He is the head of the Mouse Bioassay Team that specializes in pathology of experimental animals. His research interests include neurodegenerative disorders and animal diseases of policy relevance, particularly zoonoses.

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Candidate Cell Substrates, Vaccine Production, and Transmissible Spongiform Encephalopathies

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Transmissible spongiform encephalopathy (TSE) agents have contaminated human tissue–derived medical products, human blood components, and animal vaccines. The objective of this study was to determine the potential susceptibility to infection of 5 cell lines used or proposed for manufacture of biological products, as well as other lines. Cell lines were exposed to the infectious agents of sporadic and variant Creutzfeldt-Jakob disease and bovine spongiform encephalopathy (BSE). Exposed cultures were tested for TSE-associated prion protein (PrP^{TSE}) and TSE infectivity by assay in rodents and nonhuman primates. No PrP^{TSE} or infectivity has been detected in any exposed cell line under study so far. Animals inoculated with BSE brain homogenate developed typical spongiform encephalopathy. In contrast, animals inoculated with cells exposed to the BSE agent remained asymptomatic. All cell lines we studied resisted infection with 3 TSE agents, including the BSE agent.

Transmissible spongiform encephalopathies (TSEs or prion diseases) are a heterogeneous group of fatal neurodegenerative diseases that affect animals and humans. TSEs can be sporadic, transmitted iatrogenically, or expressed as familial disorders. TSEs include scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease in cervid ruminants, and mink encephalopathy. In humans the most common TSEs are sporadic, familial, and variant Creutzfeldt-Jakob disease (sCJD, fCJD, and vCJD, respectively). TSEs are characterized by the accumulation in the central nervous system and, less often, in lymphoid tissues of TSE-associated prion protein (PrP^{TSE}), a conformational variant of a normal host cellular prion protein (PrP^{C}). PrP^{C} is a nonessential protein but, at least in mice and cows, must be expressed by animals susceptible to TSE infection. There is compelling evidence that the BSE agent has infected humans, causing vCJD. Most cases of vCJD are attributed to exposure to contaminated beef products (*1*–*3*). In addition, vCJD infections have been transmitted by transfusions of nonleukoreduced erythrocyte concentrates and by a human-derived coagulation factor (factor VIII) (*4*–*6*).

The conclusion that PrP^{TSE} is central to the pathogenesis of TSE is based on the temporal and anatomic correlations between accumulation of PrP^{TSE} and the development of pathologic changes in tissues of the central nervous system (*1*). However, TSEs can develop in the absence of detectable PrP^{TSE} and, conversely, PrP^{TSE} might accumulate without causing either clinical illness or the neuropathologic alterations typical of TSEs (i.e., a progressive fatal illness with spongiform degeneration of the brain) (*7*,*8*). In short, the molecular basis of TSE infection and the role of PrP^{TSE} (unquestionably important in pathogenesis of TSEs) are not yet entirely clear, and both remain key issues in TSE research (*9*,*10*). The standard assay for detecting a TSE agent remains bioassay in susceptible animals.

Many investigators once believed that TSE agents infected mainly, if not exclusively, cells of neuronal and lymphoid lineages. It has become clear, however, that the susceptibility of cells to infection with TSE agents cannot be reliably predicted either from their tissue of origin or level of expression of PrP^{C} (*11*,*12*). Studies showing that murine fibroblast cell lines are susceptible to infection with mouse-adapted scrapie agent (*11*,*12*) increased concern that nonneuronal cell substrates used to propagate viruses

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DOI: http://dx.doi.org/10.3201/eid1712.110607

for vaccine production might become infected with a TSE agent contaminating some component of culture medium, especially bovine serum (*13*). The theoretical risk of contaminating vaccines or other biologic products prepared in culture cells with TSE agents from animal-derived materials in media has been considered low. However, 1) as noted above, the blood of asymptomatic humans has transmitted vCJD, and 2) in a variety of experimentally TSE-infected animals, TSE agent has been detected in blood, mainly in nucleated cells and plasma (*4–6,14–17*). Fortunately, no human vaccine has ever been implicated as a source of iatrogenic TSE. However, 2 animal tissue–derived vaccines have caused outbreaks of scrapie in sheep, and 2 medical products of human origin—dura mater allograft and human cadaveric pituitary hormones (no longer marketed in the United States)—have transmitted hundreds of cases of CJD; corneal grafts have transmitted a few cases as well (*2,18*). Since 1993, the US Food and Drug Administration has recommended against the use in the manufacture of biological products of bovine-derived materials from countries identified by the US Department of Agriculture (USDA) as having BSE or being at increased risk for BSE in native cattle (*19*).

The recognition of >20 BSE cases in North America since 2003 (most in Canada) has increased the need to determine whether cell substrates that might be accidentally exposed to the BSE agent are capable of acquiring and propagating the infectious agent and potentially transmitting infections to vaccine recipients (*20*). To address these issues, we investigated the susceptibility of cell lines used or proposed for manufacture of biologics and controls to propagate TSE agents, especially the BSE agent, under simulated worst-case conditions.

Materials and Methods

Cell Cultures Exposed to TSE Agents

We studied the following 5 actual or candidate cell substrates used or proposed for production of biologic products: CHO-K1 (Chinese hamster ovary ATCC-CCL61); Vero C1008 (African green monkey kidney ATCC-CRL1586); WI-38 (human lung diploid fibroblasts ATCC-CCL75); MDCK (dog kidney ATCC-CCL34); and HEK-293 (human embryonic kidney ATCC-CRL-1573 transformed with defective adenovirus as a surrogate for PER.C6) (Crucell, Leiden, Netherlands) (Table 1).

We used bovine-derived cell lines MBDK (bovine kidney, ATCC-CCL22); EBTR (bovine trachea, ATCC-CCL44); BT (bovine turbinate, ATCC-CRL1390); BCE C/D-1b (bovine cornea, ATCC-CRL2048); and BL3.1 (bovine B lymphocytes ATCC-CRL 2306) in an attempt to develop a cell culture assay for BSE agent. As a probable negative control, we used R9ab (rabbit fibroblasts ATCC CCL-193) known to resist infection with the scrapie agent. Finally, we used NIH-3T3 (ATCC-CRL1658) and L929 (ATCC-CCL1), both mouse embryo fibroblast cells, known to be infectable with the mouse-adapted 22L strain of scrapie agent as positive controls (*11*) (Table 1).

Table 1. Cell cultures exposed to transmissible spongiform encephalopathy agents and propagated for 30 passages*

Variable	sCJD	vCJD	BSE	22L-scrapie
Actual or candidate cell lines for vaccine production				
CHO-K1	Y	Y	Y	
Vero C1008	Y	Y	Y	
WI-38	Y	Y	Y	
MDCK	Y	Y	Y	
HEK-293	Y	Y	Y	
Other cells resistant to TSE infection				
MBDK			Y	
EBTR			Y	
BT			Y	
BCE C/D-1b			Y	
BL3.1			Y	
R9ab	Y	Y	Y	
Cells infectable with 22L scrapie				
Mo3F4-3T3				Y
L929				Y

*sCJD, sporadic Creutzfeldt-Jakob disease; vCJD, variant Creutzfeldt-Jakob disease; BSE, bovine spongiform encephalopathy; CHO-KI, Chinese hamster ovary; Vero C1008, African green monkey kidney; WI-38, human lung diploid fibroblasts; MDCK, dog kidney; HEK-293, human embryonic kidney; MBDK, bovine kidney; EBTR, bovine trachea; BT, bovine turbinate; BCE C/D-1b, bovine cornea; BL3.1, bovine B lymphocytes; R9ab, rabbit fibroblasts; Mo3F4–3T3, mouse embryo fibroblasts; L929, mouse embryo fibroblasts; Y, experiment was completed.

TSE Agents

BSE, vCJD, and sCJD agent inocula were 1% suspensions (wt/vol). Controls were similar uninfected brain suspensions. CJD-infected human brain suspensions were World Health Organization (WHO) Candidate Biologic Reference CJD Materials prepared in 0.3 mmol/L sucrose (*21*). The infectivity titer for vCJD (WHO 98–145) in transgenic mice expressing the human PrP gene (TgHu) was 6.1 $\log_{10}$ intracerebral inoculation (ic) with 50% infectious dose (ID_{50}) per 30 μL; sCJD (WHO 97–008) was 5.4 $\log_{10}$ ic ID_{50}/30 μL; and sCJD (WHO 99–009) was 6.1 $\log_{10}$ ic ID_{50}/30 μL intracerebral inoculation (L. Cervenakova, unpub. data).

The BSE-infected bovine brain was a 10% brain suspension in 250 mmol/L sucrose. The infectivity titer of that material is being determined in squirrel monkeys (>10^2/inoculum [*22*]) intracerebral 150 μL and intraperitoneally 150 μL with 10^{-1} (wt/vol) through 10^{-9} dilutions of low speed–clarified brain extracts. Nonhuman primates were also inoculated with a 10^{-2} dilution of brain extract after filtration through a 0.45-μm Millipore membrane to eliminate bacterial contamination. Three animals each

were inoculated with the BSE agent dilution 10^{-1} to 10^{-6}; 2 animals each were inoculated with the same material dilution 10^{-7} to 10^{-9}. Similar bacteria-free samples were used to expose cell substrates. The ic infectivity titer of the BSE agent in transgenic mice expressing the bovine PrP gene (TgBo) was 5.0 $\log_{10}$ LD_{50}/30 μL (L. Cervenakova, unpub. data). Brain extracts from animals with neurologic signs contained PrP^{TSE} by Western blot (WB) analysis, and TSE was confirmed neuropathologically. All BSE experiments were performed under BioSafety Level 3 containment conditions in facilities inspected and approved by the USDA. Scrapie agent used as a positive control was the 22L mouse-adapted strain (*11*).

Inoculation of Cells with TSE Agents

Cells were grown in 25-cm^2 tissue culture flasks and overlaid with 200 μL of a detergent-free homogenate of brain tissue diluted to 1% (wt/vol) in Opti-MEM (Gibco, Grand Island, NY, USA). Bacteria-free inocula were obtained by filtration of 1% brain extracts through premoistened 0.45-μm Millipore membranes (Millipore, Billerica, MA, USA). In an attempt to mimic a worst-case scenario, in some instances cells were exposed to brain extracts by gently centrifuging them in 25-cm^2 tissue culture flasks for 5 min at ≈900 × *g* to ensure maximum contact with TSE agents. After incubation of the cell cultures at 37°C for 4 h, 400 μL of fetal bovine serum/Dulbecco modified Eagle medium was added, and cells were incubated for 96 h before further passaging. To determine the presence of PrP^{TSE} in cells, WB analyses were done, usually at passages 0, 5, 10, 15, 20, and 30. The same protocol was used with NIH-3T3 and L929 murine cells exposed to mouse-adapted 22L scrapie agent (as positive controls).

Animal Models

TgHu and TgBo mice were developed at the American Red Cross (L. Cervenakova, unpub. data). TgHu mice express in the brain ≈4-fold higher levels of PrP than wild-type mice. TgBo mice express in the brain wild-type levels of PrP. These Tg mice do not develop spontaneous neurologic illness. Neuropathologic studies of selected aged mice showed no abnormalities such as vacuolation of the neuropil typical of TSEs. Conventional C57/BL6 mice were used to titrate 22L scrapie agent. Squirrel monkeys were purchased from Osage Research Primates (Kaiser, MO, USA) and housed in a USDA-approved BioSafety Level 3 animal facility. In a separate series of experiments, a work in progress, we are addressing the hypothesis, postulated by some authorities (*13*), that a TSE agent might develop spontaneously in cell cultures expressing mutated or nonmutated PrP. To that end, we are investigating whether transfected cell lines overexpressing wild-type or mutant PrP might become spontaneously infectious. Transfected cells are being bioassayed in squirrel monkeys. Although none of those monkeys showed development of any neurologic disease (7 years after inoculation), 3 animals have died of unrelated causes (1 with pneumonia and 2 culled with unexplained nontuberculous granulomatosis) without evidence of TSE neuropathologic changes. We used tissues of those 3 monkeys as negative controls. All animal experiments were reviewed and approved by the Food and Drug Administration and the American Red Cross Institutional Animal Care and Use Committees.

Preparation of Inocula for Bioassay in Mice and Primates

Samples of selected cultures at various passage levels and the final subcultures after ≥30 passages were examined for PrP^{TSE} by WB by using published protocols (*14*). Tg mice were inoculated intracerebrally with 30 μL of cell lysates from 1×10^8 cells/mL to 2×10^9 cells/mL (depending on the cell line) by 3 cycles of freezing and thawing followed either by sonication or by forcing through hypodermic needles of increasingly small gauge. Tg mice were inspected daily for signs of illness and euthanized either after illness developed or at the end of a normal expected lifespan (Tables 2, 3). The same neuropathologist, blinded to the experimental design, interpreted all stained sections used for neuropathologic studies. The same cell lysates were also inoculated into primates, 150 μL intracerebrally and 150 μL intraperitoneally. Animals were inspected daily for signs of illness and promptly euthanized when definite neurologic signs or intercurrent illnesses developed. Animals without clinical illness will be maintained for their expected normal lifespans. After euthanasia, brains were removed and portions stored frozen for WB (*22*) or in 10% formalin solution for histopathologic and immunohistochemical studies by using published protocols (*8*).

Results

TSE in TgBo Mice and Primates Inoculated with BSE agent

Neuropathologic characterization of TgBo mice inoculated with BSE agent showed spongiform degeneration in the cerebrum with variable amounts of fine-punctate, coarse, and, in some cases, plaque-like deposits in the cerebrum, cerebellum, and brain stem. Mice inoculated with the bacteria-free filtrate of 1% BSE-infected brain suspension used to expose cells also developed signs of TSE, and PrP^{TSE} was detected in brains, demonstrating that the inoculum used to expose cell cultures contained a TSE agent transmissible to mice (Figure 1).

Squirrel monkeys were inoculated with serial dilutions of the same material used to inoculate TgBo mice. At the time

Table 2. Cell lines exposed to BSE or vCJD or normal bovine or human brain suspension and bioassayed in TgBo mice*

Cell line	No. mice BSE	NB	dpi	No. mice vCJD	NB	dpi
CHO	21	9	720	17	9	730
Vero	20	10	730	ND	ND	ND
WI-38	23	10	730	ND	ND	ND
R9ab	18	9	630	ND	ND	ND
MDCK	19	10	730	20	10	730
HEK-293	20	20	730	ND	ND	ND
Mo3F4-3T3	17	10	750	ND	ND	ND

*BSE, bovine spongiform encephalopathy; vCJD, variant Creutzfeldt-Jakob disease; NB, normal brain; dpi, days postinculation at cull; ND, not done; CHO, Chinese hamster ovary; Vero, African green monkey kidney; WI-38, human lung diploid fibroblasts; R9ab, rabbit fibroblasts; MDCK, dog kidney; HEK-293, human embryonic kidney; Mo3F4-3T3, mouse fibroblasts.

this report was written, 6 monkeys have already developed neurologic signs typical of TSE. Three animals inoculated with 10^{-1} (10%) unfiltered low-speed clarified BSE reference material became ill and were euthanized ≈3.2 years after inoculation; 2 primates inoculated with 10^{-2} unfiltered, low speed–clarified BSE suspension were euthanized 3.7 years after inoculation; and 1 primate inoculated with the 0.45-μm filtered bacteria-free 10^{-2} (1%) BSE-infected brain suspension used to expose cells also developed signs of TSE and was euthanized 3.3 years after inoculation. Brain extracts from each of these monkeys contained PrP^{TSE} demonstrated by WB. Preliminary neuropathologic studies of formalin-fixed paraffin-embedded brain sections of each of the 6 animals showed severe spongiform degeneration of the cerebrum, cerebellum, and brainstem. Immunohistochemical studies showed widespread accumulations of PrP-immunopositive deposits throughout the brain of each monkey (Figure 2). Control brains from 4 other squirrel monkeys dying of nonneurologic diseases, including 1 housed with monkeys used to titrate BSE agent, showed no evidence of TSE (Figure 2). A detailed neuropathologic report of all monkeys is in press (*22*).

Attempts to Infect Actual or Candidate Cell Substrates

Analysis of Cell Cultures and Bioassay in Rodents

No PrP^{TSE} was detected in cells exposed to normal brain extracts (controls) or after passage 5 in any of the cell substrates or control TSE-resistant cells exposed to brain extracts containing TSE agents. PrP^{TSE} was detected in some samples of cells collected 96 h after inoculation (passage 0), suggesting the probable presence of residual inoculum. The consistent failure to detect PrP^{TSE} in any cell line exposed to TSE agents after ≥5 passages suggests that proteinase K–resistant PrP was not generated de novo

Table 3. Cell lines exposed to sCJD or normal human brain suspension and bioassayed in TgHu mice*

Cell line	No. mice sCJD	NB	dpi
Vero	19	10	650
CHO	18	9	580
R9ab	18	10	580

*sCJD, sporadic Creutzfeldt-Jakob disease; TgHu, transgenic mice expressing human prion protein gene; NB, normal brain; dpi, days postinoculation at cull; Vero, African green monkey kidney; CHO, Chinese hamster ovary; R9ab, rabbit fibroblasts.

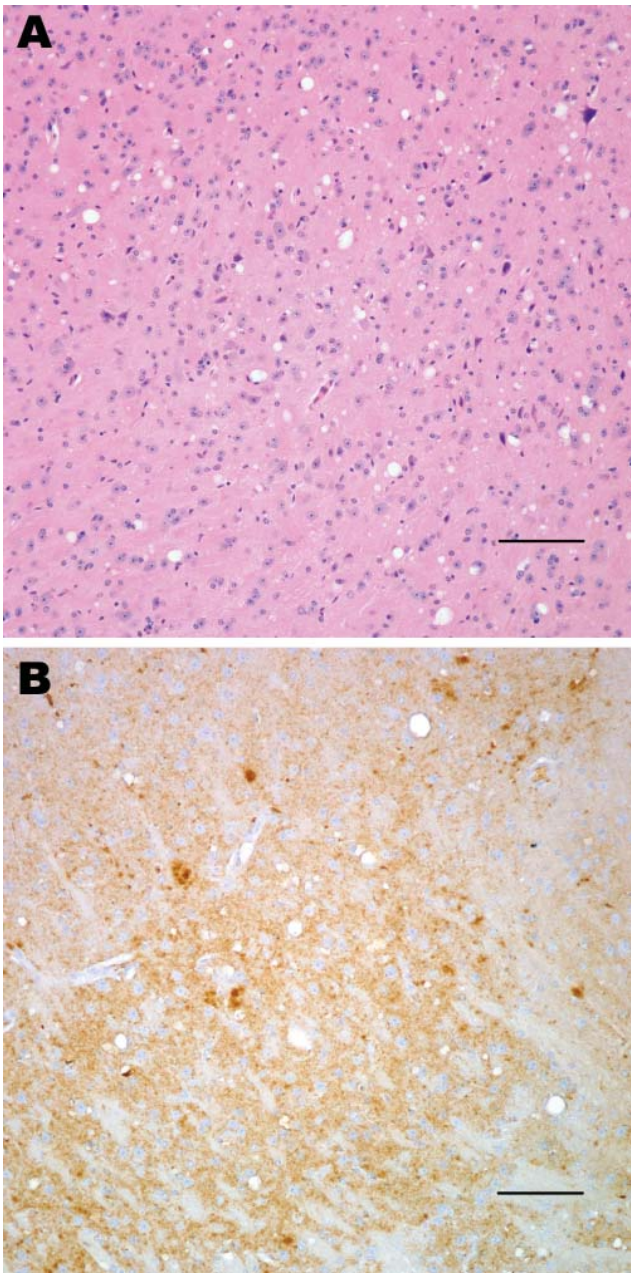

Figure 1. Histopathologic analysis of transgenic mouse expressing bovine prion protein (PrP) gene inoculated with bovine spongiform encephalopathy agent. Spongiform degeneration in the thalamus (A), adjacent section showing PrP immunopositivity (B). Panel A was stained with hematoxylin and eosin; panel B was immunostained with PrP antibody 6D11. Scale bars = 100 μm.

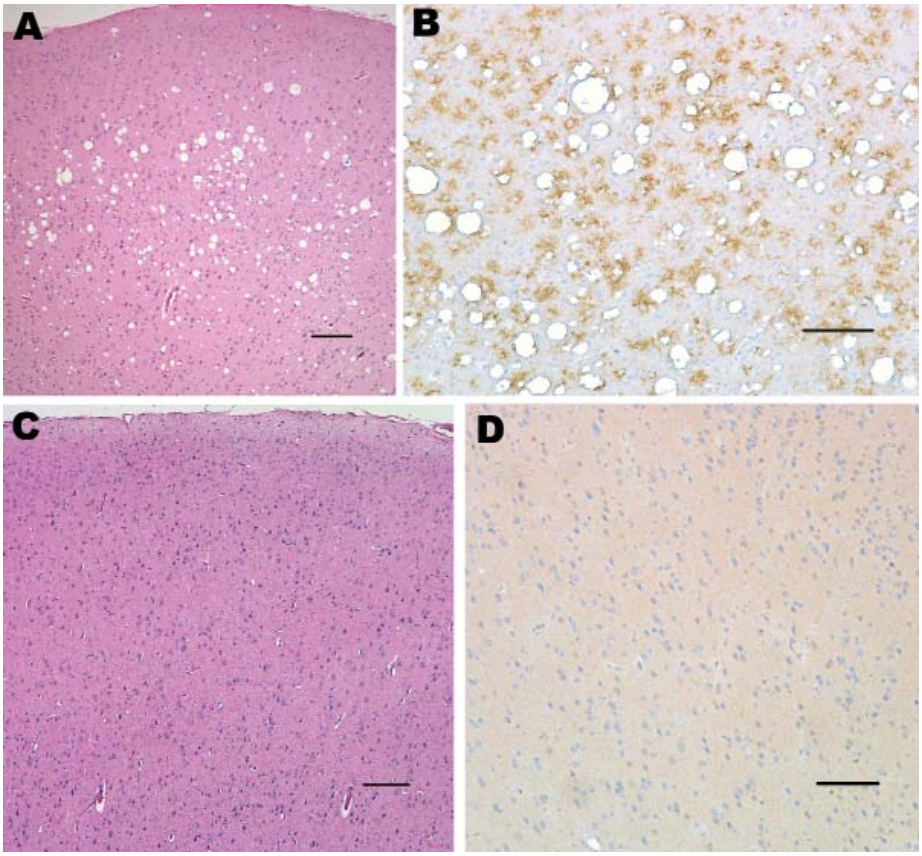

Figure 2. Histopathologic analysis of squirrel monkey inoculated with bovine spongiform encephalopathy agent (A, B). Spongiform degeneration in the cerebral cortex (A), adjacent section showing abundant prion protein (PrP) immunopositivity (B). Squirrel monkey without transmissible spongiform encephalopathy (C, D). Cerebral cortex with no spongiform degeneration (C), absence of PrP positivity in the cerebral cortex (D). Panels A and C correspond to sections stained with hematoxylin and eosin; panels B and D were sections immunostained with PrP antibody 6D11. Scale bars = 150 μm.

under these experimental conditions. WB analyses of cells collected after 30 serial passages showed no detectable PrP^{TSE} in any cell line (Figure 3). Samples of each cell line exposed to human TSE agents (sCJD, vCJD) and BSE agent were expanded after 30 passages for bioassay in TgHu and TgBo mice. At the time of this report, no mice inoculated with cells exposed to TSE agent have evidence of TSE illness during their expected lifespan (Tables 2, 3). WB with extracts of brain tissue from all culled animals showed no PrP^{TSE}. Neuropathologic analyses of selected mouse brains found no spongiform encephalopathy or accumulations of PrP (data not shown). The results confirm that no cell substrate propagated infectivity detectable by mouse bioassay, even when mice were observed for their expected lifespan.

Nonhuman Primate Bioassay

Lysates of Vero, CHO, WI-38, and R9ab cells exposed to BSE agent and passaged 30 times were inoculated into squirrel monkeys in May 2006. Lysates of MDCK and HEK-293 cells exposed to BSE agent and passaged 30 times were inoculated into squirrel monkeys in August 2007. No monkey inoculated with those cells had neurologic signs. One monkey inoculated with Vero cells exposed to the BSE agent was attacked by a cage mate; the wound became infected, suppuration increased despite treatment with antimicrobial drugs, and the injured monkey was euthanized several days later without ever showing any sign of neurologic disease. The brain showed no neuropathologic changes of TSE or PrP^{TSE} by WB (data not shown). In contrast, as noted above, primates inoculated with Swiss Reference BSE brain extracts at 10^{-1} and 10^{-2} dilutions developed typical TSE confirmed by neuropathologic results (Figure 2) and by WB (not shown). These results indicate that 1) squirrel monkeys are susceptible to infection with the BSE agent, and 2) BSE infectivity was present in the bacteria-free filtrate used to expose cell substrates.

Murine Fibroblast Cell lines Generate PrP^{TSE} after Exposure to Mouse-adapted Scrapie Agent

Several murine tissue cultures have been successfully infected with TSE agents, providing a promising alternative to assays of TSE agents by time-consuming and expensive bioassays in animals (*9*,*11*,*12*,*23*–*28*). Previous studies reported that several commonly used mouse fibroblast cell lines can be efficiently infected with the scrapie agent and support formation of PrP^{TSE} (*11*). We studied 2 such cell lines as a positive control to confirm that our protocol would have detected an infected cell line and that our failures to

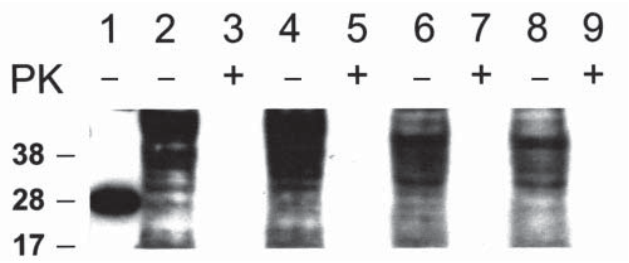

Figure 3. Western blot of recombinant prion protein (PrP) 5 ng (lane 1), CHO cells (lanes 2–5) and Vero cell (lanes 6–9). Cells exposed to normal bovine brain and passaged 30 times (lanes 2, 3, 6, 7). Cells exposed to bovine spongiform encephalopathy agent and passaged 30 times (lanes 4, 5, 8, 9). Total PrP (cell extracts without proteinase K [PK] digestion) are shown in lanes 2, 4, 6, 8; cell extracts treated with PK are shown in lanes 3, 5, 7, 9. Western blots were probed with PrP monoclonal antibody 6D11.

find PrP^{TSE} or infectivity by bioassay in cell substrates exposed to the BSE agent were more likely to have resulted from an intrinsic resistance of the cells to infection rather than to some technical problem. We exposed monolayers of NIH-3T3 and L929 murine fibroblast cells to the mouse-adapted 22L strain of scrapie agent and observed the formation of readily detectable PrP^{TSE} that persisted through 30 passages (Figure 4). We are performing bioassays of the PrP^{TSE}-positive cells in C57/BL6 mice to determine amounts of infectivity. Several mice inoculated with samples of NIH-3T3 and L929 fibroblasts collected 30 passages after exposure to 22L mouse-adapted scrapie agent have already developed spongiform encephalopathy, confirming that the agent was successfully propagated in vitro (unpub. data).

Discussion

Candidate cell substrates used to produce biologics were not infected by a simulated worst-case exposure to BSE agent. Similar more limited studies exposing the same cultures to vCJD and sCJD agents also gave negative results.

The finding of PrP^{TSE} in several cell culture samples collected at passages 0–4 probably resulted from small amounts of residual inoculum. This conclusion is reinforced by our consistent failure to detect PrP^{TSE} in any cell sample at or after passage 5. However, we cannot rule out the possibility of a transient de novo generation of PrP^{TSE} in the earliest passages of the cultures. Other investigators (*23*) have shown some immediate (acute) formation of new PrP^{TSE} in infection-resistant cell cultures exposed to scrapie agent; the new PrP formed did not depend upon the strain of TSE agent used or cell type involved and was not associated with infectivity (*23*). Whether the failure of infectivity to persist after transient formation of PrP^{TSE} resulted from death of the infected cells or the dilution of a small amount of TSE agent is unknown. We recognized no overt cytotoxicity in any cell line inoculated with a TSE-infected brain suspension. Thus, our data so far suggest that several cell substrates actually or potentially used to produce biologics were not susceptible to the propagation of TSE agents under the experimental conditions we used. In agreement with these results, others showed that MDCK cells were refractory to infection with human and mouse TSE agents (*24*). MRC5 human diploid cells also failed to support the replication of a TSE agent (*25*).

Because bioassays are time-consuming and expensive, a few lines of cells susceptible to infection with certain strains of TSE agent have been derived (*9*,*11*,*12*,*26*–*30*). However, for unknown reasons, most cell lines have resisted TSE infections. In addition, most cell lines infectable with TSE agents have been highly heterogeneous and not stable, requiring repeated subcloning of susceptible cells, and they have been successfully infected with only a few strains of TSE agent (*27*). Thus, it was vital to verify that the protocol we chose as a simulated worst-case model would successfully infect previously characterized cell lines with a TSE agent to which they were known to be susceptible. We demonstrated that the protocol we used was valid by persistently infecting 2 murine fibroblast cell lines with a mouse-adapted scrapie agent. However, we must caution that even cell lines susceptible to infection have shown widely different responses when exposed to various TSE agent strains (*27*). Furthermore, the emergence of atypical forms of BSE raises a new concern, i.e., that cell substrates resistant to infection with the original classic BSE agent (*31*) might not resist infection with newer strains of BSE agent (if new strains are implicated in atypical BSE).

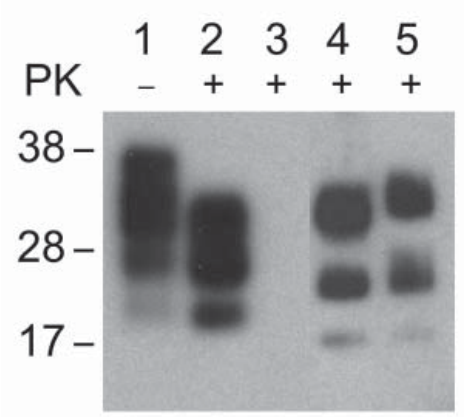

Figure 4. Western blot of brain extract from C57/Bl mouse inoculated with 22L mouse-adapted scrapie agent (lanes 1, 2); NIH-3T3 cells exposed to normal mouse brain and passaged 30 times (lane 3); NIH-3T3 (lane 4) and L929 (lane 5) cells exposed to 22L scrapie agent and passaged 30 times. Non–proteinase K [PK]–treated samples (lane 1), PK-treated samples (lanes 2–5). Western blots were probed with prion protein monoclonal antibody 6H4.

Although BSE has been transmitted to many animal species, the efficiency of transmission between species has been difficult to predict. Low transmission rates and long incubation periods are often observed when TSEs are transmitted to a new species—commonly known as a species barrier (*32*)—but experience with squirrel monkeys (sensitive to almost all TSE agents affecting humans and to several animal TSEs as well) suggests that they are an especially useful model species (*33*). To that end we initiated titration experiments of the BSE agent and found at the time of writing this report that squirrel monkeys develop TSE when inoculated with BSE brain suspensions at high concentration 10^{-1} and 10^{-2} dilutions (work in progress). Given the long incubation time (years) needed to elicit disease in this primate model, we will continue the observation of monkeys inoculated with cell cultures exposed to the BSE agent in an attempt to determine whether low infectivity could potentially be detected in this model.

Because few cell lines used as substrates for biologics are of bovine origin, it is tempting to speculate that the potential for contaminating biologics with the BSE agent is low. However, an observation that cells derived from a pheochromocytoma of the rat adrenal medulla (PC12 cells) could be infected with a mouse-adapted scrapie agent and murine hypothalamic cells with a human TSE agent demonstrates that cross-species transmissions of TSE agents in cells in culture are possible (*29*). Recent studies showed that passage of TSE agents through different animal species altered key characteristics of the agent, sometimes producing variants with increased virulence or broader host range, as happened when BSE agent was passaged through sheep (*34*). Thus, further experiments are needed to evaluate the potential susceptibility of various cultured cell lines to infection with new emerging TSE strains.

Acknowledgments

We thank S.A. Priola for providing 22L scrapie agent; T. Seuberlich and D. Heim for providing brain tissue from a cow with confirmed BSE; J. Cooper, K. Sadhani, and P. Minor for providing WHO Candidate Biological Reference CJD Material; K. O'Rourke for providing normal cattle brain; and A.M. Lewis for encouragement in the development of this project.

This work was generously funded by the National Institutes of Health, National Institute of Allergy and Infectious Diseases, agreement no. Y1-AI-4893-02; and FDA agreement no. 224-05-1307.

The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any agency determination or policy.

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Molecular Epidemiology of Rift Valley Fever Virus

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Phylogenetic relationships were examined for 198 Rift Valley fever virus isolates and 5 derived strains obtained from various sources in Saudi Arabia and 16 countries in Africa during a 67-year period (1944–2010). A maximum-likelihood tree prepared with sequence data for a 490-nt section of the Gn glycoprotein gene showed that 95 unique sequences sorted into 15 lineages. A 2010 isolate from a patient in South Africa potentially exposed to co-infection with live animal vaccine and wild virus was a reassortant. The potential influence of large-scale use of live animal vaccine on evolution of Rift Valley fever virus is discussed.

Rift Valley fever (RVF) is an acute disease of domestic ruminants in Africa and the Arabian Peninsula. This disease is caused by a mosquito-borne virus of the family *Bunyaviridae* and genus *Phlebovirus*. Large outbreaks occur at irregular intervals when heavy rains favor breeding of mosquito vectors of the virus and are characterized by deaths of newborn animals and abortion in pregnant sheep, goats, and cattle. Humans become infected by contact with tissues of infected animals or from mosquito bites and usually show development of mildly to moderately severe febrile illness. However, severe complications, including ocular sequelae, encephalitis, and fatal hemorrhagic disease, occur in some patients (*1*).

Rift Valley fever virus (RVFV) has a negative-sense, single-stranded RNA genome comprising large (L), medium (M), and small (S) segments. The L segment encodes viral RNA polymerase. The M segment encodes envelope glycoproteins Gc and Gn, a nonstructural protein, and a 78-kDa fusion protein of nonstructural and Gn proteins. The S segment shows an ambisense strategy and encodes nucleocapsid protein N and a nonstructural protein (*2*).

Early genetic analysis involved nucleotide sequencing of M segment RNA fragments encoding glycoprotein that induced neutralizing antibody response in 22 isolates obtained over 34 years in 6 countries (*3*). This analysis showed remarkable stability of sections of the genome expected to be under greatest immune selection pressure. The diversity observed in isolates from Zimbabwe indicated that outbreaks do not invariably involve a single genotype of virus but can result from intensified transmission of multiple strains already circulating in RVF-endemic areas. Nevertheless, neutralization of isolates with monoclonal antibodies confirmed that a single vaccine should suffice to control the disease (*3*). Slightly greater variability was observed among isolates when nonstructural protein sequence data were analyzed (*4*). Partial sequences determined for all 3 RNA segments of the genome of 20 isolates sorted into 3 geographically linked lineages associated with western Africa, Egypt, and eastcentral Africa and showed evidence of reassortment of genome segments between some sub-Saharan isolates (*5*).

High-throughput technology facilitated whole-genome sequence analysis of 33 isolates and resulted in confirmation of low genetic diversity of the virus and separation of the isolates into 7 lineages. There was no mutually exclusive correlation between genotype and geographic origin; representatives of geographic areas tended to cluster, but isolates from distant locations occurred in each lineage, indicating continuous widespread dispersal of virus (*6*). The remarkable congruence of phylogenetic trees for the 3 genome segments suggested

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DOI: http://dx.doi.org/10.3201/eid1712.111035

that reassortment was not common, but convergence of some lineages within genome segments implied that reassortment had played an evolutionary role in the history of RVFV. Bayesian analysis suggested that the time of divergence of RVFV isolates from a most recent common ancestor dated to 1880–1890, the colonial period when the introduction of large concentrations of susceptible sheep and cattle would have facilitated exploitation of a new niche by an unknown progenitor virus. The evolutionary rate of the virus was similar to that of other RNA viruses. Thus, low nucleotide diversity probably relates to recent derivation from a common ancestor rather than stability of the genome.

Whole-genome sequencing and Bayesian analysis of 31 isolates associated with the 2006–2007 outbreak of RVF in Kenya showed that 2 sublineages of virus had evolved separately before or during a large outbreak during 1997–1998, with continued expansion of 1 sublineage dating from 2–4 years before 2006, confirming that outbreaks in disease-endemic areas might be associated with multiple lineages of virus, and that virus activity and evolution can occur below the threshold of detection by public health or animal authorities during interepidemic periods (*7*). Other genetic studies have been more limited in scope and concerned with either locating and investigating mechanisms of pathogenicity or determining phylogenetic relationships of isolates involved in particular outbreaks (*8–14*).

Analysis of partial M segment sequence data for a large collection of isolates and derived strains obtained from various sources in Saudi Arabia and 16 countries in Africa during 1944–2010 showed phylogenetic relationships not apparent in studies involving a smaller range of isolates. A 2010 isolate from a patient in South Africa potentially exposed to co-infection with live animal vaccine and wild virus from a needle injury while vaccinating sheep plus selected other isolates were subjected to limited sequencing of all 3 segments of the genome to obtain evidence of reassortment. We present and discuss the epidemiologic implications of these findings.

Materials and Methods

Viruses

Sequence data for 33 viruses were obtained from GenBank. The remaining 170 viruses for which we determined partial nucleotide sequences were obtained from the various institutions (online Appendix Table, wwwnc.cdc.gov/EID/article/17/12/11-1035-TA1.htm).

RNA Extraction

Viral RNA was extracted directly from 140 μL of infected human or livestock serum, clarified 10% organ suspensions, reconstituted freeze-dried mouse brain suspensions, or Vero cell culture supernatant fluids. Extraction was performed by using a QIAamp Viral RNA Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions.

Reverse Transcription PCR and Nucleotide Sequencing of PCR Products

A 10-μL RNA aliquot was analyzed by using reverse transcription PCR with the Titan One Tube RT-PCR Kit (Roche Diagnostics, Penzburg, Germany) in a final volume of 50 μL as described (*15*). The forward primer FD1 (771/5′-CCAAATGACTACCAGTCAGC-3′/790) (*3*) and the reverse primer RVF E (1342/5′-CCTGAC CCATTAGCATG-3′/1326) were selected to amplify a portion of the Gn glycoprotein gene of the virus; the primer positions correspond to the viral complementary DNA sequence of the M segment of the ZH501 human RVF virus isolate (*16*).

Amplicons were purified by using the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Nucleotide sequences of PCR products were determined by using BigDye version 3.1 Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. For confirmatory purposes, sequences were obtained for both strands of PCR products by using primers FD1 and RVF E. Products were purified by using CentriSep spin columns (Princeton Separations Inc., Adelphia, NJ, USA) and analyzed by using a 377 GenAmp Sequencer and a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

On the basis of findings for M segment data, partial sequences were determined for S and L RNA segments of isolate SA184/10 from a patient potentially co-infected with live animal vaccine and wild virus; isolate SA54/10 from another patient in the same area; the batch of vaccine used (Smithburn neurotropic strain [SNS] 105/2010); SNS vaccine master seed virus; 95EG vaccine; and historical isolates 95EG Cow-2509, H1739, and H1825 (online Appendix Table) to test for reassortment by using specifically designed primers. Primers F1 (1/5′-ACACAAAGACCCCCTAGTGC-3′/20) and R4 (1690/5′-ACACAAAGCTCCCTAGAGATAC-3′/1669) were used to amplify the S segment and the N gene (735 nt). Primers RVFL10 (4237/5′-GGTGTTGTGTCA TCATTG-3′/4254)andL2(4730/5′-GTGTGAGCTAGAGT TGCTTC-3′/4711) were used to amplify a 494-nt region of the L segment.

Sequence Analysis

Nucleotide and amino acid sequence alignments were generated by using ClustalW Multiple Alignment analysis

software as implemented in BioEdit version 7.0.5.3 (*17*). Unique sequences generated in the study were submitted to GenBank and assigned accession numbers indicated in the online Appendix Table. Preliminary phylogenetic analysis was performed by using a neighbor-joining distance method in MEGA4 that applied a Jukes-Cantor model under 1,000 bootstrap iterations (*18*). Sequence divergence was determined by using MEGA4 to calculate mean pairwise distances within groups. A phylogenetic tree was constructed for 95 isolates exhibiting unique sequences and for 16 isolates that exhibited duplicate sequences but that were isolated in different years or countries, by using the maximum-likelihood method in PAUP* version 4.0b2 (Sinauer Associates, Inc., Sunderland, MA, USA).

Further analysis of partial M segment sequence data was performed by using the Bayesian software package, which included BEAST, BEAUTI, Tracer, TreeAnnotator, and FigTree (*19*), by using a Markov Chain Monte Carlo chain length of 3.0×10^7, a 3.0×10^6 burn in, a generalized time reversible plus gamma plus invariant nucleotide substitution model, a relaxed uncorrelated logarithmic normal molecular clock, and sampling every 1,000 states.

To check for recombination events within the RVFV alignment, the alignment was examined by using a set of 6 detection methods implemented in RDP3 (*20*). Partial nucleotide sequence data for S, M, and L RNA segments of 33 isolates for which whole-genome sequences have been reported (*6*) (all included in the online Appendix Table) were obtained from GenBank and used with sequences determined in the present study for 5 isolates plus the 3 animal vaccine viruses to perform analyses as described above to test whether genetic reassortment had occurred.

Results

Diversity of partial M segment sequences was low; pairwise differences ranged from 0% to 5.4% for nucleotides and from 0% to 2.8% for deduced amino acids. These values are similar to those identified for 33 whole-genome sequences (*6*). Neighbor-joining, maximum-likelihood, and Bayesian phylogenies were similar. Bayesian analysis indicated that divergence of isolates included in the study from a most recent common ancestor dated to 1892, which is similar to the estimate of 1880–1890 deduced earlier from 33 whole-genome sequences. However, posterior support values for the tree nodes were weak because a small nucleotide segment was analyzed. Despite low genetic diversity, the 5 derived strains and 198 isolates of RVFV circulating over the past 67 years produced 95 unique sequences that resolved into 15 lineages (A–O) (online Appendix Figure, wwwnc.cdc.gov/EID/article/17/12/11-1035-FA1.htm; online Appendix Table) with mean pairwise distances ≤0.017 within lineages and bootstrap values ≥70% (*21*).

Five lineages (B, D, F, J, and O) contained single isolates, and lineage I contained 2 isolates from South Africa. Six other lineages (A, C, E, G, H and N) contained clusters of isolates associated with outbreaks in individual countries or regions, but each also included isolates from distant locations or separate outbreaks. Thus, lineage A comprised isolates from outbreaks in Zimbabwe in 1978, Madagascar in 1979, and Egypt in 1977–1978 and 1993 and a 1974 isolate from an interepidemic period in Zimbabwe (online Appendix Figure; online Appendix Table).

Lineage C was most widely distributed and contained isolates from major outbreaks in Zimbabwe in 1978, Madagascar in 1991, eastern Africa (Kenya, Tanzania, and Somalia) during 1997–1998, Saudi Arabia during 2000–2001, Kenya in 2007, and South Africa during 2008–2009 and lesser outbreaks and interepidemic periods in the same countries and Mauritania during 1976–2009. It was also associated with an isolated fatal infection in Angola in 1985 in a visitor from South Africa ostensibly exposed to mosquito bites (*22*). Lineage E, which was found in the Central African Republic and Zimbabwe during 1973–1978, was isolated from 2 patients referred to South Africa for treatment in 1985 after a relative had died of a similar disease in Zambia during an outbreak of RVF in livestock confirmed serologically (*22*,*23*). Lineage G contained isolates from the Central African Republic, Zimbabwe, Guinea, and Senegal during 1969–1986, and lineage H contained isolates from the 2009–2010 outbreak in South Africa and an apparent antecedent from Namibia in 2004. Lineage N, originally designated West African (*5*), contained isolates from Senegal, Burkina Faso, and Mauritania during 1975–1993.

Lineages K, L, and M with a common root node contained the SNS neurotropic and the KCS hepatotropic strains derived from the same mosquito isolate (*24*) and isolates from Kenya, Zimbabwe, South Africa, and Egypt during 1951–2010, countries that used the SNS animal vaccine on a large scale during major outbreaks of RVF. Isolate KEN57 Rintoul, obtained from a cow in Kenya in 1951, when the first batch of SNS vaccine was sent from South Africa to Kenya, had a partial M segment sequence identical to that of the vaccine virus. The SNS 105/2010 batch of vaccine and Egyptian 95EG vaccine also had the same sequence as the SNS vaccine master seed stock produced in 1987 (online Appendix Table).

Isolate SA184/10, from a patient potentially exposed to wild virus and SNS 105/2010 animal vaccine, grouped with the parent vaccine strain in lineage K, albeit with a divergence of 11 (2.2%) of 490 nt (2 aa), and distantly from 46 other human isolates from the 2008–2010 outbreaks in South Africa. Moreover, isolate 95EG Cow-2509 from the fetus of a cow that aborted after the administration of 95EG vaccine in Egypt grouped in lineage L distantly

from all other isolates from Egypt in lineage A (*12*) (online Appendix Figure; online Appendix Table). Isolates H1739 and H1825, which also grouped in lineage L, came from the first human deaths caused by RVF during a major outbreak in South Africa during 1974–1976 during which animal vaccine was used on an unprecedented scale (*25*; National Institute for Communicable Diseases [NICD], unpub data) (Figure 1). These 5 isolates were investigated for genetic reassortment.

Partial nucleotide sequence data for S, M, and L RNA segments derived from whole-genome sequences of 33 isolates produced phylogenetic trees with the same topologies as those published for the complete sequences (*6*). Isolate SA184/10 from the patient who sustained a needle injury while vaccinating sheep sorted with SNS vaccine virus in the M segment tree but with isolate SA54/10 in the other segments, and was clearly a reassortant. SNS 105/2010 and 95EG batches of vaccine corresponded with the SNS master seed stock for all 3 segments of the genome. Analysis of isolates H1739 and H1825 obtained from the first recorded human deaths caused by RVF in 1975 and isolate 95EG Cow-2509 from Egypt did not show clear-cut evidence of reassortment but fell into a group that manifested convergence of lineages D and E in the M RNA segment tree in the 33 whole-genome study, corresponding to lineages L plus K, the vaccine lineage, in the present study. This phenomenon was interpreted as evidence of historical reassortment (*6*). Isolate SA75 also ostensibly came from a human infected in South Africa in 1975 (*6*), but we could not relate the designation to records at NICD. Analysis of partial M segment sequence data did not show evidence of recombination.

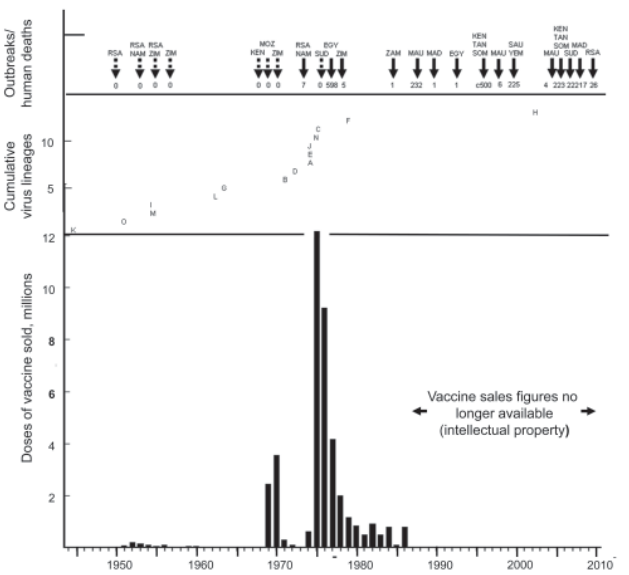

Figure 1. Annual sales of Smithburn neurotropic strain animal vaccine produced in South Africa in relation to cumulative viral lineages isolated and human deaths in major outbreaks of Rift Valley fever (RVF) in Africa and Saudi Arabia, 1944–2010. Broken arrows indicate RVF outbreaks without human deaths recorded, and solid arrows indicate RVF outbreaks with human deaths. RSA, Republic of South Africa; NAM, Namibia; ZIM, Zimbabwe; MOZ, Mozambique; KEN, Kenya; EGY, Egypt; SUD, Sudan; ZAM, Zambia; MAU, Mauritania; MAD, Madagascar; TAN, Tanzania; SOM, Somalia; SAU, Saudi Arabia; YEM, Yemen.

Discussion

Historical developments in RVF include spread beyond sub-Saharan Africa during 1977–2007 and fatal human infections during a large outbreak in South Africa during 1974–1976. Since that time, large outbreaks of the disease in livestock have invariably been associated with human deaths (*1*). Mechanisms for dispersal of RVFV fall beyond the scope of this report. However, refinements to phylogenetics, such as Bayesian-based and population-based genetic analysis, have shown that translocated virus does not necessarily arrive in receptive circumstances to trigger epidemics, but can initiate smoldering infection or seeding of the ground, which remains undetected until suitable climatic conditions precipitate outbreaks, as occurred ahead of the 2000–2001 and 2006–2007 outbreaks in the Arabian Peninsula and Kenya (*6*,*7*).

Recent outbreaks in South Africa showed an analogous pattern. After heavy rains in 2008, lineage C virus, which had been isolated during a limited disease outbreak in Kruger National Park in 1999, was associated with scattered outbreaks of RVF in adjacent parts of northeastern South Africa. In the first half of 2009, the same lineage caused limited outbreaks to the south in KwaZulu-Natal Province. In the second half of 2009, lineage H virus, which had been encountered in the Caprivi Strip of Namibia in 2004, caused focal outbreaks in Northern Cape Province and was progressively identified in coalescing outbreaks over much of interior South Africa in 2010 (Figure 2). Diversity of genotypes observed among recent isolates of the 2 lineages (online Appendix Figure), and results of our limited Bayesian analysis imply that RVFV had progressively reinfiltrated the interior plateau of South Africa during a period of increasing rainfall, 3 decades after the major outbreak of 1974–1976. There were 26 deaths among 244 persons infected with lineage H virus, and while no deaths were recorded in areas where lineage C virus was active, only 22 cases were diagnosed (NICD, unpub. data).

Reassortment between SNS vaccine virus and wild virus has implications for the safety of the vaccine and its possible role in evolution of RVFV. The SNS virus from Uganda (online Appendix Table) was taken to mouse intracranial passage 102 and embryonated chicken egg passage 54 to produce avianized (decreased infectivity

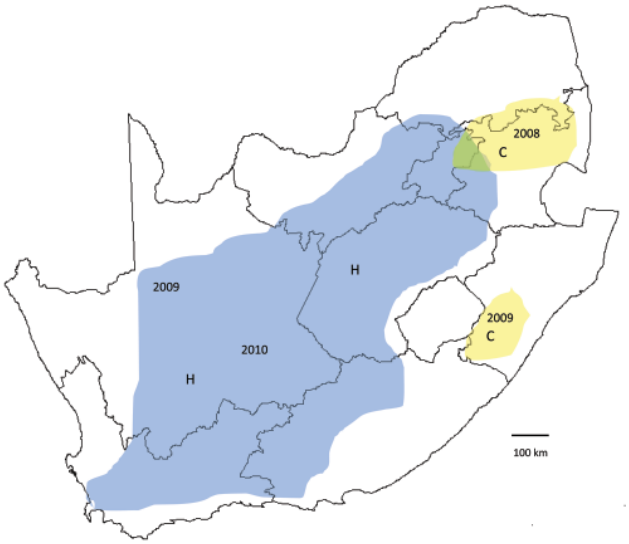

Figure 2. Recent outbreaks of Rift Valley fever in South Africa. Lineage C virus (yellow areas), which caused a small outbreak in Kruger National Park in 1999, was associated with scattered outbreaks of disease in adjacent parts of northeastern South Africa in 2008 and limited outbreaks to the south in KwaZulu-Natal Province early in 2009. Lineage H virus (blue area), which was first encountered in the Caprivi Strip of Namibia in 2004, caused focal outbreaks in the Northern Cape Province late in 2009, and was associated with coalescing outbreaks over much of interior South Africa in 2010. Lines indicate province boundaries.

produced by repeated culture in chick embryo) animal vaccine in South Africa in 1951. After several adjustments, reversion was made in 1958 to SNS virus passed 103 times in mice only. Since 1971, the same virus has been propagated in BHK21 cells for preparation of freeze-dried vaccine (*24*,*26*–*29*). In Kenya, animal vaccine was initially produced from avianized virus from South Africa, but since 1960, SNS virus has been used at mouse passage 106 (*30*–*32*). SNS seed virus from South Africa was used to produce vaccine in Egypt in 1994, and South Africa and Egypt have used field isolates to produce inactivated animal vaccine (*12*,*27*,*29*). SNS vaccine is only partially attenuated, but accurate estimates for abortigenicity and teratogenicity were never determined because conflicting results were initially obtained in different sheep breeds and because pathogenicity varies with stage of pregnancy (*26*,*33*). However, there has been a tendency to regard risks associated with vaccine as acceptable in the face of an outbreak of RVF (*34*).

During 1951–1968, ≈1 million doses of SNS animal vaccine were sold in South Africa; sales were similar in Kenya (*28*,*32*). The vaccine was first used on a large scale in 1969–1970 when 6 million doses were issued, mainly to Zimbabwe, where a large outbreak occurred (*29*) (Figure 1). Sales decreased sharply by 1973 but increased to 22 million doses during the major outbreak in South Africa and Namibia during 1974–1976.

During the same period, 4.7 million doses of newly developed inactivated RVF vaccine and ≈14 million doses of Wesselsbron virus vaccine were sold (*27*,*28*,*35*). Wesselsbron is a mosquito-borne flavivirus initially thought to cause outbreaks analogous to RVF, and vaccine was produced by similar empirical attenuation of virus by intracranial passage in mice (*33*,*35*). Thus, >40 million doses of either vaccine were administered over 3 years in South Africa and Namibia, where the combined sheep, cattle, and goat population was <60 million. However, fewer than two thirds of the animals were in RVF-affected areas. Approximately 4.2 million doses of SNS vaccine were sold in 1977, much of it to South Africa, but also to Israel and Egypt. Approximately 3 million doses were sold in 1978–1979, mostly to Zimbabwe where an additional large outbreak of RVF had occurred. Subsequently sales remained <1 million doses per year until 1986, after which figures were no longer made public (*28*). However, substantial quantities of vaccine, mainly from South Africa, were used in eastern Africa and Saudi Arabia in recent years. In all locations except Israel and the Sinai Peninsula, where the threat of RVF was not proven, large numbers of animals were vaccinated only after onset of outbreaks (*28*,*29*).

Livestock vaccines are sold in multidose vials and commonly administered with automatic syringes and intermittent changes of needles. Vaccine is likely to be administered to some animals that are already infected. Thus, in view of intense viremia that occurs in RVF, serial transfer of wild virus by needle is a recognized hazard of vaccinating livestock during outbreaks (*29*). This vaccination likely resulted in co-infections with vaccine and wild virus, or even different wild genotypes, on multiple occasions over decades, particularly during 1969–1979, with an implied potential for the generation of recombinant and reassortant genotypes. Uptake and transmission of virus by vectors would have increased co-infections and potential for genetic interaction (*36*).

Although there is no corresponding evidence that reassortment or recombination occurred on a large scale, few isolates remain available from the 1969–1970 and 1974–1976 outbreaks in Zimbabwe and South Africa. Nevertheless, multiple new lineages were encountered during 1969–1981, particularly in Zimbabwe where intensive monitoring was instituted during 1971–1979 and where lineage A and C isolates were obtained (*37*) (online Appendix Figure; Figure 1). Moreover, it can be deduced from reported Bayesian analysis (*6*) that emergence of new lineages and genotypes surged during this period. An alternative interpretation is that reassortment and recombination occur infrequently but that mutations result from as yet inadequately explored interactions during

replication in co-infections or from the generation of neutralizing antibody–escape mutants (*38*).

Thus, the partial M segment nucleotide sequence of reassortant isolate SA184/10 differs from that of vaccine virus, and that of isolate 95EG Cow-2509 from the aborted fetus of a vaccinated cow differs even more markedly from that of vaccine virus and from all other known isolates from Egypt but has some relationship with isolates H1739 and H1825 in lineage L (online Appendix Figure), which caused human deaths. The implication is that further investigation of evolutionary relationships in lineages K, L, and M are warranted, including whole-genome sequencing of additional isolates identified in the present study. It could be relevant that viruses attenuated through intracranial passage in mice may acquire new tissue tropisms and pathogenic properties (*39*,*40*).

New vaccines, some with natural or induced attenuating deletions or mutations in all 3 segments of the genome, are being developed (*1*). However, interactions of replication-competent strains with wild virus should be investigated, especially because some vaccines were deliberately modeled on viruses considered to be particularly virulent.

Acknowledgments

We thank J. Morvan, B.A. Botros, R.R. Arthur, F.G. Davies, H.C.W. Mbugua, G.H. Gerdes, C.A. Potgieter, C.J Peters, J.C. Morrill, and B.J. Erasmus for providing RVFV isolates and strains.

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Novel Multiplexed HIV/Simian Immunodeficiency Virus Antibody Detection Assay

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Like most emerging infectious disease viruses, HIV is also of zoonotic origin. To assess the risk for cross-species transmission of simian immunodeficiency viruses (SIVs) from nonhuman primates to humans in the Democratic Republic of the Congo, we collected 330 samples derived from nonhuman primate bushmeat at 3 remote forest sites. SIV prevalences were estimated by using a novel high-throughput assay that included 34 HIV and SIV antigens in a single well. Overall, 19% of nonhuman primate bushmeat was infected with SIVs, and new SIV lineages were identified. Highest SIV prevalences were seen in red-tailed guenons (25%) and Tshuapa red colobus monkeys (24%), representing the most common hunted primate species, thus increasing the likelihood for cross-species transmission. Additional studies are needed to determine whether other SIVs crossed the species barrier. With the newly developed assay, large-scale screening against many antigens is now easier and faster.

Like many emerging infectious disease viruses, HIV is also of zoonotic origin (*1*). The closest relatives of HIV-1 are simian immunodeficiency viruses (SIVs), specifically SIVcpz and SIVgor in chimpanzees (*Pan troglodytes troglodytes*) and gorillas (*Gorilla gorilla*), respectively, from west-central Africa (*2*,*3*). SIVsmm in sooty mangabeys (*Cercocebus atys*) from west Africa are the closest relatives of HIV-2 (*4*,*5*). SIVs from mangabeys, gorillas, and chimpanzees crossed the species barrier ≥12 times (*1*,*6*). Exposure to blood, secretions, or tissues from infected primates through hunting and butchering of bushmeat represents the most plausible source for human infection.

Humans are still hunting and butchering a wide diversity of primate species, and the possibility of additional cross-species transfers of viruses has to be considered (*7*,*8*). Recent reports showed ongoing transmission of simian retroviruses to humans in central Africa, i.e., a wide variety of simian foamy viruses and new human T-lymphotropic virus variants, closely related to viruses in co-habiting nonhuman primates, have been observed in humans who report primate hunting and butchering (*9*–*12*). The description in 2009 of HIV-1 group P, closely related to SIVgor, in a patient from Cameroon living in France, shows also that our knowledge on HIV diversity and possible cross-species transmissions is still incomplete and illustrates how rapidly new viruses can spread today to other continents (*6*).

Given the potential pathogenicity of these lentiviruses, as illustrated by the actual HIV-1 group M pandemic that resulted from a single cross-species transmission, it is necessary to estimate to what extent humans are exposed to SIVs and whether other viruses crossed the species-barrier. SIV infection has already been identified in >40 nonhuman primate (NHP) species from Africa but our knowledge on prevalence and geographic distribution remains limited; few large-scale studies on retroviral infections in wild

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DOI: http://dx.doi.org/10.3201/eid1712.110783

primate populations have been conducted (*13*). SIV prevalences can vary among species and within species according to geographic areas (*2*,*14*,*15*), and exposure to infected primates and subsequent risk for cross-species transmission can thus differ across Africa.

SIV infections were initially identified on the basis of cross-reactivity with HIV antigens (*8*), but to increase sensitivity, SIV lineage-specific ELISAs have been developed. These assays must be regularly updated when new SIV lineages are discovered (*14*–*17*). Therefore, they become time-consuming and bench work–consuming, use relatively large volumes of scarce biological material, and are not adapted for large-scale surveillance studies. We adapted the Multiple Analyte Profiling technology (xMAP; Luminex Inc., Austin, TX, USA), which is a flow cytometry–based system (*18*), for simultaneous antibody detection against 34 peptides representing the actual known HIV/SIV diversity. This new assay was used to study SIV infection in primate bushmeat in the Democratic Republic of Congo (DRC), home to a wide diversity of primate species.

Materials and Methods

NHP Samples

For the validation of the HIV/SIV xMAP assay, we used 142 well-characterized samples from our NHP reference panel in which SIV infection was either confirmed or ruled out by highly sensitive PCR approaches, and for which sufficient plasma was available (*14*,*15*). The panel included 93 SIV-negative samples from 8 species and 49 SIV-positive samples from 9 species (Table 1). For SIV prevalence studies, 330 samples were collected during May 2009–2010 as dried blood spots (DBSs) around 3 rural cities in DRC (Figure 1). Whole blood, collected from primate bushmeat, was spotted onto a filter 903 FTA card (Whatman Plc, Kent, UK). After air-drying at ambient temperature, DBSs were stored into individual envelopes at ambient temperature. Animals died 6–78 hours before sampling. All NHP samples were obtained with approval from the Ministry of Environment and Health and the National Ethics Committee. Similar to our previous studies, bushmeat samples were obtained through a strategy specifically designed not to increase demand (*8*,*15*).

Screening for Cross-Reactive HIV/SIV Antibodies

All DBS samples were screened with the new HIV/SIV multiplex microbead immunoassay technology, i.e., xMAP. Similarly as for the SIV ELISAs, we used peptides covering the immunodominant region of the gp41 transmembrane and V3-loop region from all major SIV/HIV lineages known at the time we conducted this study (Table 2). To avoid interpeptide and intrapeptide cross-linking, the 2 cysteins of the gp41 peptides were cyclicized during synthesis. For SIVcol, gp41 peptides could not be synthesized because of their low solubility; the V3-loop peptide was used to identify corresponding antibodies (*14*,*15*). Peptides were covalently coupled on carboxyl-functionalized fluorescent polystyrene beads (Luminex Inc.) by using the Bio-Plex Amine Coupling Kit (Bio-Rad Laboratories, Marnes-la-Coquette, France) according to the manufacturer's instructions. Unreacted sites were blocked with blocking buffer from the Amine Coupling Kit (Bio-Rad Laboratories). Peptide-coupled microsphere preparations were counted by using a hemocytometer and stored in the dark at 4°C. Before use, peptide-coupled beads were vortexed (30 s), sonicated (30 s), and diluted to 4,000 beads/µL. Dilution and washing buffer consisted of phosphate-buffered saline (PBS) containing 0.75 mol/L NaCl, 1% (wt/vol) bovine serum albumin (Sigma Aldrich, St. Quentin Fallavier, France), 5% (vol/vol) fetal bovine serum (Gibco-Invitrogen, Cergy Pontoise, France), and 0.05% (vol/vol) Tween-20 (Sigma-Aldrich).

Assays were performed in 96 well flat-bottomed filter plates (Millipore, Tullagreen, Ireland). Plates were prewet with 100 µL assay buffer; 50 µL of bead mixture was subsequently added to each well. Liquid was aspirated with a vacuum manifold and wells were washed with 100 µL of assay buffer. Wells were then incubated with 100 µL plasma (diluted 1:200) for 60 min at room temperature in the dark on a plate shaker (300 rpm/min). For DBSs, 100 µL of eluate, obtained after overnight incubation of two 6-mm disks in 1 mL of hypertonic PBS, was added to

Table 1. Panel of reference serum samples from SIV-infected and -noninfected nonhuman primate species*

Species (common name)	SIV lineage	No. samples SIV+	No. samples SIV–
Pan troglodytes troglodytes (west central chimpanzee)	SIVcpz*Ptt*	2	1
Pan troglodytes schweinfurthii (eastern chimpanzee)	SIVcpz*Pts*	1	NA
Cercopithecus nictitans (greater spot-nosed monkey)	SIVgsn	6	45
Cercopithecus cephus (mustached monkey)	SIVmus	7	30
Cercopithecus mona (Mona monkey)	SIVmon	NA	1
Miopithecus ogouensis (northern talapoin)	SIVtal	1	6
Cercopithecus neglectus (De Brazza monkey)	SIVdeb	7	1
Cercocebus torquatus (red-capped mangabey)	SIVrcm	4	NA
Chlorocebus tantalus (African green monkey)	SIVagm	1	NA
Mandrillus sphinx (mandrill)	SIVmnd-2	10	2
Colobus guereza (mantled guereza)	SIVcol	10	7
Total		49	93

*SIV, simian immunodefiency virus; NA, none available.

Figure 1. Sites in the Democratic Republic of the Congo where dried blood spots of nonhuman primates were collected (red circles).

peptide-coated beads. After washing, plates were incubated with 50 µL/well of 4 µg/mL biotinylated mouse antihuman IgG (BD Pharmingen, Le Pont de Claix, France) for 30 min at room temperature in the dark under continuous shaking. After washing, wells were incubated with 50 µL of 1 µg/mL streptavidin-R-phycoerythrin conjugate/well (Invitrogen/Molecular Probes, Cergy Pontoise, France) for 10 min in the dark while shaking. After 2 final washes with reading buffer (PBS containing 1% [wt/vol] bovine serum albumin), beads were resuspended in 125 µL reading buffer/well and analyzed by using BioPlex-200 (Bio-Rad Laboratories).

Data were analyzed by using BioPlex Software Manager version 5.0 (Bio-Rad Laboratories). For each bead set, ≥100 events were read and results were expressed as median fluorescence intensity (MFI) per 100 beads. The cutoff value was calculated for each peptide as the mean MFI for all antibody-negative reference serum samples plus 5 SD as an adaptation of the strategy defined for ELISA and was set at 200 MFI corresponding to a consensus value for all peptides (*19*). Sensitivity and specificity were calculated for homologous (same species) and heterologous (different species) antibody detection.

DBS samples were also tested for HIV cross-reactive antibodies by using the INNO-LIA HIV Confirmation Test (Innogenetics, Gent, Belgium) as described (*10*). This test configuration includes HIV-1 and HIV-2 recombinant proteins and synthetic peptides that are coated as discrete lines on a nylon strip.

DNA Extraction and NHP Species Confirmation

Total DNA was extracted from all DBSs by using the Nuclisens MiniMAG Extraction Kit (Biomerieux, Craponne, France) according to the manufacturer's instructions. Minor changes consisted of increasing the incubation time (2 h) of the viral lysis step to increase DNA release (*20*). Species identification recorded in the field was confirmed on all samples by amplifying a 386-bp mitochondrial DNA fragment of the 12S rRNA gene with

Table 2. Amino acid sequences of the 34 SIV/HIV peptides used to develop the xMAP gp41 and V3-loop multiplex Luminex assay

Peptide	SIV lineage	gp41 peptide sequences	V3-loop peptide sequences
HIV-1 M	HIV1/SIVcpz/gor	LAVERYLKDQQLLGIWGCSGKLIC	NNTRKSVRIGPGQAFYATGDIIGDIRQAYC
HIV-1 O	HIV1/SIVcpz/gor	LALGTLIQNQQLLNLWGCKGKLIC	NLTVQEIKIGPMAWYSMGLAAGNGSRAYC
HIV-1 N	HIV1/SIVcpz/gor	LAIGRYLRDQQILSLWGCSGKTIC	NNTGGQVGIGPAMTFYNIGKIVGDIRKAYC
SIVcpz*Pts*	HIV1/SIVcpz/gor	LAVEKYLRDQQLLSLWGCADKVTC	NRTVRNLQIGPGMTFYNVEIATGDTRKAFC
SIVcpz*Ptt*	HIV1/SIVcpz/gor	LAVERYLQDQQILGLWGCSGKAVC	NNTRGEVQIGPGMTFYNIENVVGDTRSAYC
SIVgor	HIV1/SIVcpz/gor	LAIETYLRDQQLLGLWGCTGKLIC	NNTRGQIQIGPMTIYNSERIIGNTRKAYC
HIV-2/SIVsmm	HIV-2/SIVsmm	TAIEKYLKDQAQLNSWGCAFRQVC	GNKTVVPITLMSGLVFMSQPINKRPRQAWC
SIVrcm	SIVrcm	TAIEKYLADQSLLNTFGCAWRQVC	SNRTVKGISLAIGVFISLRVEKRPKGAWC
SIVagm	SIVagm	TALEKYLEDQARLNAWGCAWKQVC	GNKTVLPVTIMAGLVFHSQKYNTLLRQAWC
SIVgsn	SIVgsn/mus/mon†	SSLEKYLRDQTILQAWGCANRPIC	GNKTIRNLQIGAGMTFYSQVIVGGNTRKAYC
SIVmus	SIVgsn/mus/mon†	TALEKFVKDQAILNLWGCANRQIC	†
SIVmon	SIVgsn/mus/mon†	TAVEKFIKDQTLLNAWGCANKAVC	†
SIVdeb	SIVdeb	TAIEKYLKDQAKLNEWGCAFKQIC	GNKTYRAVHMATGLSFYTTFIPRLRIKRAHC
SIVtal	SIVtal‡	TALEKYLEDQAKLNSWGCAWKQIC	*RTIKDLQIAAGLMFHSQIIAGKDLKRAY*
SIVsyk	SIVsyk	TALETYLRDQAIMSNWGCAFKQIC	GNESIKNIQLAAGYFLPVIQGKLKTGRDAKRAFC
SIVlho	SIVlho/sun	TAIEEYLKDQALLASWGCQWKQVC	GNRSEVSTISSTGLLFYYGLEHGSRLRLAQC
SIVmnd-2	SIVmnd	TALEDYVADQSRLAVWGCSFSQVC	GNRSVVSTPSATGLLFYHLGPGKNLKKGMC
SIVwrc	SIVwrc	SAIEGFLEDQLKLKQWGCELTQVC	GNRSVVSVNSASGLIYYAGLEPHRNIRKGLC
SIVcol	SIVcol‡	*ATIEGYLEEQAKLASIGCANMQIC*	GNSSHRNLNTANGAKFYYELIPYSKGIYGRC

*SIV, simian immunodeficiency virus.
†V3 amino acid sequences of SIVgsn, SIVmus, and SIVmon were identical, and only the SIVgsn above the V3-loop peptide was synthesized.
‡Synthesis of SIVcol gp41 and SIVtal V3-loop peptides was unsuccessful.

primers 12S-L1091 and 12S-H1478 (*21*). PCR products were purified by electrophoresis on a 1% agarose gel and directly sequenced (ABI PRISM Big Dye Terminator Cycle sequencing Ready Reaction Kit with amplitaq FS DNA polymerase) on an automated sequencer (ABI 3130XL, Applied Biosystems, Courtaboeuf, France). Sequences were then assembled by using the software package Lasergene (DNASTAR, Inc, Madison, WI, USA).

Molecular Characterization and Phylogenetic Analyses of SIVs

PCR analyses were performed on SIV antibody-positive samples by using described conditions with universal HIV/SIV and SIV lineage-specific primers in *pol* or *env* shown in Table 3 (*8*,*15*,*22*–*24*). PCR products were purified by electrophoresis on a 1% agarose gel and directly sequenced as described above. Newly derived SIV nucleotide sequences were aligned with reference sequences of the different HIV/SIV lineages with MEGA4 and ClustalX version 2 (*25*) and minor manual adjustments when necessary. Nucleotide sites that could not be unambiguously aligned were excluded from the analyses. Appropriate models of evolution were selected for each data set by using Topali software (*26*) and maximum-likelihood phylogenies were reconstructed by using PhyML (*27*). The analyses were performed by using discrete gamma distribution and generalized time-reversible model. The starting tree was obtained by using PhyML. One hundred bootstrap replications were performed to assess confidence in topology. New sequences have been deposited in GenBank under accession nos. JN020273–JN020279 and GU989632.

Table 3. Primers used to amplify simian immunodeficiency virus from dried blood spot samples

Primer	Sequences, 5′ → 3′*	Region targeted	Estimated amplicon size, bp	References
DR1	TRCAYACAGGRGCWGAYGA	*pol*	800	(*8*,*22*)
DR2	AIADRTCATCCATRTAYTG			
DR4	GGIATWCCICAYCCDGCAGG		200	
DR5	GGIGAYCCYTTCCAYCCYTGHGG			
polis4†	CCAGCNCACAAAGGNATAGGAGG	*pol*	800	(*8*,*22*)
polOR†	ACBACYGCNCCTTCHCCTTTC			
polis2†	TGGCARATRGAYTGYACNCAYNTRGAA		400	
uni2†	CCCCTATTCCTCCCCTTCTTTTAAAA			
polis4†	CCAGCNCACAAAGGNATAGGAGG	*pol*	800	(*8*,*22*)
polOR†	ACBACYGCNCCTTCHCCTTTC			
polis4†	CCAGCNCACAAAGGNATAGGAGG		650	
uni2†	CCCCTATTCCTCCCCTTCTTTTAAAA			
CNMF1	TATCCYTCCYTGTCATCYCTCTTT	*pol*	2,750	(*23*)
POLor2	ACBACWGCTCCTTCWCCTTTCCA			
CNMF2	AATGGAGAATGYTMATAGATTTCAG		2,050	
CNMR	CCCCYATTCCTCCCTTTTTTTTA			
SPBS	GGCGCCCGAACAGGGACTTG	*gag-pol*	2,500	(*23*)
2500P1	CCTCCTATGTTCCCCTATTTCTCTG			
CNM.G1	CGAGGCACTCGGCGATGCTGA		2,200	
2500P2	GGAACTGAGAAGGCTGTGTAAGGC			
2500L1	CTATCCCCAAACGCATCCGC	*env-gag*	2,000	(*23*)
CNM.G1rev	TCAGCATCGCCGAGTGCCTCG			
2500L2	AGAAAAGGGAGGACTGGAAGGGAT		800	
SPBSrev	CAAGTCCCTGTTCGGGCGCC			
CNMenvF1	TGTGTSAAAYTRACHCCNATGTGTGT	*env*	2,480	(*23*)
CNMenvR1	AACATNNCYTCYAGTCCTCYCTTTTYT			
CNMenvF2	TCCTTYAAYCAGACYACAGARTTYAGRGA		2,140	
CNMenvR1	GGGATAGCCANGAATTNTCNCCAT			
wrcpolF1	TAGGGACAGAAAGTATAGTAATHTGG	*pol*	1,100	(*24*)
wrcpolR1	GCCATWGCYAA TGCTGTTTC			
wrcpolF2	AGAGACAGTAAGGAAGGGAAAGCAGG		650	
wrcpolR2	GTTCWATTCCTAACCACCAGCADA			
wrcenvF1	TGGC AGTGGGACAAAAATATAAAC	*env*	750	(*24*)
wrcenvR1	CTGGCAGTCCCTCTTCCA AGTT GT			
wrcenvF2	TGATAGGGMTGGCTCCTGGTGATG		550	
wrcenvR2	AATCCCCATTTYAACCAGTTCCA			

*R = A or G; M = A or C; W = A or T; S = G or C; Y = C or T; B = C, G, or T; H = A, C, or T; and N = A, C, G, or T.
†Primers that amplifie simian immunodeficiency virus from dried blood spot samples in this study.

Results

Performance of the HIV/SIV Lineage Specific xMAP Assay on a Reference Panel of NHP Samples

Table 4 summarizes the sensitivity and specificity of homologous and heterologous antibody detection of the xMAP assay on the same reference panel that was used for the SIV lineage–specific ELISAs (*17*,*18*). The homologous gp41 peptide was available for 39 samples; 34 (87.2%) reacted with their gp41 peptide counterpart. Similarly, 46 (93.9%) of the 49 samples for which the homologous V3 peptide was available reacted with their V3 peptide counterpart. The combination of homologous gp41 and V3 peptides identified 47 (95.9%) of the 49 SIV-positive

Table 4. Sensitivity and specificity of SIV/HIV peptides used in the xMAP assay to detect SIV infection in human and nonhuman primate samples*

Species (common name)	Peptide	Homologous detection						Homologous and heterologous detection			
		SIV+			SIV–			SIV+		SIV–	
		gp41	V3	gp41 + V3	gp41	V3	gp41 + V3	gp41	V3	gp41	V3
Pan troglodytes troglodytes (west central chimpanzee)	SIVcpz*Ptt*	1/2	1/2	1/2	0/1	0/1	0/1	3/49	4/49	0/93	0/93
Pan troglodytes schweinfurthii (eastern chimpanzee)	SIVcpz*Pts*	1/1	1/1	−/−	−/−	−/−	−/−	8/49	13/49	0/93	0/93
Gorilla gorilla gorilla (western lowland gorilla)	SIVgor	−/−	−/−	−/−	−/−	−/−	−/−	1/49	0/49	0/93	0/93
Cercopithecus nictitans (greater spot-nosed monkey)	SIVgsn	5/6	6/6	6/6	0/45	0/45	0/45	14/49	15/49	0/93	0/93
Cercopithecus cephus (mustached monkey)	SIVmus	7/7	7/7	7/7	0/30	0/30	0/30	23/49	15/49	0/93	0/93
Cercopithecus mona (Mona monkey)	SIVmon	−/−	−/−	−/−	0/1	0/1	0/1	12/49	15/49	0/93	0/93
Miopithecus ogouensis (northern talapoin)	SIVtal	1/1	−/−	−/−	0/6	0/6	0/6	6/49	−/−	0/93	0/93
Cercopithecus neglectus (De Brazza monkey)	SIVdeb	7/7	6/7	7/7	0/1	0/1	0/1	19/49	6/49	0/93	0/93
Cercopithecus albogularis (Sykes' monkey)	SIVsyk	−/−	−/−	−/−	−/−	−/−	−/−	11/49	1/49	0/93	0/93
Cercocebus atys (sooty mangabey)	SIVsmm	−/−	−/−	−/−	−/−	−/−	−/−	30/49	1/49	0/93	0/93
Cercocebus torquatus (red-capped mangabey)	SIVrcm	4/4	4/4	4/4	−/−	−/−	−/−	7/49	4/49	0/93	0/93
Chlorocebus tantalus (African green monkey)	SIVagm	1/1	1/1	1/1	−/−	−/−	−/−	5/49	3/49	0/93	2/93
Mandrillus sphinx (mandrill)	SIVmnd-2	7/10	10/10	10/10	0/2	0/2	0/2	13/49	11/49	0/93	0/93
Cercopithecus lhoesti (L'Hoest's monkey)	SIVlho	−/−	−/−	−/−	−/−	−/−	−/−	6/49	6/49	0/93	0/93
Procolobus badius (western red colobus)	SIVwrc	−/−	−/−	−/−	−/−	−/−	−/−	1/49	6/49	0/93	0/93
Colobus guereza (mantled guereza)	SIVcol	−/−	9/10	9/10	−/−	0/7	0/7	−/−	9/49	0/93	0/93
*Homo sapiens (*human)	HIV-1M	−/−	−/−	−/−	−/−	−/−	−/−	4/49	1/49	0/93	0/93
	HIV-1-O	−/−	−/−	−/−	−/−	−/−	−/−	1/49	0/49	0/93	0/93
	HIV-1N	−/−	−/−	−/−	−/−	−/−	−/−	9/49	1/49	0/93	0/93
Total		34/39	46/49	47/49	0/87	0/87	0/87	49/49	47/49	0/93	2//93
Sensitivity		87.2%	93.9%	95.9%	NA	NA	NA	100%	95.5%	NA	NA
Specificity		NA	NA	NA	100%	100%	100%	NA	NA	100%	97.9%

*SIV, simian immunodeficiency virus; −/−, not calculated because homologous serum and/or peptide not available; NA, not applicable.

samples. All 49 SIV positive samples were identified by combining homologous and heterologous gp41 reactivities, including SIVcol positive samples for which no homologous gp41peptide was available, resulting in 100% sensitivity. The 3 SIVmnd samples that were not detected by the homologous gp41 peptide were all detected with the SIVmnd V3 peptide, and the SIVcpz*Ptt* sample that was not detected by the SIVcpz peptides was reactive with the HIV-1 N gp41 peptide. Each gp41 peptide cross-reacted with ≥1 sample from a different primate species (data not shown); highest cross-reactivities were for SIVmus (23/48, 47.9%) and SIVsmm (30/48, 62.5%) peptides. Finally, none of the negative serum samples showed positive results with homologous gp41 or V3 peptides. However, 2 (1 *Cercopithecus nictitans* and 1 *C. cephus* monkey) reacted weakly (MFI/cutoff ratio <2) with a single heterologous V3 peptide from SIVagm, resulting in an overall 100% and 97.9% specificity of homologous and heterologous antibody detection, respectively. Given the extraordinary SIV diversity, few false-negative samples were observed, and the combination of all peptides in a single well resulted in 100% sensitivity and 97.5% specificity. Thus, the new assay should enable detection of most SIV infections.

NHP Species Collected as Bushmeat at the Different Localities in DRC

DBS samples were obtained from 330 NHPs in 3 sites, but most (258/330, 78.2%) were collected around Kole (Figure 1). Species were identified in the field by pictographs and confirmed by sequence analysis of the 12S rRNA gene. This analysis identified 7 species: 147 yellow-nosed red-tailed guenons (*C. ascanius whitesidei*), 79 Tshuapa red colobus monkeys (*Piliocolobus tholloni)*, 33 Wolf's monkeys (*C. mona wolfi)*, 33 black mangabeys (*Lophocebus atterrimus atterrimus)*, 25 Angolan pied colobus (*Colobus angolensis angolensis)*, 10 De Brazza monkeys (*C. neglectus),* and 3 Allen swamp monkeys (*Allenopithecus negroviridis*) (*28*). Four of the 7 species or subspecies are only present in DRC, i.e., red-tailed guenons, tshuapa red colobus, black mangabeys, and Wolf's monkeys (*28*).

Prevalence and Genetic Diversity of SIVs in DRC

Because 4 of the 7 NHP species or subspecies are only present in DRC, they are most likely infected with SIVs that have not been documented, and antibody detection will thus depend on extent of cross-reactivity with antigens of known HIV/SIV lineages. Therefore, we screened all samples with the new SIV/HIV xMAP assay and with the HIV line immune assay (INNO-LIA) confirmation assay, which we previously used to detect a large diversity of SIV infections (*8*). Table 5 shows the number of SIV-positive and indeterminate samples per species. SIV infection was documented in 6 species and the overall prevalence was 19% (64/330) ranging from 0% to 25% per species. Highest SIV prevalences were seen in red-tailed guenons (25%) and Tshuapa red colobus (24%), which represent 70% (226/330) of the primate bushmeat

Table 5. Number and percentage of SIV antibody–positive samples per species and number of samples confirmed by PCR and sequence analysis per species and per site*

Species (common name)	Antibody detection, no. (%)											PCR, no. pos/no. tested
	Kole			Malebo			Monkoto†		Total			
	Tested	Pos	Ind	Tested	Pos	Ind	Tested	Pos	Tested	Pos	Ind	
Allopithecus negroviridis (Allen's swamp monkey)	1	0	0	NA	NA	NA	2	0	3	0	0	Not done
Colobus angolensis (Angolan colobus)	21	1 (5.0)	0	NA	NA	NA	4	1 (25.0)	25	1 (4.0)	0	0/1
Cercopithecus ascanius (red-tailed monkey)	94	23 (24.4)	4 (4.2)	40	12 (30.0)	3 (7.5)	13	3 (23.0)	147	37 (25.0)	7 (4.7)	4/37
Cercopithecus neglectus (De Brazza monkey)	7	2 (28.0)	0	2	0	0	1	0	10	2 (20)	0	1/2
Cercopithecus wolfi (Wolf's monkey)	27	5 (19.0)	3 (11.0)	1	0	0	5	3 (60.0)	33	4 (12.1)	3 (9.1)	1/4
Lophocebus atterimus (black mangabey)	30	2 (6.0)	0	NA	NA	NA	3	0	33	1 (3.0)	0	0/1
Piliocolobus tholloni (Tshuapa red colobus)	78	19 (24.0)	0	NA	NA	NA	1	0	79	19 (24.1)	0	2/19
Total	258	52 (20.0)	7 (3.0)	43	12 (27.0)	3 (7.0)	29	7 (21.0)	330	64 (19.0)	10 (3.0)	8/64

*SIV, simian immunodeficiency virus; pos, positive; ind, indeterminate; NA, not applicable (samples not available). Samples were scored as SIV positive when both assays (xMAP and HIV INNO-LIA) were clearly positive or positive in 1 assay (MFI/cutoff>2 or reactivity with at least 1 HIV antigen with a band intensity equal to or greater than the assay cutoff). Samples were scored indeterminate (ind) when reactive in HIV INNO-LIA with at least one HIV antigen with a band intensity equal to or greater than the assay cut-off and weakly positive (MFI/cut-off ratio between 1 and 2) in the other assay or when both assays were indeterminate; samples were considered as negative when both test results were negative.
†No samples from Monkoto were indeterminate.

collected in this study. We also observed HIV/SIV cross-reactive antibodies in De Brazza monkeys (20%), Wolf's monkeys (12 %), black mangabeys (3%), and Angola pied colobus (4%). In addition, 3% (10/330) of the samples were considered as indeterminate for SIV. Notably, all samples from Tsuapa red colobus were only reactive in the xMAP assay and showed strong cross-reactivity with SIVwrc antigens from western red colobus (*Piliocolobus badius*), illustrating clearly the need for including a wide variety of SIV antigens to uncover new SIV lineages.

Genetic Diversity of SIVS in DRC

To confirm SIV infection and document SIV diversity, all SIV-positive and indeterminate samples were subjected to PCR amplification. Although DNA integrity was sufficient to confirm the primate species in all DBSs, proviral SIV DNA could only be amplified in *pol* (400 bp) for 8 samples, most likely because of DNA degradation related to long and suboptimal storage at ambient temperature in the field and the fact that animals died several days before sampling. SIV infection was confirmed in 4 red-tailed guenons, 1 Wolf's monkey, 1 De Brazza monkey, and 2 Tshuapa red colobus. Phylogenetic tree analysis shows the presence of new SIV lineages in Wolf's monkeys and Tshuapa red colobus (Figure 2). SIVwol is close to SIVden obtained from Dent's monkeys (*C. mona denti*), which are found in eastern DRC but without overlapping habitats with Wolf's monkeys in central DRC (*28*). SIVtrc from Tshuapa red colobus forms a separate lineage although related to SIVkrc from Kibale red colobus in eastern Africa (*29*). SIVasc from red-tailed guenons forms a species-specific lineage with SIVasc described in a capitive animal, but a high genetic diversity is seen (*30*). The reported *pol* sequence from a captive black mangabey housed in the zoo in Kinshasa, also falls within the SIVasc radiation (*31*). Finally, SIVdeb clustered within the species-specific SIVdeb lineage observed for De Brazza monkeys across central Africa (*32*).

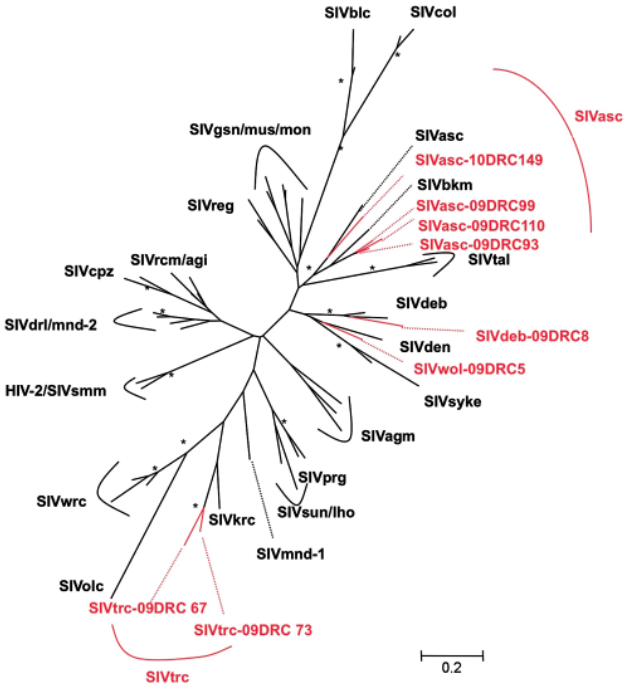

Figure 2. Phylogenetic relationships of the newly derived simian immunodeficiency virus (SIV) sequences in *pol* to representatives of the other SIV lineages. Newly identified strains in this study are in red and reference strains are in black. Unrooted trees were inferred from 350-bp nucleotides. Analyses were performed by using discrete gamma distribution and a generalized time reversible model. The starting tree was obtained by using phyML (*27*). One hundred bootstrap replications were performed to assess confidence in topology. Numbers at nodes are from 100 bootstrap replicates; only those ≥90% are shown with an asterisk. Scale bar represents nucleotide replacements per site.

Discussion

In this study, we used a novel high throughput immune assay that included 34 HIV and SIV antigens in a single well to evaluate prevalence and genetic diversity of SIVs from NHPs at the primate/human interface in DRC. Overall, we showed that ≈20% of NHP bushmeat is infected with SIVs and identified new SIV lineages. Highest SIV prevalences were seen among the most commonly hunted primate species.

Although SIV lineage-specific ELISAs were highly sensitive and specific (*14*–*17*), with the increasing number of new SIV lineages and the high genetic diversity within SIV lineages, a large number of antigens must be included. Using a large set of SIV antigens is necessary, especially when new species are tested for which no SIVs have been reported and when antibody detection is based on cross-reactivity with antigens from heterologous SIV/HIV lineages. To reduce increasing workload and volumes of scarce biological material, we adapted xMAP technology to enable a single sample to be tested simultaneously for multiple peptides (*18*). We used the same gp41 and V3-loop peptides as in the SIV lineage-specific ELISAs and the same reference panel of NHP samples to validate the assay (*14*,*15*). We updated the assay with antigens of SIVgor from gorillas (*3*) and HIV-1 groups M, N, and O. The homologous reactivity for some gp41 peptides, especially SIVmnd, was lower in the xMAP assay (95.9%) compared with that of ELISAs (97.5%) (*15*). However, these samples were also only weakly reactive in the gp41 ELISA, suggesting that all antigens in a single well could slightly reduce sensitivity when mismatches are present in corresponding gp41 sequences. The combination of 34 peptides in a single well detected SIV infection in the reference panel with 100%

sensitivity and 97.9% specificity and reduced workload and volumes of biological material. The need for including a wide diversity of SIV antigens was clearly illustrated by identification of SIVtrc in Tshuapa red colobus samples, which showed negative results with the INNO-LIA HIV confirmatory assay.

No extensive studies have been conducted on SIV infection in monkeys from DRC, which harbors many species because of the geographic barriers constituted by the Congo, Ubangui, and Kasai Rivers (*28*). Overall, ≈20% of primate bushmeat was SIV infected, and as observed in previous studies, prevalences varied per species (*17*,*18*). We confirmed SIV infection in De Brazza monkeys and red-tailed guenons (*14*,*15*,*30*) and identified new SIV lineages in Wolf's monkeys (SIVwol) and Tshuapa red colobus (SIVtrc). De Brazza monkeys seem widely infected with SIVdeb across central Africa: 20% in DRC and 40% in Cameroon (*15*). A high genetic diversity is seen in the SIVasc lineage, and this lineage also includes the previously reported SIVbkm sequence from a captive black mangabey from the zoo in Kinshasa, DRC (*31*). Attempts to amplify an SIV in a wild black mangabey in our study were unsuccesful, and more studies are needed to clarify whether the initial SIVbkm infection is caused by contamination from a red-tailed monkey in captivity or in the wild because black mangabeys share habitats with *Cercopithecus* species. Finally, only full-length genome sequences will enable understanding of the evolutionary history of the new SIVwol and SIVtrc viruses.

In addition to many other factors, risk for cross-species transmissions most likely depends on frequency of human contacts with infected primates and on prevalences in frequently hunted species (*33*). For example, SIVcpz*Ptt* and SIVsmm prevalences are highest (30% and 50%, respectively) in areas in west-central and western Africa where precursors of HIV-1 M (M and N) and HIV-2 (A and B) have been identified in chimpanzees and mangabeys, respectively (*2*,*34*). In contrast to our study on SIV prevalences in primate bushmeat in Cameroon, in which we showed <3% seroprevalence (*15*), we observed in DRC high rates of SIV infection (19%) and highest prevalences in the 2 most commonly hunted species. This observation shows clearly that exposure to SIV differs across Africa and that the likelihood for SIVs to cross the species barrier could be higher in DRC than in Cameroon. In addition, contemporary simian foamy virus infections from Angolan pied colobus and red- tailed guenons have been identified in persons living around an area where we collected samples from primate bushmeat, thus confirming ongoing cross-species transmission with simian retroviruses (*35*).

Given the enormous size of the country and absence of road infrastructure, persons in DRC rely on bushmeat for subsistence in many areas. Among ≈70 million inhabitants, 60% live in rural areas (*36*). DRC is recognized as the area where the HIV-1 group M epidemic originated (*37*), but exact conditions associated with epidemic spread of HIV in this part of the world are still incomplete. The internal and regional armed conflicts in DRC that started in 1997 have led to profound socioeconomic changes and internal displacement of human populations (*38*). The long period of civil unrest that followed the outbreak of those conflicts damaged the health care system, and today 70% of the population has little or no access to health care, including HIV/AIDS services (*36*). New epidemic outbreaks, especially with diseases having a long incubation period, can go unrecognized for a long period. In addition, these SIV strains are not recognized by commercial HIV-1/HIV-2 screening assays. A multiplying factor is the presence of logging and mining industries in remote areas that provide favorable conditions for increased human contact with primates and exchanges between urban and rural settlements. High HIV prevalence was reported around logging industries and in displaced population groups (*39*,*40*), which could lead to recombinants between HIVs and SIVs and enable more efficient adaptation and replication in the new host in addition to rapid and further spread to other geographic areas.

Prevalences and high exposure are among the factors that most likely play a role in the transmission of certain SIVs and other simian retroviruses to humans, but viral and host factors also play a role in establishing efficient infection and disease. Given the ongoing contacts between infected NHP and African populations through hunting and butchering, it is likely that SIV cross-transmissions are still occurring. Viruses can remain unrecognized because of a weak health infrastructure and because they are not detected by commercial HIV screening assays. With increasing travel, new viruses can reach other areas rapidly, which have favorable conditions for epidemic spread. It will be necessary to determine whether other SIVs crossed the species barrier, especially in human populations exposed to highly infected primates and in populations with risk behavior that is associated with high epidemic spread. With the new assay that we developed in this study, large-scale screening against a wide variety of antigens is now easier and faster.

Acknowledgments

We thank the Ministries of Health and Environment and the National Ethics committee from the DRC for permission to conduct this study; the field staff in the DRC for assistance; and the staff of World Wildlife Fund for Nature in the DRC and Didier Mazongo, Lisette Makaya, Gerry Makaya, Frederic Besomba, and Vincent Ntshikala for their collaboration and participation in this study.

This study was supported in part by grants from the National Institute of Health (RO1 AI 50529) and the Agence Nationale de Recherches sur le SIDA (ANRS 12125/12182). S.A.-M. is supported by a grant from Infectiopole Sud, France.

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Astroviruses in Rabbits

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By screening rabbits with enterocolitis or enteritis complex and asymptomatic rabbits, we identified a novel astrovirus. The virus was distantly related (19.3%–23.7% aa identity) in the capsid precursor to other mammalian astroviruses within the *Mamastrovirus* genus**.** By using real-time reverse transcription PCR, with specific primers and probes and targeting a conserved stretch in open reading frame 1b, we found rabbit astrovirus in 10 (43%) of 23 samples from animals with enteric disease and in 25 (18%) of 139 samples from asymptomatic animals in Italy during 2005–2008. The mean and median titers in the positive animals were $10^2\times$ and $10^3\times$ greater, respectively, in the symptomatic animals than in the asymptomatic animals. These findings support the idea that rabbit astroviruses should be included in the diagnostic algorithm of rabbit enteric disease and animal experiments to increase information obtained about their epidemiology and potential pathogenic role.

Astroviruses (AstVs) (family *Astroviridae*) are nonenveloped, and their genome is composed of a plus-sense single-stranded RNA of 6.4–7.3 kb, containing 3 open reading frames (ORFs) and a 3′ poly-A tail (*1*). Two ORFs, located at the 5′ end of the genome (ORF1a and ORF1b), encode nonstructural proteins, and ORF2, located at the 3′ end, encodes the capsid protein (*1*). AstVs were first identified by electron microscopy (EM) in 1975 in Scotland in fecal specimens of infants hospitalized with diarrhea (*2*). Subsequently, similar viruses were identified from several mammalian and avian species (*3*–*12*), including bats (*13*) and aquatic mammals (*14*). AstV infection is associated with gastroenteritis in most animal species and humans. AstVs are regarded as the second or third most common cause of viral diarrhea in children (*1*). Avian AstVs have also been associated with extraintestinal diseases, such as nephritis in chickens (*12*) and hepatitis in ducks (*11*). Even more notably, recently AstVs have been detected in the nervous tissues of minks with shaking disease (*15*) and in the central nervous system of a child with encephalitis (*16*). Also, novel human AstVs (MLB1, MLB2, VA1, HMO-C, HMO-B, HMO-A, VA-2) have been identified that are genetically unrelated to classical human AstVs (*17*–*19*) and more closely related to animal AstVs.

Rabbit enteritis, also referred to as enteritis complex (EC) or rabbit enterocolitis (REC), is a multiform enteric disease, characterized by a variety of symptoms. The syndrome can be caused by bacteria, viruses, and parasites. Moreover, environmental factors can alter rabbit physiology and impair rabbit welfare, thus increasing the effects EC/REC syndrome would have on rabbit production. Several different viruses have been isolated from rabbits with diarrhea, such as rotavirus, coronavirus, parvovirus, adenovirus, and caliciviruses (*20*). Whether natural outbreaks of enteritis can be caused by these viral agents alone or in conjunction/synergism with other pathogens is not clear, and the mechanisms of persistence/transmission are also not known.

Although AstVs have a peculiar star shape when purified fractions are observed in EM, which distinguishes them from other small, rounded viruses (SRVs), such as enteroviruses and caliciviruses, identifying them can be difficult when examining biologic samples because their typical morphologic features tend to be altered easily. During 1997–2005 surveillance by the National (Italian)

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DOI: http://dx.doi.org/10.3201/eid1712.110967

Reference Centre for Viral Diseases of Rabbits, SRVs were identified by EM in 18 (3.49%) of 515 fecal samples from rabbits with enteric disease (*20*,*21*). In this study, we report the detection and characterization of AstVs in the intestinal contents of rabbits affected by EC/REC.

Materials and Methods

Samples from Animals with Enteritis (Collection A)

A total of 23 pooled (2–5 animals) and single samples (various tracts of small and large intestine and/or intestinal contents of rabbits with enteritis) were collected from 23 commercial rabbitries in Italy during 2005–2008. EC/REC of various degrees of severity was described in the herds, with animals ranging in age from 35 to 55 days (Table 1). The samples were sent to the laboratories of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy. After routine laboratory investigations (bacteriologic and parasitologic analysis for enteric pathogens), the samples were stored at –80°C. For bacteriologic analysis, samples were inoculated on MacConkey and Columbia blood agar (Liofilchem, Teramo, Italy), under aerobic and anaerobic conditions, at 37°C for 48 h. The presence of parasites was investigated by microscopy observation of smears made from the fecal or intestinal content specimens, both directly and after concentration by flotation.

Samples from Asymptomatic Animals (Collection B)

A total of 139 fecal samples were collected from postweaning rabbits (30–35 days of age) from 15 herds. EC/REC disease was not reported in the history of the herds, and the animals were overtly healthy at the time of sampling. The samples were stored at –80°C until use.

RNA Extraction and Screening for AstVs by Reverse Transcription PCR

RNA extracts were prepared from 10% homogenates in phosphate-buffered saline, pH 7.3, after clarification by centrifugation at 10,000 × *g* for 1 min. Viral RNA was extracted by using the QIAamp viral RNA kit (QIAGEN GmbH, Hilden, Germany). The samples from collection A were used for an initial screening with a broadly reactive primer pair, targeted to the ORF1b region of AstV (*13*). The initial screening showed PCR amplicons of the expected size (409 bp). Sequence analysis indicated that the sequences displayed 91.9%–96.6% nt identity to each other. BLAST (www.ncbi.nlm.nih.gov) and FASTA (www.ebi.ac.uk/fasta33) with default values were used to find homologous hits in the sequence databases. The sequences displayed the highest identity (69.0%–71.3% nt) to an AstV from a California sea lion (GenBank accession no. FJ890353), thus confirming AstV infection.

Table 1. Astrovirus-positive samples from commercial rabbitries with enteric diseases and results of electron microscopy, clinical observations, and pathologic, bacteriologic, and parasitologic investigations, Italy, 2008*

Sample no.	Place of origin	GE/mL feces	Electron microscopy results†	Clinical observations	Pathologic findings	Bacteriologic findings (intestinal tract)	Parasitologic findings (intestinal tract)
1	Pavia	3.1×10^8	Rotavirus ++ Coronavirus +++	Age 48 d, enteric syndrome	Enterocolitis	*Escherichia coli, Clostridium perfrigens*	Negative
2	Brescia	8.5×10^3	Negative	Age 35 d, enteric syndrome	Enterocolitis	*E. coli*	Negative
3	Brescia	1.5×10^6	Negative	Age 55 d, enteric syndrome; fatal in 24 h	Enteritis with tracts containing fluids and tract filled with feces	Negative	*Coccidia*
4	Nuoro	1.0×10^8	Rotavirus +/–	NA	Catarral enteritis	*E. coli*	*Coccidia*
5	Brescia	9.7×10^7	Rotavirus ++ Phages ++	Age 51 d, enteric syndrome, high mortality	Enterocolitis with swollen colon	*E. coli*	*Coccidia*
6	Brescia	1.0×10^9	Coronavirus+	Age 51 d, enteric syndrome, high mortality	Enteritis	*E. coli*	*Coccidia*
7	Brescia	3.8×10^7	Rotavirus +++ Phages +++	Age 51 d, enteric syndrome	Enterocolitis, with swollen colon	*E. coli*	Negative
8	Padova	7.3×10^3	Rotavirus +++	NA	Catarral enteritis	*E. coli*	Negative
9	Lecco	1.4×10^{10}	Rotavirus +++	NA	Typhlitis and colitis, swollen tracts of the gut with fluid content	*E. coli* (gut), *Streptococcus* spp. (liver and spleen)	*Coccidia*
10	Cagliari	6.5×10^7	Negative	NA	Catharral enteritis	*E. coli*	*Coccidia*

*GE, genome equivalent; NA, not available.
†+/–, very low positivity; +, low positivity; ++, discrete positivity; +++, strong positivity.

Real-Time Quantitative PCR for Rabbit AstVs

The partial ORF1b sequences generated with the AstV broadly reactive ORF1b primers were used to design more specific primer sets and probes for reverse transcription PCR and RT real-time quantitative PCR (RT-qPCR) able to identify and quantify the rabbit AstVs (Table 2). Primers and TaqMan probes were designed by using Beacon Design software version 2.0 (Premier Biosoft International, Palo Alto, CA, USA). The RT-qPCR was performed by using a 2-step protocol and a real-time thermocycler (i-Cycler iQTM Real-Time Detection, Bio-Rad Laboratories, Hercules, CA, USA). The ORF1b amplicon (409 bp) of the rabbit AstV strain Nausika/08/ITA was cloned into pCR4-TOPO vector (TOPO TA cloning, Invitrogen, Milan, Italy) and transcribed in vitro with Ribo-MAXTM Large Scale RNA Production System-T7 (Promega Italia, Milan, Italy) from the T7 promoter, according to the manufacturer's guidelines. The transcribed RNA was quantified and used to generate an RNA standard curve. The detection limit was 10 genomic equivalents (GEs)/50 μL-reaction (cycle threshold = 42.67), corresponding to 3.6×10^2 GE/g of fecal sample. No other enteric viruses, including rabbit rotaviruses and human, canine, porcine, and avian AstV strains, were detected. This RT-qPCR is sensitive and specific for the detection of rabbit AstV.

EM Observation

The samples that contained AstV RNA were processed for EM observation (*22*). Briefly, the feces were diluted 1:10 in distilled water, vortexed, and centrifuged for 20 min at 4,000 × *g* and again for 10 min at 9.300 × *g* for clarification. The supernatant was then ultracentrifuged (Beckman Airfuge, Fullerton, CA, USA) for 15 min at 82.000 × *g*. After negative staining with 2% sodium phosphotungstate (pH 6.8), samples were examined by using a Philips CM10 electron microscope.

Molecular Characterization of Rabbit AstV Strain Nausika/08/ITA

To determine the sequence and genomic organization of the novel rabbit AstV, we selected a sample containing 1.3×10^{10} GE/*g* fecal sample (strain Nausika/ITA/08). A 3.4-kb region at the 3′ end of the genome was amplified by RT-PCR as described by Wang et al. (*23*). cDNA was synthesized by SuperScript III First-Strand cDNA synthesis kit (Invitrogen Ltd, Paisley, UK) with primer VN3T20. PCR was then performed with TaKaRa La Taq polymerase (TaKaRa Bio Europe SAS, Saint-Germain-en-Laye, France) with forward primer and VN3T20. Finally, the amplicon was purified and cloned by using TOPO XL Cloning Kit (Invitrogen Ltd). Additional primers also were designed to determine the complete 3.4-kb sequence by an overlapping strategy. The sequence was deposited in GenBank under accession no. JN052023.

Sequence editing and multiple alignments were performed with Bioedit software package version 2.1 (*24*). Phylogenetic analysis (neighbor-joining and unweighted pair group method) with arithmetic mean with bootstrap analysis (1,000 replicates) and no-distance correction was conducted by using MEGA4 software (*25*).

Analysis of RNA-Dependent RNA Polymerase (ORF1b) and Capsid Protein (ORF2) of Strain Nausika/08/ITA

Pair-wise identity in the ORF1b and full-length capsid protein of strain Nausika/08/ITA to a selection of AstV strains was determined by using multiple alignments generated with Bioedit software package version 2.1 (*24*). The values were calculated with the uncorrected distance method by using amino acid sequence alignment without removing the gaps, including sequences of human and animal AstVs. The strain and sequences used are listed in Table 3.

Results

Screening of Samples from Collections A and B for rabbit AstV by RT-qPCR

Rabbit AstV was detected by RT-qPCR in 10 (43.49%) of 23 samples from collection A (Table 1) and in 25 (17.98%) of 139 from collection B. Rabbit AstV was detected in 12 (80%) of the 15 rabbit herds sampled, with the herd prevalence ranging from 9% to 50%. Virus titers (GE/μL RNA extract) in collection A ranged from 2.0×10^1 to 3.8×10^7 (mean value 4.3×10^6, median value 2.1×10^5), and in collection B from 1.2×10^1 to 1.7×10^6 (mean value 7.6×10^4, median value 1.5×10^2). By comparing the 2 groups using Software R version 2.8.1 (www.r-project.org), by the χ^2 test, the positivity rates differed significantly between groups A and B ($p = 0.0132$; $p<0.05$). To assess

Table 2. Primers used for detection and sequencing analysis of rabbit astroviruses, Italy, 2008

Primer	Sequence, 5′ → 3′	Sense	Reference
702VM-Pb	6FAM-TCTCAACAGGTATGTCGTCCTCCCTTCTGG-BHQ1	+	This study
683VM-F	CCATATAYAAGTGGTATTGCAARCA	+	This study
684VM-R	TTCCGCTGRATGGTRACCTC	–	This study
panAstVFor1	GARTTYGATTGGRCKCGKTAYGA	+	(*13*)
panAstVFor2	GARTTYGATTGGRCKAGGTAYGA	+	(*13*)
panAstVRev	GGYTTKACCCACATNCCRAA	–	(*13*)
VN3T20	$GAGTGACCGCGGCCGCT_{20}$	–	(*23*)

Table 3. Comparison of full-length capsid protein of strain rabbit/Nausika/08/ITA and that of various mammalian and avian astroviruses*

GenBank accession no./ species/strain	% aa identity to rabbit/Nausika/08/ITA† RdRp (ORF1b)	Capsid (ORF2)
AY720892/human/AstV1	62.7	21.8
L06802/human/AstV2	–	22.3
DQ630763/human/AstV3	–	22.5
DQ070852/human/AstV4	–	22.2
U15136/human/AstV5	–	22.0
GQ495608/human/AstV6	61.4	21.9
AF248738/human/AstV7	52.8	22.8
AF260508/human/AstV8	61.4	22.7
FJ973620/human/VA1	–	20.1
GQ502193/human/VA2	52.5	19.7
FJ222451/human/MLB1	61.4	19.6
AF056197/cat	–	21.7
Y15937/sheep	52.9	20.5
AY179509/mink	54.1	20.0
AB037272/pig	–	23.5
FJ890351/CSL/AstV1	52.1	19.3
FJ890352/CSL/AstV2	60.6	23.3
FJ890355/bottlenose dolphin	61.4	23.7
EU847155/bat/AstV1	–	22.8
FJ57174/bat/LC03	–	22.7
FJ57065/bat/LD38	50.9	22.3
HM045005/dog/Bari/08	57.7	23.7
HM450382/rat/RS126/HKG/07	57.7	20.6
GU985458/mink/SMS-AstV/Swe/01	54.1	19.6
AB033998/chicken/ANV-1	36.8	14.9
AB046864/chicken/ANV-2	36.4	14.5
Y15936/turkey/AstV1	36.8	15.5
AF206663/turkey/AstV2	35.6	14.2
FJ434664/duck/C-NGB/China/08	37.2	13.4

*RdRP, RNA-dependent RNA polymerase; ORF, open reading frame; AstV, astrovirus; –, values not calculated; CSL, California sea lion; ANV, avian nephritis virus.
†Full-length aa capsid sequence and partial (245-aa residues) RdRp at the C-terminus were used to calculate identities.

whether a significant difference in virus shedding (titers) between symptomatic and asymptomatic animals, the virus titers of group A and B were compared by the nonparametric Mann-Whitney U test. In this analysis, the 2 animal groups differed significantly ($p = 0.0137$; $p<0.05$).

EM Observation

Upon EM observation, none of the AstV-positive samples contained SRV-like particles. Rotaviruses, coronaviruses, and phages were detected in 8 samples in various combinations, and 2 samples did not contain viral particles (Table 1).

Molecular Characterization of Rabbit AstV Strain Nausika/08/ITA

A 3.4-kb (3,395 nt) sequence at the 3′ end of the genome of strain Nausika/08/ITA was determined. The sequence spanned the 3′ end of ORF1b, the full-length ORF2 and the 3′ noncoding region (NCR) to the poly-A tail. The 3′ end of ORF1b comprised 759 nt, encoding for a 252-aa polypeptide fragment at the C-terminus of the RdRp. By pair-wise comparison in the partial RdRp, the highest aa identity (62.7%) was to human AstV type 1 (GenBank accession no. AY720892). Identity to other mammalian AstVs ranged from 50.9% to 61.4% aa, whereas identity to avian AstVs ranged from 31.0% to 37.5% aa.

An 8-nt overlap occurred between the termination codon of ORF1b and the initiation codon of ORF2. The highly conserved nt stretch upstream of ORF2, ATTTGGAGNGGNGGACCNAAN$_{5–8}$ATGNC, which is believed to be part of a promoter region for synthesis of subgenomic RNA (*26*), was nearly completely conserved in the sequence of strain Nausika/08/ITA. The ORF2 was 2,559 nt and encoded for a capsid protein of 852 aa, with a predicted molecular mass of 84.7 kDa. The NCR was 85 nt. By pair-wise comparison, the highest identity (23.7%) in the capsid protein was found to a canine AstV, strain Dog/Italy05 and to a bottlenose dolphin AstV. Identity to other mammalian AstVs ranged from 19.3% to 23.5% aa, whereas identity to avian AstVs ranged from 13.4% to 14.9% aa (Table 2). The highly conserved motive SRGHAE at the C-terminus of VP1 was not present (*27*). By phylogenetic analysis, the strain was found to segregate in the *Mamastrovirus* genogroup (Figure).

With some exception, most AstVs have a conserved RNA secondary structure, referred to as the stem-loop II-like motif (s2m), located at the 3′ end of the genome in the 3′ NCR (*28*). Nucleotide alignment of the 150 nt at the 3′ terminus of the rabbit AstV Nausika/08/ITA genome and other viruses known to contain the stem-loop motif suggested that Nausika/08/ITA does not have this conserved nucleotide motif (data not shown).

Discussion

Rabbit EC/REC syndrome is a multiform enteric disease characterized by a variety of symptoms. The syndrome may be multifactorial with several microorganisms acting in synergy and with environmental factors also altering or influencing rabbit physiology, metabolism, and immune response. Within this miscellaneous group of enteric pathologies, some diseases appear to possess peculiar features, such as the epizootic rabbit enteropathy, enterotoxemia caused by *Clostridium spiriforme* and *C. perfrigens* (*29*), *C. difficile* (*30*) infection, Tyzzer disease (caused by *C. piliformis)*, mucoid enteropathy, *Escherichia coli* enteritis, and coccidiosis (*31*).

Several viruses have been identified from rabbits with diarrhea (*20*), but whether viruses can act as primary agent of enteritis is not clear. Experimental infection of rabbits with rotavirus has shown that the rotavirus-induced disease

is age restricted to the neonatal period (<2 weeks), although natural infection has been associated with disease in animals after weaning (28–45 days of age) (*32*). Also, maternally derived immunity protects young rabbits up to 2 months of age and may influence the evolution of virus infection or disease. Accordingly, much additional work remains to elucidate the viral pathogenicity and immunology of most enteric viruses of rabbits.

By EM observation, SRV-like viral particles have been seen sporadically in rabbits with EC/REC disease (*20*,*21*), but the exact nature of the observed SRVs, was not investigated. By using an AstV broadly reactive set of primers, we could detect AstV RNA in the intestinal contents of rabbits affected by EC/REC syndrome and the sequences obtained were used to generate more specific diagnostic tools. By rescreening the samples (collection A) with a RT-qPCR, AstV RNA was detected in 43.49% (10/23) of the samples tested. The mean titer in the AstV-positive samples from collection A was 4.3×10^6 GE/μL RNA extract, corresponding to $\approx 1.5 \times 10^9$ GE/mL feces. Notably, by EM observation, none of the AstV-positive samples was clearly found to contain SRV-like particles (Table 1). Immune EM that uses specific hyperimmune or convalescent-phase serum specimens could be necessary to improve the sensitivity of the EM technique. Also enzyme- or pH-mediated alterations in virus morphologic features could be triggered during conservation of samples and therefore hamper recognition of these SRVs. Overall, rabbit AstVs can be assumed to be easily undetected in EM-based surveys, thus leading to underestimation of the potential role of SRVs in rabbit EC/REC syndrome.

To better assess the epidemiology of these viruses, we analyzed a collection of samples obtained from asymptomatic animals (at 30–35 days of age). In these samples, rabbit AstV RNA was detected in 17.98% (25/139) of the samples from 12 of 15 herds. The mean titer in samples from collection B was 7.6×10^4 GE/μL RNA extract, corresponding to 2.7×10^7 GE/mL feces, and this value (mean) was $\approx 10^2$ times lower than in the samples from collection A. Accordingly, the prevalence rates and the virus shedding titers differed markedly and significantly between the 2 sample groups.

The rate of detection of enteric viruses (noroviruses) in humans is significantly higher for symptomatic (37.2%) than asymptomatic patients (14.1%) (*33*). Also, increased viral load in the feces has been associated with greater severity of gastroenteric disease in children infected by group A rotavirus (*34*) and with longer duration of diarrhea, but not with greater disease severity, in children infected with AstVs (*35*). However, the differences observed in rabbit AstV prevalence, and titers between the 2 sample collections are not necessarily suggestive of a pathogenic attitude or role for rabbit AstVs and must be interpreted with caution. Bias in AstV distribution could be accounted for by the sampling inclusion criteria (age of group B animals) or by the relatively small number of samples analyzed.

Regardless, the samples from collection A and B were from herds of different regions in Italy (Emilia Romagna, Lombardia, Sardegna, Umbria, and Veneto). Accordingly,

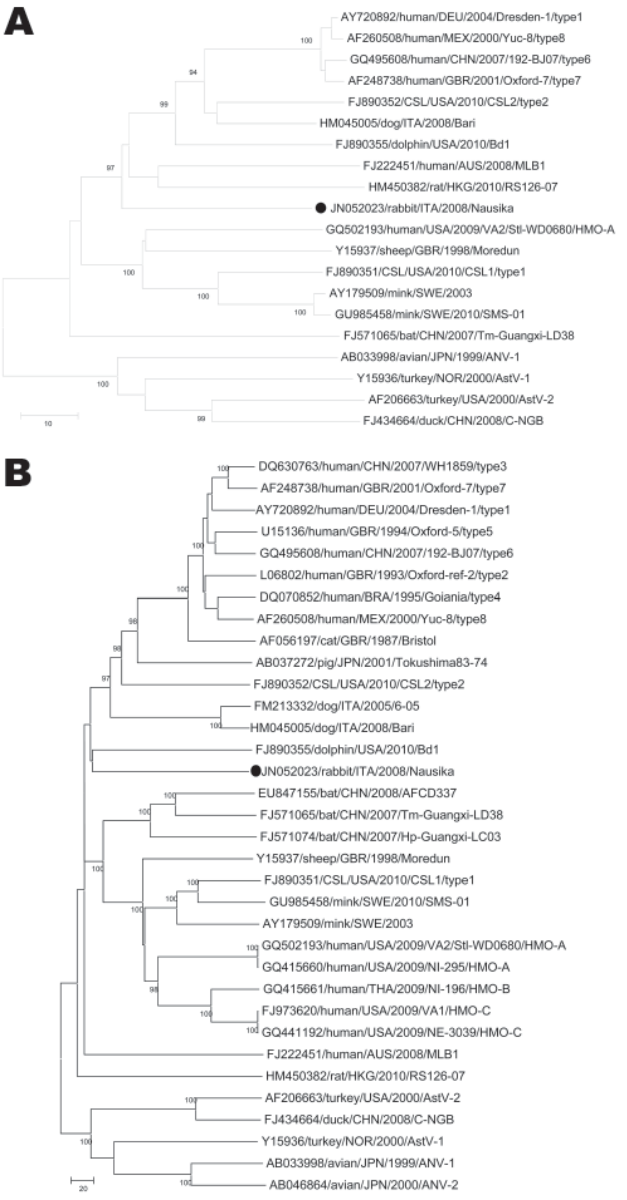

Figure. Phylogenetic trees constructed on the partial (245 aa) RNA-dependent RNA polymerase (panel A, RdRp) (open reading frame [ORF] 1b) and the full-length capsid precursor (panel B, ORF2) amino acid sequences. Black circles indicate strain identified in this study. The trees were constructed by using a selection of astrovirus (AstV) strains. Country names are abbreviated. Scale bars indicate the number of amino acid substitutions per 100 residues. Bootstrap values <90% are not shown. CSL, California sea lion; ANV, avian nephritis virus.

our findings suggest that rabbit AstVs are common in rabbit herds. Cycles of infection in newly susceptible animals, coupled with stability/resistance of SRVs and management conditions (high density of animals), could account for the high prevalence rates observed.

Upon sequence analysis of the full-length capsid sequence, the rabbit strain Nausika/08/ITA was distantly related (19.3%–23.7%-aa identity) to other mammalian AstVs. Similar ranges of genetic diversity/heterogeneity in this portion of AstV genome (ORF2) have been observed in other AstVs found in mammals (*36*). Notably, the 5′ end of ORF2 appeared to be more conserved than the central region and the 3′ end. AstVs contain a conserved region at the junction between ORF1b and ORF2 (*26*). The exact role of this sequence is not known, but it may be a regulatory element of the subgenomic RNA that encodes for ORF2. This region appeared nearly completely conserved in the sequence of strain Nausika/08/ITA. Unlike the situation with other mammalian AstVs, but not unusually, the highly conserved motif SRGHAE at the C-terminus of the capsid protein (*27*) was not found. In addition, the stem-loop II-like motif (s2m) at the 3′ end of the genome in the 3′ nontranslated region (*28*) was not present in strain Nausika/08/ITA. This motif is present in most AstVs, with the exception of turkey AstV 2, human AstV MLB1, and rat AstV (*36*,*37*). This motif is also found in some coronaviruses and equine rhinovirus serotype 2. The conservation of such a sequence motif across multiple viral families suggests that it may play a broad role in the biology of positive-stranded RNA viruses (*28*), although the exact function of this conserved region is still unknown.

In conclusion, we identified a novel AstV in rabbits. Also, we developed an RT-qPCR useful for detection and quantification of rabbit AstV and gathered evidence that AstVs are common in the intestinal content/feces of both symptomatic and asymptomatic rabbits. Including rabbit AstV in the diagnostic algorithms of rabbit enteritis and animal experiments will be useful in clarifying whether these enteric viruses play a role in rabbit EC/REC syndrome.

Acknowledgments

We are grateful to Valeriana Colao for assistance with statistical analysis.

This work was financed by grants from the University of Bari, Italy, and from the Italian Ministry of University and Research.

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A Pilot Study of Host Genetic Variants Associated with Influenza-associated Deaths among Children and Young Adults[1]

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We compared the prevalence of 8 polymorphisms in the tumor necrosis factor and mannose-binding lectin genes among 105 children and young adults with fatal influenza with US population estimates and determined in subanalyses whether these polymorphisms were associated with sudden death and bacterial co-infection among persons with fatal influenza. No differences were observed in genotype prevalence or minor allele frequencies between persons with fatal influenza and the reference sample. Fatal cases with low-producing *MBL2* genotypes had a 7-fold increased risk for invasive methicillin-resistant *Staphylococcus aureus* (MRSA) co-infection compared with fatal cases with high- and intermediate-producing *MBL2* genotypes (odds ratio 7.1, 95% confidence interval 1.6–32.1). Limited analysis of 2 genes important to the innate immune response found no association between genetic variants and fatal influenza infection. Among children and young adults who died of influenza, low-producing *MBL2* genotypes may have increased risk for MRSA co-infection.

It is unknown why some apparently healthy persons become severely ill after influenza infection while others infected by the same strain remain asymptomatic or become only mildly ill. The presence of neutralizing antibody to a specific influenza strain is protective, and certain chronic medical conditions increase the risk for severe outcomes of influenza infections, but the risk factors for influenza-associated deaths among previously healthy persons remain largely unknown (*1*).

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DOI: http://dx.doi.org/10.3201/eid1712.111002

Infectious disease mortality risk has a heritable component; children of parents who died of an infectious disease are ≈6× more likely to die of an infectious cause compared with the general population (*2*). A recent large family study that used genealogic databases found an elevated risk for influenza death among relatives of persons who died of influenza (*3*). By comparing the influenza mortality rate for relatives of persons who died of influenza with the influenza mortality rate for relatives of spouses of persons who died, the authors showed that the increased risk was not explained by shared exposure to influenza virus and thus may have a genetic component. However, to our knowledge, no published studies have examined the association between specific host genetic variants and severe influenza disease outcomes.

To address the paucity of research on host genomics and influenza, the Centers for Disease Control and Prevention (CDC) convened a meeting of experts in 2007 to solicit opinions on how to explore the role of host genomics in public health activities for influenza conducted by the agency. A study of host genomic factors related to severe influenza outcomes in children was recommended as an activity that CDC was well positioned to pursue. This article reports the findings of the study implemented in response to that recommendation.

We conducted a hypothesis-generating pilot study to examine if host genetic variants were associated with fatal influenza virus infection by comparing prevalence of selected host genetic variants among children and

[1]Portions of this study were presented at the Options for the Control of Influenza VII meeting, September 3–7, 2010, Hong Kong.

young adults who died of influenza with population-based prevalence estimates. We focused on 8 single-nucleotide polymorphisms (SNPs) in 2 candidate genes important in the innate immune response to influenza infection and for which national prevalence estimates were available: the gene for tumor necrosis factor superfamily, member 2 (official symbol *TNF*) and the mannose-binding lectin gene (official symbol *MBL2*).

Methods

Study Population

Because influenza-associated deaths in children, but not adults, are nationally reportable in the United States, most cases in this study were pediatric cases reported to CDC through the Influenza-associated Pediatric Mortality Surveillance system. This system requires state public health authorities to report to CDC any influenza-associated death among persons <18 years old that occurred within their jurisdiction. Information collected by this surveillance system constitutes the primary phenotypic information used in this study and includes underlying health status and chronic medical conditions, influenza vaccination status, clinical course and features, and results of microbiologic and virologic testing. Reporting to this surveillance system does not require submission of tissue samples; however, CDC routinely receives tissue samples for a subset of fatal pediatric influenza cases for diagnostic confirmation. For some cases, medical records and autopsy reports provided additional information.

A total of 442 influenza-associated deaths among children (<18 years old) and young adults (18–40 years old) residing in the United States were reported to CDC for the 1998–99 through 2007–08 influenza seasons; of these, 105 cases with laboratory-confirmed influenza infection had sufficient tissue specimens available for DNA extraction and constitute the analytic dataset for this study. Fatal influenza cases were considered laboratory confirmed if a positive test result for influenza by viral culture, immunohistochemical analysis, or reverse transcription PCR (RT-PCR) had been documented. These represented 1) fatal pediatric cases reported to CDC during the 2003–04 influenza season when CDC conducted surveillance for influenza-associated pediatric deaths as part of an emergency response effort; 2) fatal pediatric cases identified through national surveillance since 2004 when pediatric influenza–associated death was made nationally notifiable in the United States; or 3) fatal cases of influenza among young adults at any point in time or among children before 2003 whose case reports and specimens were received by the CDC Infectious Diseases Pathology Branch on a case-by-case basis.

Genotyping

To obtain DNA for genotyping, a 10-μm section from blocks containing formalin-fixed, paraffin-embedded tissues was deparaffinized with xylene and washed twice with absolute ethanol. After residual ethanol evaporated, tissues were digested overnight at 56°C in 200 μL Buffer PKD with 20 μL proteinase K (QIAGEN, Valencia, CA, USA). Extraction of the supernatant was completed with an EZ1 DNA Tissue Kit or a MagAttract DNA Mini M48 Kit (QIAGEN), with DNA eluted into a final 100-μL volume. DNA quality was assessed with a human RNase P real-time PCR in 25-μL volumes by using Agilent Brilliant II QPCR Master Mix as described (*4*). Validated TaqMan assays were used to genotype each SNP (protocols, primers, and probes available at http://snp500cancer.nci.nih.gov). Each 25-μL real-time PCR consisted of 12.5 μL of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 900 nmol of assay-specific primer, 200 nmol of assay-specific probe, and 5 μL of DNA. All controls (extraction blanks, no template controls, and positive controls for each genotype used at 5 ng per PCR; Coriell Institute for Medical Research, Camden, NJ, USA) and unknown samples were assayed in duplicate. Thermal cycling conditions consisted of 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 50 cycles of 92°C for 30 s and 60°C for 1 min. Data were collected during the annealing plateau.

Genotype Definitions

For *TNF*, we examined 3 promoter SNPs: −308G>A (rs1800629), −238G>A (rs361525), and −555G>A (rs1800750) (*5*,*6*); we were unable to infer *TNF* haplotypes. For *MBL2*, we examined 5 SNPS, 3 in the coding region of exon 1 and 2 in the promoter region. The 3 structural SNPs in *MBL2* that we examined encode variant alleles known as *D* (codon 52, rs5030737), *B* (codon 54, rs1800450), and *C* (codon 57, rs1800451); the wild-type is *A* (*7*,*8*). These variants are typically pooled and designated as the *O* allele. The *MBL2* genotype *A/A* refers to wild-type homozygotes, *A/O* refers to heterozygotes, and *O/O* refers to homozygotes or compound heterozygotes. Promoter polymorphisms at positions −550 (*H/L* variant, rs11003125) and −221 (*X/Y* variant, rs7096206) encode variants that mediate *MBL2* expression. Case-patients were classified as low, intermediate, or high producers of MBL on the basis of their structural and promoter variants (referred to as a "truncated haplotype") (*7*). Case-patients homozygous or compound heterozygous for any of the 3 variant structural alleles and case-patients with a variant structural allele on 1 chromosome and the *X* variant on the other were categorized as low MBL producers. Case-patients homozygous for the wild-type structural allele were categorized as high MBL producers except for those also homozygous for

the *X* variant, who were classified as intermediate MBL producers on the basis of evidence that possession of the *X/X* promoter genotype significantly down-regulates MBL production (*9*). Case-patients with the *YA/O* genotype were classified as intermediate MBL producers on the basis of analyses indicating that this genotype confers intermediate levels of functional MBL (*9*). For some analyses, the intermediate and high producers were combined into 1 group and compared with MBL low-producers.

Reference Sample

The prevalence of genetic variants among cases was compared with population-based prevalence estimates for the same genetic variants for the 12–19-year age group available from the National Health and Nutrition Examination Survey (NHANES) III CDC–National Cancer Institute Collaborative Genomics Project databank (*10*). NHANES is a nationally representative survey of the US population conducted by the CDC National Center for Health Statistics. During the second phase of NHANES III (1991–1994), leukocytes from participants were used to create a DNA bank maintained by CDC's National Center for Environmental Health that contains specimens from >7,000 participants, including ≈1,200 children. To our knowledge, the NHANES DNA bank is the only currently available source of nationally representative prevalence estimates for genetic variants among US residents. The 12–19-year age group is the youngest age group available in the NHANES DNA bank.

Variable Definitions

Cases were stratified by presence or absence of any chronic medical conditions in the patients known to increase the risk for influenza-associated complications (including moderate to severe developmental delay; hemoglobinopathy, immunosuppressive disorders, asthma or reactive airway disease, diabetes mellitus, history of febrile seizures, seizure disorder, cystic fibrosis, or cardiac, renal, chronic pulmonary, metabolic, or neuromuscular disorders) (*11*). Case-patients without chronic medical conditions were classified as "previously healthy." Case-patients who were admitted to an inpatient ward or intensive care unit were classified as "hospitalized." Length of illness was defined as the duration of time between the reported date of illness onset and death. Case-patients with length of illness ≤3 days were classified as having "sudden death." Bacterial co-infection was defined as at least 1 positive culture for a bacterial pathogen from a normally sterile site (e.g., blood, cerebrospinal fluid).

Statistical Analyses

Minor allele frequencies between groups were compared with a test of binomial proportions. The null hypothesis was that there was no difference in minor allele frequency between the cases and the reference sample. A priori groups examined in subgroup analyses included previously healthy case-patients, case-patients <5 years old, case-patients with invasive bacterial co-infection, and case-patients with sudden death. Differences in length of illness were evaluated with the Kaplan-Meier estimator with differences tested with the log-rank statistic. Tests of significance were based on a 2-sided test with $\alpha = 0.05$. Tests of departure from Hardy-Weinberg equilibrium for the reference sample have been published (*10*). Analyses were conducted in SAS version 9.2 (SAS Institute, Cary, NC, USA).

Human Subjects

This study was exempted from institutional review board review for approval of human subjects research. Data were obtained only from deceased case-patients, and reference sample data were used only in a de-identified and aggregate manner.

Results

Participant Characteristics

Of 442 cases of fatal influenza in children and young adults reported to CDC during the 1998–99 through 2007–08 influenza seasons, 105 (24%) cases had available autopsy specimens with sufficient DNA for genotyping. Case-patient characteristics are summarized in Table 1. Genotyped case-patients had a median age of 6.0 years (range 1 month–40 years) and 52% were female. Sixty-one percent of case-patients were white, and 17% were black. Seventy-four percent of cases occurred during 3 influenza seasons: 2003–04 (31%), 2006–07 (21%), and 2007–08 (22%). Eighty-one (77%) of 105 case-patients were infected with influenza A and 24 (23%) with influenza B. There were no significant differences in the distribution of influenza types by season between cases and the national pattern of types found in the US viral surveillance system (data not shown).

Compared with case-patients who were not genotyped, the 105 case-patients with DNA available for genotyping were slightly older (median age 6 years vs. 4 years; $p<0.05$), less likely to have had a preexisting medical condition (28% vs. 61%; $p<0.001$), and less likely to have been vaccinated for influenza during the season of death (7% vs. 16%; $p<0.01$). Case-patients genotyped were more likely to have experienced sudden death (31% vs. 22%; $p<0.05$) and to have died before reaching medical care (34% vs. 22%; $p<0.001$). It is not surprising that case-patients with sudden death were more likely to have undergone autopsy and, hence, to have had tissues available for DNA extraction. Genotyped case-patients were less likely to have had pneumonia evident on chest radiograph (22% vs. 46%; $p<0.05$) and about equally likely to have had invasive

bacterial co-infection (21% vs. 23%; not significant), but differences in these characteristics are difficult to interpret because genotyped case-patients were less likely to have received medical care for their illnesses (presumably because of a greater frequency of sudden death).

Genotyping Results

Genotype and minor allele frequencies among case-patients are summarized in Table 2. Minor allele frequencies comparing case-patients to the NHANES reference sample are shown in Figure 1.

TNF

No statistically significant differences were observed in minor allele frequencies or genotype prevalence between the case-patients and the NHANES reference sample for the 3 *TNF* variants with all case-patients examined together or with black and white racial groups examined separately.

Table 1. Characteristics of and course of illness for children and young adults who died of influenza, reported to the Centers for Disease Control and Prevention during influenza seasons 1998–99 through 2007–08, United States*

Characteristic	Genotyping status		All, n = 442
	Genotyped, n = 105	Not genotyped, n = 337	
Patient demographics			
Median age, y (range)†	6.0 (0.1–40.0)	4.0 (0.0–17.3)	4.3 (0.0–40.0)
Female sex	55 (52)	158 (47)	213 (48)
Race			
White	64 (61)	208 (62)	272 (62)
Black	18 (17)	62 (18)	80 (18)
Asian	7 (7)	17 (5)	24 (5)
Other	3 (3)	9 (3)	12 (3)
Unknown/missing	13 (12)	41 (12)	54 (12)
Influenza season			
1998–99	1 (1)	0	1 (<1)
1999–00	0	0	0
2000–01	1 (1)	0	1 (<1)
2001–02	2 (2)	0	2 (<1)
2002–03	10 (10)	0	10 (2)
2003–04	33 (31)	121 (36)	154 (35)
2004–05	7 (7)	44 (13)	51 (12)
2005–06	6 (6)	42 (12)	48 (11)
2006–07	22 (21)	60 (18)	82 (19)
2007–08	23 (22)	70 (21)	93 (21)
Influenza type			
A	81 (77)	233 (69)	314 (71)
B	24 (23)	65 (19)	89 (20)
A/B not distinguished	0	36 (11)	36 (8)
Unknown/missing	0	3 (<1)	3 (<1)
Preexisting medical condition‡	29 (28)	205 (61)	234 (53)
Vaccinated season of death§	7 (7)	54 (16)	61 (14)
Sudden death†	33 (31)	74 (22)	107 (24)
Length of illness, d, median (range)‡	3.0 (0–15)	5.0 (0–194)	5.0 (0–194)
Location of death‡			
ICU/inpatient	28 (27)	216 (64)	244 (55)
Emergency department	25 (24)	48 (14)	73 (17)
Outside hospital	36 (34)	73 (22)	109 (25)
Unknown/missing	16 (15)	0	16 (4)
Mechanical ventilation†	41 (39)	216 (64)	257 (58)
ICU admission‡	28 (27)	165 (49)	193 (44)
Invasive bacterial co-infection	22 (21)	79 (23)	101 (23)
Invasive MRSA co-infection	8 (8)	24 (7)	32 (7)
Pneumonia evident on chest radiograph†	23 (22)	154 (46)	177 (40)

*Values are no. (%) patients except as indicated. A χ^2 test of difference in proportion was used to test differences in proportion between genotyped and ungenotyped cases except for patient age, season, and length of illness, for which the Kruskal-Wallis nonparametric test of difference was used. A total of 442 possible or confirmed influenza-related deaths among children (<18 years old) and young adults (18–40 years old) were reported to the Centers for Disease Control and Prevention (CDC) from 1998–99 through 2007–08. Of these, 105 case-patients had laboratory-confirmed influenza infection and sufficient tissue specimen available at CDC for genotyping. ICU, intensive care unit; MRSA, methicillin-resistant *Staphylococcus aureus*.
†p<0.05.
‡p<0.001.
§p<0.01.

Length of illness was shorter among case-patients with the *TNF* rs1800750 AG genotype than among those with the GG genotype (median length of illness, 1 day vs. 3 days, p = 0.001); no case-patients had the AA genotype. The estimated odds ratio for sudden death was 15.0 for case-patients with the AG genotype compared with case-patients with the GG genotype (p = 0.04 by Fisher exact test). No significant associations were found between any *TNF* variant examined and bacterial co-infection.

Table 2. Genotype and minor allele frequencies among 105 children and young adults who died of influenza, United States, 1998–99 through 2007–08 influenza seasons*

Gene	Variant	Genotype	Genotype frequency, no. (%) case-patients			
			All, n = 105	White, n = 64	Black, n = 18	Asian, n = 7
TNF	rs1800629	–308GG	74 (70)	47 (73)	10 (56)	6 (86)
		–308GA	30 (29)	16 (25)	8 (44)	1 (14)
		–308AA	1 (1)	1 (2)	0	0
		A allele	0.152	0.141	0.222	0.071
	rs1800750	–555GG	101 (96)	62 (97)	16 (89)	7 (100)
		–555GA	4 (4)	2 (3)	2 (11)	0
		–555AA	0	0	0	0
		A allele	0.019	0.016	0.056	0
	rs361525	–238GG	95 (90)	59 (92)	14 (78)	6 (86)
		–238GA	10 (10)	5 (8)	4 (22)	1 (14)
		–238AA	0	0	0	0
		A allele	0.048	0.039	0.111	0.071
MBL2	rs1800450 (*B* variant)	27GG	77 (73)	47 (73)	15 (83)	5 (71)
		27GA	27 (26)	17 (27)	3 (17)	2 (29)
		27AA	1 (1)	0	0	0
		A allele	0.138	0.133	0.083	0.143
	rs1800451 (*C* variant)†	18GG	95 (90)	61 (95)	12 (67)	7 (100)
		18GA	9 (9)	3 (5)	5 (28)	0
		18AA	1 (1)	0	1 (6)	0
		A allele	0.052	0.023	0.194	0
	rs5030737 (*D* variant)	34CC	95 (90)	55 (86)	17 (94)	7 (100)
		34CT	9 (9)	8 (13)	1 (6)	0
		34TT	1 (1)	1 (2)	0	0
		T allele	0.052	0.078	0.028	0
	rs7096206 (X/Y variant)	–221CC	71 (68)	43 (67)	14 (78)	4 (57)
		–221CG	32 (30)	20 (31)	3 (17)	3 (43)
		–221GG	2 (2)	1 (2)	1 (6)	0
		G allele	0.171	0.172	0.139	0.214
	rs11003125 (H/L variant)	–550CC	43 (41)	21 (33)	12 (67)	2 (29)
		–550CG	46 (44)	34 (53)	6 (33)	4 (57)
		–550GG	16 (15)	9 (14)	0	1 (14)
		G allele	0.371	0.406	0.167	0.429
	Pooled structural variants	AA	59 (56)	37 (58)	8 (44)	5 (71)
		AO	41 (39)	24 (38)	9 (50)	2 (29)
		OO	5 (5)	1 (2)	1 (6)	0
	Truncated haplotypes	YA/YA	36 (34)	21 (33)	5 (28)	3 (43)
		XA/YA	21 (20)	15 (23)	2 (11)	2 (29)
		XA/XA	2 (2)	1 (2)	1 (6)	0
		YA/O	30 (29)	19 (30)	8 (44)	1 (14)
		XA/O	11 (10)	5 (8)	1 (6)	1 (14)
		O/O	5 (5)	3 (5)	1 (6)	0
	MBL production	Low	16 (15)	8 (13)	2 (11)	1 (14)
		Intermediate	32 (31)	20 (31)	9 (50)	1 (14)
		High	57 (54)	36 (56)	7 (39)	5 (71)

*Cases include 16 patients with missing/unknown or other race. Per common designation, for *MBL2*, A refers to the wild-type structural allele, O refers to any of the 3 variant structural alleles, and O/O refers to any combination of structural variant alleles. Low producers included truncated haplotypes O/O and XA/O. *MBL2*, mannose-binding lectin gene.
†p<0.01 by Fisher exact test of difference in proportion across racial group.

MBL2

No statistically significant differences were observed in minor allele frequencies for the 5 *MBL2* SNPs examined (Figure 1) or the prevalence of pooled *MBL2* genotypes (Figure 2) between the case-patients and the NHANES reference sample with all case-patients examined together or with black and white racial groups examined separately. In a subgroup analysis, the minor allele frequency of rs5030737 was significantly less common among case-patients ≤5 years old than in the reference sample (2% vs. 7.2%; p = 0.02). Among low producers of MBL, we observed an estimated odds ratio of 7.1 (95% confidence interval [CI] 1.6–32.1) for invasive methicillin-resistant *Staphylococcus aureus* (MRSA) co-infection compared with case-patients with high or intermediate MBL production, according to *MBL2* genotype (p = 0.02 by Fisher exact test; Table 3). Low-producing *MBL2* genotypes were also associated with an approximate 3-fold increased risk for bacterial co-infection in general and with *S. aureus* infection overall, but these associations did not reach statistical significance. Characteristics of case-patients with invasive MRSA co-infection are shown in Table 4.

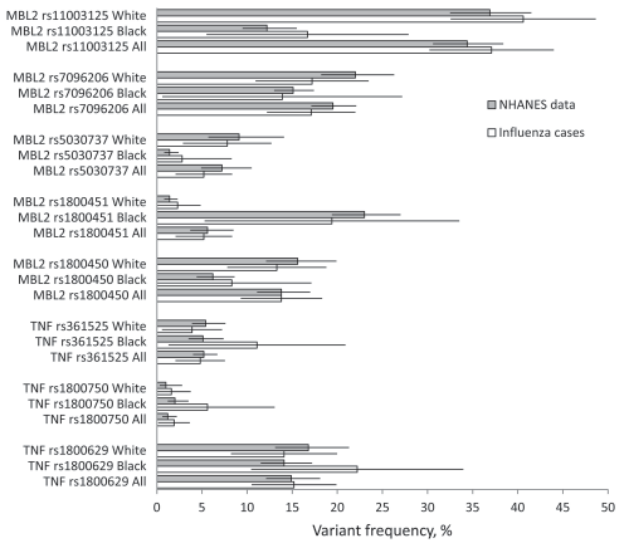

Figure 1. Variant frequency and 95% confidence intervals for fatal influenza cases compared with the NHANES reference group for 8 single-nucleotide polymorphisms. Allele frequency did not differ significantly between cases and the reference group for any single-nucleotide polymorphism. NHANES, National Health and Nutrition Examination Survey. Error bars represent confidence intervals.

Discussion

We found no significant differences in allele frequencies or genotype prevalence for variants in the *TNF* and *MBL2* genes between fatal influenza cases in patients <40 years old and a nationally representative reference sample. However, among the case-patients who died, most of whom died in childhood, variants of *MBL2* responsible for low production of MBL were associated with MRSA co-infection. This observation should be viewed cautiously as a hypothesis for further exploration, given the small number of case-patients with MRSA in our study (n = 8). This finding is consistent with results from previous studies that found associations between MBL insufficiency (defined by genotype) and respiratory infection in children (*12–14*), severe and fatal sepsis (*9*,*15–17*), and systemic inflammatory response syndrome in children (*18*).

TNF is a potent proinflammatory cytokine produced early in the innate immune response to infection that promotes a wide range of immunologic responses. Excessive systemic TNF is responsible for many symptoms of clinical infection and may lead to fatal complications. Studies have demonstrated a significant genetic contribution to circulating TNF levels, with 50%–60% of variance in TNF levels genetically determined (*19–21*). The most studied SNP is at position −308 (rs1800629), with the A allele associated with 20%–40% greater TNF production (*22–24*) and with susceptibility to and severity of numerous infectious diseases (*20*,*22*,*25*,*26*). Carriage of the A allele at the −238 position (rs361525) also has been associated with a variety of diseases (*20*,*22*).

MBL, another key component of the innate immune system, is a soluble protein of the collectin family that binds to microbial surfaces and promotes phago-opsonization directly and indirectly by activating the lectin complement pathway. Low serum MBL levels are common and associated with an increased risk for a variety of infections and autoimmune diseases (*15*,*27–29*), including acute respiratory infection in young children (*12*). MBL levels are strongly influenced by genetic factors, with >75% of variation in MBL levels explained by a small number of polymorphisms in the *MBL2* gene (*30*). Variant proteins are unstable and of lower oligomeric form, which decreases affinity for microbial ligands and complement-activating ability. Each variant produces significantly reduced serum MBL levels.

MBL has been shown to strongly bind *S. aureus* (*31*) and susceptibility to fatal *S. aureus* infection due to MBL deficiency has been convincingly demonstrated in murine models (*32*). Phase I clinical trials of MBL replacement therapy indicate that this therapy is well tolerated and effective at improving MBL deficiency in healthy persons (*33*). Reports of MBL replacement therapy administered to severely ill persons (*34–36*) or to patients with *S. aureus* sepsis (*37*) suggest that therapy can improve clinical conditions, although results of these studies were mixed, and in some cases, clinical improvements were temporary. The clinical implications of MBL replacement therapy for influenza treatment or prevention are unknown.

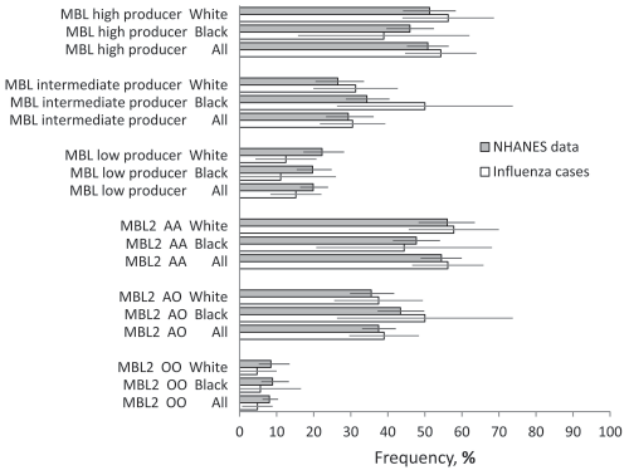

Figure 2. Frequency and 95% confidence intervals for fatal influenza cases (n = 105) compared with the NHANES reference group for pooled structural genotypes (AA/AO/OO) of *MBL2* and categorization of MBL production on the basis of genotype (low/intermediate/high). Frequencies did not differ significantly between cases and the reference group. MBL, mannose-binding lectin; NHANES, National Health and Nutrition Examination Survey; *MBL*2, mannose-binding lectin gene. Error bars represent confidence intervals.

Among persons with fatal cases, we observed an increased risk for sudden death in carriers of the variant allele of *TNF* rs1800750. We are unaware of previous literature reporting a similar association; there is no obvious biologic mechanism to explain the finding. The *TNF* rs1800750 variant is in linkage disequilibrium with other *TNF* variants (http://pga.gs.washington.edu), some of which (including *TNF* rs361525) have been associated with increased TNF serum levels. Therefore, it is possible that the observed association may be due to linkage disequilibrium with unmeasured polymorphisms that are the causal variants, and more exhaustive analysis of *TNF* variants is worthy of future study.

A strength of this study is its use of a cohort of case-patients particularly well-suited for investigation of potential host genetic risk factors—these case-patients died with active influenza infections, yet were predominantly children and young adults without severe preexisting medical conditions. In such a group, other factors associated with severe influenza are less likely to obscure possible genetic associations. An additional strength was access to postmortem lung tissue for immunohistochemistry and/or RT-PCR confirmation of influenza infection.

We recognize that this study has several limitations. Although the study cohort is, to our knowledge, the largest sample of fatal influenza cases in children and young adults, the analysis has limited statistical power to detect associations because of small sample sizes, especially when examining subsamples. We had access to limited information about racial and ethnic background of case-patients. Clinical data were obtained primarily from a US surveillance system and were not validated with medical chart review. Although we were able to infer truncated haplotypes for *MBL2*, haplotype information for *TNF* was unavailable. Despite these shortcomings, the possibility that specific variants of the *MBL2* gene known to influence serum MBL levels appear to be associated with severe bacterial co-infection is an intriguing finding deserving of additional study, especially given the prevalence of co-infection among case-patients who died of pandemic (H1N1) 2009 virus infection (*38*) and observations that children co-infected with influenza and *S. aureus* may have higher case-fatality rates (*39*).

That we observed a stronger relationship between low-producing MBL genotypes and MRSA infection than between those genotypes and *S. aureus* infection in general is puzzling. We are unaware of an obvious physiologic explanation for why low MBL would predispose more strongly to infection with methicillin-resistant versus methicillin-sensitive *S. aureus*. One possibility is that MRSA is a marker for other strain characteristics. For example, such an association could arise if MRSA infections were predominantly the USA300 strain while other *S. aureus* infections were predominantly the USA100 strain. Unfortunately, we do not have data on *S. aureus* genetic strain types. We also found that of the 4 fatal influenza cases in which patients had both MRSA co-infection and low-producing MBL genotypes, 2 patients reportedly also had asthma. It is well-established that asthma increases the risk for serious complications of influenza, and although we know of no evidence suggesting that low-producing

Table 3. Associations between *MBL2* haplotypes and invasive bacterial co-infection in children and young adults with fatal influenza, United States, 1998–99 through 2007–08 influenza seasons*

Outcome	Estimated odds ratio (95% CI)		p value
	Low producing *MBL2* haplotype†	Intermediate or high producing *MBL2* haplotype‡	
MRSA, n = 8	7.1 (1.6–32.1)	Referent	0.02
Staphylococcus aureus, n = 13	3.0 (0.8–11.2)	Referent	0.11
Bacterial co-infection, n = 22	2.7 (0.9–8.6)	Referent	0.10

**MBL2*, mannose-binding lectin gene; MRSA, methicillin-resistant *Staphylococcus aureus*.
†includes *MBL2* haplotypes O/O and XA/O.
‡includes *MBL2* intermediate producer haplotypes XA/XA and YA/O and high producer haplotypes XA/YA and YA/YA.

Table 4. Characteristics of 8 children and young adults who died of influenza and had invasive methicillin-resistant *Staphylococcus aureus* co-infection, United States, 1998–99 through 2007–08 influenza seasons

Patient age, y/sex	Season	Influenza type	Previous conditions	Sudden death*	*MBL2* haplotype†	MBL production haplotype‡	*MBL2* structural variant
29/M	2004–05	A	None	Yes	YA/O	Intermediate	rs1800450 A/G
14/M	2006–07	A	None	No	XA/YA	High	None
11/F	2006–07	B	None	Yes	YA/YA	High	None
13/M	2006–07	B	None	Yes	XA/O	Low	rs1800450 A/G
12/F	2006–07	B	None	No	O/O	Low	rs1800450 A/A
8/F	2007–08	A	Asthma	Yes	XA/O	Low	rs1800450 A/G
18/M	2007–08	A	Asthma	Yes	XA/O	Low	rs1800450 A/G
32/M	2007–08	B	Asthma	No	XA/YA	High	None

*Sudden death is defined as death occurring less than 3 days after symptom onset. *MBL2*, mannose-binding lectin gene.
†Per common designation for *MBL2*, A refers to the wild-type structural allele, O refers to any of the 3 variant structural alleles, and O/O refers to any combination of structural variant alleles. Y refers to the wild-type allele and X to the variant allele at promoter locus rs7096206 (the X/Y variant).
‡Low mannose-binding lectin (MBL) producers included *MBL2* haplotypes O/O and XA/O. Intermediate producers included *MBL2* haplotypes XA/XA and YA/O. High producers included *MBL2* haplotypes XA/YA and YA/YA.

MBL genotypes are associated with increased risk for asthma (*40*), this finding may be worth further exploration in future studies.

Our findings suggest several opportunities for additional influenza-related research. An obvious next step is examination of all functional variants of the *MBL2* gene in conjunction with gene expression and functional assays in a larger group of severely ill influenza case-patients with sufficiently detailed clinical data to define important phenotypes (e.g., MRSA co-infection). Interest in association studies of rare variants, the availability of new sequencing technologies that dramatically decrease the cost of sequencing, and access to reference human sequence data suggest that investigating rare variants in candidate genes (including *MBL2* and *TNF*) and their functional effects may be a promising avenue of research. Large-scale genotyping of a sample of case-patients to look for common variants by using methods such as genomewide association studies may be possible if a network of collaborators capable of pooling a sufficient number of case-patients is developed. Recent initiatives such as the Genome-based Research and Population Health International Network (www.graphint.org/ver2) are aimed at encouraging such networks. Given the rapid acceleration in laboratory technologies, enhancement in bioinformatics methods and capacity, and trends toward collaborative research within large consortia, exploration of the role of host genomic factors in serious illness associated with influenza and other viral pathogens is increasingly feasible. We believe that host genomics is a promising area for future research regarding who is at risk for severe complications of acute infectious diseases, including influenza.

Acknowledgments

We thank Muin Khoury, Nancy Cox, Venkatachalam Udhayakumar, Rosaline Dhara, Man-Huei Chang, Lyna Zhang, Ajay Yesupriya, Tara Jones, and Eric Shattuck for their assistance.

This study was funded by the Centers for Disease Control and Prevention and the Atlanta Research and Education Foundation, Veterans Administration Medical Center, Atlanta, Georgia, USA.

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Severe Human Bocavirus Infection, Germany

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Human bocavirus (HBoV), discovered in 2005, can cause respiratory disease or no symptoms at all. We confirmed HBoV infection in an 8-month-old girl with hypoxia, respiratory distress, wheezing, cough, and fever. This case demonstrates that lower respiratory tract infection caused by HBoV can lead to severe and life-threatening disease.

Human bocavirus (HBoV; family *Parvoviridae*; genus *Bocavirus*) was discovered in 2005 and is distributed worldwide (*1*). Four species of HBoV have been identified (HBoV1–4). Increasing evidence indicates that HBoV causes infections of the respiratory tract. Numerous studies depict HBoV as a co-pathogen but also show that its prevalence in asymptomatic patients is high.

HBoV has gained considerable clinical relevance since its discovery. It has been detected in respiratory specimens, and when it causes disease, it seems to have a broad spectrum of signs and symptoms. Although certain features make HBoV infection distinguishable from other viral infections, a diagnosis cannot be made clinically. We report a case of severe HBoV infection that led to hypoxia, respiratory distress, wheezing, cough, and fever.

The Patient

On January 2, 2011, an 8-month-old girl with acute respiratory distress was seen in the emergency department of the Children's Hospital of the University of Cologne. The girl's parents had noticed labored breathing, intercostal retractions, and fever for 1 day. Further medical history was unremarkable. No one else who had contact with the girl was sick.

At admission, the girl was lethargic and inappetent with a fever of 38.3°C and oxygen saturation of 86%. Expiration was prolonged, and an expiratory wheeze was heard. Breath sounds over the right upper lung lobe were remarkably diminished. The patient had submandibular lymphadenopathy and mild pharyngitis but no rash or abnormalities of the heart or abdomen. The clinical signs were typical of an upper respiratory tract infection combined with severe obstructive bronchitis.

Chest radiographs showed diffuse bilateral infiltrates and total atelectasis of the right upper lung lobe. Blood analysis indicated partial CO_2 concentration within normal limits and slightly elevated C-reactive protein level (10 mg/L). Electrolytes and renal and hepatic markers were within reference ranges, as was complete blood count except for thrombocytosis (744,000 platelets/μL).

Treatment with prednisolone and inhalation therapy delivering albuterol and ipratropium by vaporizor was initiated. Because of the lung atelectasis, the child received cefuroxime as prophylaxis for 2 weeks. Oxygen saturation could be restored to within reference limits with nasal cannula oxygen delivery at 4 L/min.

During the next 2 days, the patient's body temperature was 38.0°C–39.0°C, and she remained lethargic. A nasopharyngeal aspirate was obtained 4 days after symptom onset and screened for respiratory viruses by real-time PCR (*2*) and Luminex xTAG RVP panel (Abbott, Wiesbaden, Germany). Each assay had positive results for HBoV DNA and negative results for influenza A virus; influenza A virus subtype H1N1; influenza B virus; parainfluenza viruses types 1–4; adenovirus; human metapneumovirus; coronaviruses NL63, HKU1, 229E, and OC43; respiratory syncytial virus (RSV); rhinovirus; and enterovirus. The aspirate was not tested for bacteria. Cultures of blood collected on the day of admission, before antimicrobial drug treatment was started, were negative. A second chest radiograph showed partial atelectasis of the left upper lung lobe, a retrocardial infiltrate, and pneumomediastinum. On hospitalization day 5, oxygen saturation could be maintained at a lower flow rate. On day 6, chest radiograph showed regression of atelectasis in both lobes and of the pneumomediastinum. Temperature dropped to subfebrile levels, and the child's general condition was improving. On day 9, no additional oxygen was needed. On day 10, chest radiograph showed residual atelectasis in both upper lobes and residual infiltrates in both lungs, and the patient was discharged. Two weeks later, at follow-up examination, the child was doing well, breath sounds were unremarkable, and the pathologic radiographic findings had resolved completely.

To verify that the disease was caused by an HBoV infection, we performed a real-time PCR targeting the nonstructural protein 1 region of HBoV; we detected 5,819 copies/mL HBoV DNA in the patient's serum 30 days after symptom onset (*3*). The PCR was conducted with primers OS1 and OS2 as described (*4*), and an HBoV subtype

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DOI: http://dx.doi.org/10.3201/eid1712.110574

2 plasmid dilution series (kindly provided by Tobias Allander, Karolinska Institute, Stockholm, Sweden) was used as a quantitative control. Sequencing was performed by an external sequencing service (Eurofins MWG, Munich, Germany); the same primers from both directions were used.

Sequencing and BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) confirmed the virus to be HBoV1, not HBoV2–4. Additionally, HBoV-specific IgM and IgG and avidity of IgG were measured by sensitive and specific enzyme immunoassays (*5*,*6*). Results were clearly positive for both: IgM absorbance 0.385 and cutoff 0.167; IgG absorbance 2.971 and cutoff 0.188. IgG was of low avidity (4%, cutoff 15%), indicating acute primary HBoV infection. Control samples were tested as described (*5*–*8*).

Conclusions

HBoV has been detected worldwide in nasopharyngeal aspirates collected for screening (*9*). Large-scale studies showed that HBoV can be detected in children with signs and symptoms of respiratory tract infection. Studies have detected HBoV in 9% and 19.3% of all samples, indicating that it is the second or third most commonly detected virus, after RSV and rhinovirus (*2*,*10*). Thus, the overall contribution of HBoV to all detected respiratory viruses lies well below that of RSV. High prevalence does not necessarily mean high clinical relevance, and proving its causative role has been difficult because the virus is often detected along with other viruses; co-detection rates are as high as 75% (*11*,*12*).

HBoV can also persist for months in the respiratory tract after resolution of disease, further complicating diagnosis (*12*), which therefore should be based on detection of HBoV DNA in blood and measurement of HBoV-specific antibodies. HBoV is the most probable cause of respiratory tract disease if the patient has a high viral load in nasopharyngeal aspirates accompanied by viremia, if HBoV is the only pathogen detected, and if an acute primary HBoV infection is diagnosed by serologic testing (*5*,*11*,*13*). We detected HBoV DNA in nasopharyngeal aspirate and serum. The serologic results strongly suggest that this child had an acute primary HBoV infection. The serologic assay developed earlier was validated in a series of studies and shown to reliably measure serologic response against HBoV (*5*–*8*). Inflammatory markers were not elevated, thereby indicating no bacterial infection. However, the contribution of bacteria to the course of the disease cannot be completely ruled out.

Signs and symptoms for the patient reported here have been described as the most common signs and symptoms of HBoV-infected children (*14*) and are typical of lower respiratory tract viral infections in general. In 1 study (*14*), 18 patients had an HBoV single infection and 2 had lobar atelectasis, as did the patient reported here. Similarly, another case report described HBoV infection that resulted in pneumomediastinum (*15*). The age of the patient reported here is also typical of HBoV, which mostly infects children 6 months to 3 years of age, but it does not usually infect young infants, as RSV does (*14*). The case reported here shows that a lower respiratory tract infection caused by HBoV can lead to severe and life-threatening disease.

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Continuing Threat of Influenza (H5N1) Virus Circulation in Egypt

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Reservoirs for the continuing influenza (H5N1) outbreaks in Egypt are ill-defined. Through active surveillance, we detected highly pathogenic influenza subtype H5 viruses in all poultry sectors; incidence was 5%. No other subtypes were found. Continued circulation of influenza (H5N1) viruses in various regions and poultry sectors perpetuates human exposure in Egypt.

After 150 confirmed human cases and continuous outbreaks in its different poultry production sectors, Egypt became an epicenter for highly pathogenic avian influenza (H5N1) virus activity and one of the few countries where this virus is endemic. The long-term endemicity of influenza (H5N1) virus in poultry in Egypt has generated substantial viral genetic and antigenic diversity, as has been seen in other areas (*1*–*3*), yet the ecology and epizootology of the virus in the various poultry sectors remains unknown. To determine the incidence and diversity of influenza viruses among poultry in 6 governorates in Egypt, we conducted surveillance for 1 year.

The Study

From August 2009 through July 2010, we collected 5,562 cloacal and oropharyngeal swab samples from poultry at 58 sites in 6 governorates in Egypt (Cairo, 4 sites, 24 birds/100,000 inhabitants; Qalubiya, 12 sites, 317,000 birds/100,000 inhabitants; Menofiya, 9 sites, 436,000 birds/100,000 inhabitants; Sharkiya, 2 sites, 375,000 birds/100,000 inhabitants; Fayyoum, 22 sites, 98,000 birds/100,000 inhabitants; and Beni Suef, 9 sites, 108,000 birds/100,000 inhabitants) (*4*). The selected governorates represent the main foci of the poultry industry in Egypt. In each governorate, 2–6 sites were routinely sampled monthly; samples were also collected from other sites and villages in the same governorate. Sample collection, handling, transport, screening, and subtyping by reverse transcription PCR (RT-PCR) were performed according to published protocols (*5*–*7*).

Of the cloacal swab samples, 5.0% were positive for influenza (H5N1) virus by matrix gene RT-PCR; of the oropharyngeal swab samples, 4.9% were positive (Table). All positive samples contained hemagglutinin subtype H5, determined by H5-specific RT-PCR. The percentages of positive samples by governorate were 0%–13.1% ($p<0.001$, Pearson χ^2 test). Positivity rates were higher for governorates in the Nile Delta region (Qalubiya, Sharkiya, and Menofiya) and Cairo (6%) than for those in southern Egypt (Beni Suef and Fayyoum, 0%–3.8%).

A total of 243–764 samples were collected monthly, depending on the number of poultry available at the sites during the sample collection visit (Figure 1). At least 2 samples tested positive in every surveillance month except June 2010. The positivity rate was highest (11.1%) in October 2009. We were not able to detect a seasonal pattern

Table. Epizootologic data for avian influenza (H5N1) virus, Egypt, August 2009–July 2010*

Variable	No. (%) samples		p value†
	Collected	Positive	
Swab type			NS
Cloacal	4,353 (78.3)	217 (5.0)	
Oropharyngeal	1,209 (21.7)	59 (4.9)	
Governorate			<0.001
Cairo	979 (17.6)	58 (5.9)	
Qalubiya	916 (16.5)	120 (13.1)	
Menofiya	1,636 (29.4)	27 (1.7)	
Sharkiya	280 (5.0)	21 (7.5)	
Fayyoum	1,323 (23.8)	50 (3.8)	
Beni Suef	428 (7.7)	0	
Species			<0.001
Breeder chickens	50 (0.9)	5 (10.0)	
Broiler chickens	3,803 (68.4)	163 (4.3)	
Layer chickens	710 (12.8)	97 (13.7)	
Ducks	819 (14.7)	10 (1.2)	
Geese	55 (1.0)	0	
Pigeons	51 (0.9)	1 (2.0)	
Turkeys	74 (1.3)	0	
Location			<0.001
Abattoir	330 (5.9)	38 (11.5)	
Commercial farm	2,827 (50.8)	192 (6.8)	
Backyard flock	1,381 (24.8)	12 (0.9)	
Live-bird market	1,024 (18.4)	34 (3.3)	
Bird health status			<0.001
Healthy	5,255 (94.5)	235 (4.5)	
Sick	214 (3.8)	35 (16.4)	
Dead	93 (1.7)	6 (6.5)	

*NS, not significant.
†Pearson χ^2.

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DOI: http://dx.doi.org/10.3201/eid1712.110683

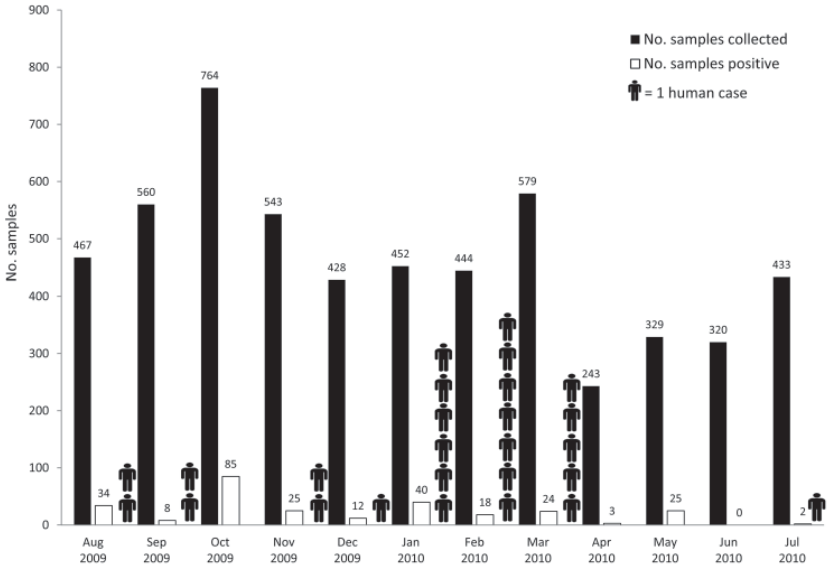

Figure 1. Number of samples collected from poultry and number positive for influenza (H5N1) virus, Egypt, August 2009–July 2010.

of influenza outbreaks in poultry (Figure 1); however, during our surveillance period, human cases of influenza (H5N1) virus infection were reported throughout the year and peaked in January and February 2009 (*8*) (Figure 1).

By species, ≈82% of the swab samples were collected from chickens, followed by ducks (14.7%) and other species of domestic birds (3.2%). Positivity rates differed significantly ($p<0.001$, Pearson χ^2 test). Among chickens, 13.7% of the samples from layers, 10.0% from breeders, and 4.3% from broilers were positive. Among ducks, 1.2% of samples were positive. Only 1 pigeon swab sample was positive (Figure 2).

By collection location, the highest positivity rate (≈12%) came from poultry abattoirs ($p<0.001$, Pearson χ^2 test). The next highest rates came from commercial farms (6.8%), followed by live-bird markets (3.3%). Only 0.9% of swab samples from backyard flocks were positive. Most (94.5%) samples were collected from apparently healthy birds; of those, 4.5% were positive. In contrast, 13.4% of samples from sick or dead birds were positive ($p<0.001$, Pearson χ^2 test).

To identify other putative sources of human infection with influenza (H5N1) virus, we also examined a population of wild egrets (*Bubulcus ibis*) in urban greater Cairo. These birds congregate on trees next to the Giza Zoo in a heavy traffic area with a dense human population. RT-PCR detected influenza (H5N1) viruses in the feces of these wild egrets. Influenza (H5N1) virus shedding by the egrets threatens the exotic bird population at the zoo as well as humans in that area.

Conclusions

In Egypt, most swab samples positive for influenza (H5N1) virus were from chickens. Among ducks, the positivity rate was as low as 1.2%, although in other regions, ducks have been shown to play a key role in avian influenza transmission (*9*). All samples with positive results by RT-PCR contained highly pathogenic influenza (H5N1) viruses. The surprising lack of detection of other influenza subtypes in our surveillance may be explained by establishment of subtype H5N1 as the dominant influenza strain in poultry in Egypt. Alternatively, low-pathogenicity viruses may be circulating in different regions or different host populations not covered by our surveillance. Whatever the reason, the lack of substantial cocirculation of multiple influenza viruses reduces the chances of influenza (H5N1) virus evolution occurring in Egypt by reassortment.

In Egypt, commercial farms are major reservoirs for influenza (H5N1) virus; the positivity rate was higher for those farms (7.2%) than for backyard farms (0.9%). Because the sampled poultry at commercial farms, where biosecurity measures were generally lax, were vaccinated with commercially available subtype H5 vaccines, the effectiveness of such vaccines becomes highly questionable.

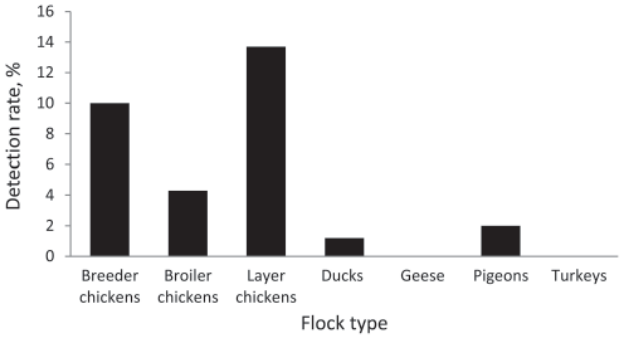

Figure 2. Rates of detection of influenza A (H5N1) virus by reverse transcription PCR, Egypt, August 2009–July 2010.

The lower positivity rate among backyard poultry may be explained by the fact that the growers slaughter these birds at the first sign of disease.

Reports of influenza (H5N1) virus infections in humans in Egypt show that most of these persons had had contact with sick poultry, primarily in backyards (*8*,*10*), as has been reported in Asia (*11*–*13*). Our findings indicate that the threat to humans in Egypt is much more widespread than previously reported. We detected influenza (H5N1) viruses in poultry from all production sectors and from wild egrets in Cairo. Among specimens collected from live-bird markets and slaughterhouses in Cairo, ≈6% had positive results; these birds usually come from commercial farms in rural areas. This finding indicates that the public health concern applies not only to rural poultry growers but also to persons in urban areas.

Although we were able to detect influenza virus among poultry continuously during surveillance, we did not establish a clear seasonal pattern of outbreaks, an indicator of continuous evolution of subtype H5N1 viruses endemic to Egypt. During the same period covered by our surveillance, human cases were reported during 8 of the 12 months; incidence was highest in January and February, reflecting a seasonal pattern conforming to the climate, with influenza activity peaks in the colder months. These data suggest that the seasonality of influenza (H5N1) in humans is not explained by increased virus activity in the associated poultry population but rather by other unidentified behavioral or environmental factors.

Our surveillance findings reveal that highly pathogenic influenza (H5N1) viruses are abundant and persistent in Egypt. Closer surveillance of avian influenza viruses in domestic poultry and expansion of such surveillance to include wild and migratory birds is warranted in an effort to continuously monitor the evolution of subtype H5N1 and other influenza viruses in Egypt.

This work was funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, US Department of Health and Human Services, contract no. HHSN266200700005C, and supported by the American Lebanese Syrian Associated Charities.

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Hepatitis E Virus Antibodies in Blood Donors, France

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Using a validated sensitive assay, we found hepatitis E virus (HEV) IgG in 52.5% of voluntary blood donors in southwestern France. This finding suggests HEV is highly endemic to this region. The high HEV prevalence may reflect local dietary practices, such as eating uncooked pork and game products.

It is now recognized that hepatitis E virus infection is not confined to developing countries. HEV infection is a growing public health concern in industrialized countries where the disease is mainly autochthonous, caused by HEV genotypes 3 (Europe) and 4 (People's Republic of China and Japan), and is thought to be zoonotic (*1*).

In a previous study, we estimated that 16.6% of blood donors in the Midi-Pyrénées region of southwestern France have HEV antibodies (*2*). This rate was much higher than that measured in northern France (*3*), which suggests differences between these 2 populations and their exposure to HEV that we wished to explore further. However, it is difficult to make wider comparisons with seroprevalence studies from other areas because the various assays used differed in sensitivity and specificity (*4*). Because a recent study suggested that the HEV IgG assay we used in our original study lacks sensitivity (*5*), we repeated and extended the study using a more sensitive assay that has been validated by using serum from PCR-proven HEV genotype 3 infections (*5*).

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DOI: http://dx.doi.org/10.3201/eid1712.110371

The Study

During September 2003 through May 2004, serum samples were collected from 512 adult blood donors 18–64 years old (median 42 years) and 188 children 2–4 years old. The blood donors were unpaid voluntary donors; the children were hospitalized in Toulouse for surgery or trauma. All were residents of the Midi-Pyrénées region. The prevalence of HEV IgG was determined by using the Wantai HEV IgG enzyme immunoassay (Wantai Biologic Pharmacy Enterprise, Beijing, People's Republic of China), according to the manufacturer's instructions. Details of baseline demographic data and putative risk factors were collected from blood donors by using a structured questionnaire. In addition, to assess the risk for foodborne infection, we tested 18 local pig-liver sausages for HEV RNA using a quantitative real-time PCR based on the open reading frame 2 region of the HEV genome (*6*).

HEV IgG was detected in 268 (52.5%) of 512 (95% confidence interval [CI] 48.2%–56.8%) of the blood donors. Seroprevalence increased with age (Figure 1). The ranges of optical density/cutoff ratios for positive and negative samples showed a clear bimodal distribution (Figure 2). Of 244 rural donors, 63.1% (95% CI 57%–69.2%) were anti-HEV positive compared with 42.9% (95% CI 37–48.8) of 268 urban donors ($p<0.01$). For children, seroprevalence was 3.7% (95% CI 1.0%–6.5%). The mean ± SD optical density/cutoff ratio of the positive samples was 5.43 ± 3.93 for children and 5.99 ± 3.52 for adults. Although several factors were associated with the presence of HEV IgG after univariate analysis, multivariate analysis identified only age, rural residence, hunting, and contact with cats as factors independently associated with HEV IgG positivity (Table 1).

HEV RNA was found in 8 (44%) of the 18 sausages tested by real-time PCR (Table 2). The virus load ranged from 100 (the limit of detection for this assay) to 668,520

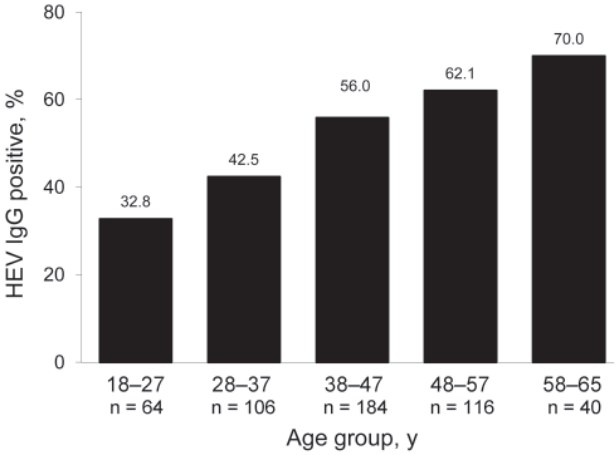

Figure 1. Prevalence of hepatitis E virus (HEV) IgG in 512 blood donors by age group, Midi-Pyrénées region, France, 2003–2004.

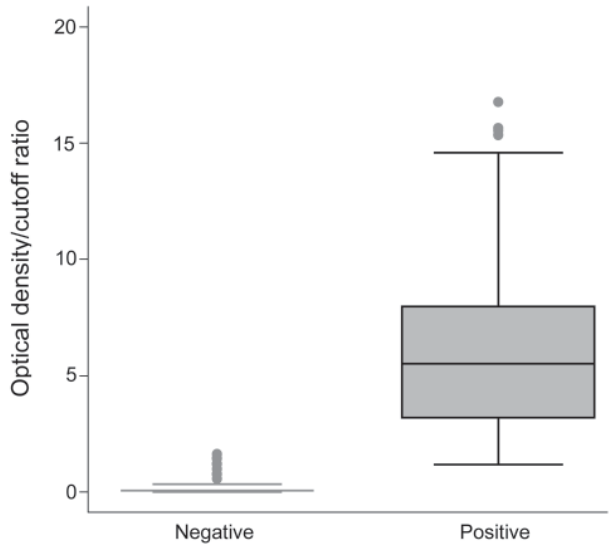

Figure 2. Distribution of optical density/cut off ratios for hepatitis E virus IgG in positive and negative samples from 512 blood donors, Midi-Pyrénées region, France, 2003–2004. Whiskers represent percentiles.

copies/g. We attempted to genotype HEV RNA–positive samples by sequencing a 189-nt fragment of the open reading frame 2 gene (*7*). This was successful only for the sample with the highest virus load. The virus was identified as HEV genotype 3.

Conclusions

We determined that the HEV IgG prevalence among blood donors in Midi-Pyrénées is 52.5%, the highest seroprevalence reported in an industrialized country. This rate is 3.1 times higher than our previous estimate (16.6%) for the same population (*2*). The implication is that HEV is hyperendemic to Midi-Pyrénées.

Although surprising, we believe these results are valid for several reasons. First, the Wantai assay used to assess HEV seroprevalence has been validated for this purpose in the United Kingdom, another region where HEV-3 predominates (*5*). The greater proportion of reactive serum seen with this assay is unlikely to have resulted from nonspecific reactivity because the assay produced a clear distinction between negative and positive samples, and only a small proportion of young children tested positive. Our findings agree with those of another study that found a much increased HEV seroprevalence when the more accurate test was used (*5*). Second, autochthonous HEV genotype 3 hepatitis in Midi-Pyrénées is common. The estimated rate of acquisition of HEV infection in organ transplant recipients in Toulouse is 3.2 per 100 person-years (*8*). This figure is derived from regular monitoring by using sensitive molecular techniques and does not depend on serologic assays. As noted in other countries (*9*,*10*), the percentage of HEV-positive serum increased with age, which is consistent with cumulative exposure to infection over time.

HEV is usually transmitted orally, and foodborne transmission of zoonotic strains has been demonstrated. Hunting of wild boar and deer is popular in Midi-Pyrénées, particularly in rural areas. Both species have been identified as sources of human infection (*11*). The consumption of uncooked game meat, which is traditional in this area, could explain the high HEV antibody prevalence in 20 (80%) of 25 hunters. Further evidence comes from a case–control study among organ transplant recipients in Midi-Pyrénées

Table 1. Prevalence of HEV IgG, demographics, and potential risk factors for 512 blood donors, Midi-Pyrénées region, France, 2003–2004*

Analysis and risk factor	% Donors with HEV IgG	Odds ratio (95% CI)	p value
Univariate analysis			
Age		1.24 (1.14–1.35)	<0.001
Sex, M/F	51/55	1.14 (0.79–1.64)	NS
Rural residence	59	2.27 (1.60–3.25)	<0.001
Gardening	61	1.75 (1.22–2.5)	<0.01
Kitchen gardening	69	2.23 (1.30–3.87)	<0.001
Hunting	80	3.82 (1.41–10.4)	<0.01
Contact with farm animals	57	1.6 (1.12–2.29)	<0.01
Contact with dogs	58	1.42 (0.99–2.0)	NS
Contact with cats	59	1.49 (0.04–2.13)	<0.05
Contact with horses	54	1.05 (0.35–3.17)	NS
Contact with pigs	50	0.89 (0.25–3.11)	NS
Contact with poultry	51	0.93 (0.49–1.80)	NS
Contact with wild animals	74	2.73 (1.20–6.23)	<0.05
Travel outside France	52	0.54 (0.35–0.83)	<0.01
Travel outside Europe	50	0.58 (0.38–0.89)	<0.05
Multivariate analysis			
Age		1.20 (1.10–1.31)	<0.01
Rural residence		1.80 (1.24–2.62)	<0.01
Hunting		4.11 (1.35–12.5)	<0.05
Contact with cats		1.6 (1.10–2.34)	<0.05

*HEV, hepatitis E virus; CI, confidence interval; NS, not significant.

Table 2. Detection and quantification by real-time PCR of HEV RNA in pig-liver sausages purchased from markets in the Midi-Pyrénées region, France, 2003–2004*

Sample	Market	HEV RNA	HEV RNA concentration†	Genotype
1	A1	Negative		
2	A2	Negative		
3	A3	Negative		
4	A4	Positive	4,100	NA
5	A5	Positive	175	NA
6	A6	Positive	240	NA
7	A7	Negative		
8	B	Positive	100	NA
9	C	Negative		
10	D	Positive	8,200	NA
11	E	Negative		
12	F	Positive	668,520	3
13	G	Negative		
14	H	Negative		
15	I	Positive	120	NA
16	J	Negative		
17	K	Negative		
18	L	Positive	48,550	NA

*A1–A7 indicate different shops in the same market. HEV, hepatitis E virus; NA, no PCR amplification with primers used for genotyping.
†Copies/g.

that demonstrated that the only factor independently associated with HEV infection was consumption of game meat (*6*). Some of these foods have been shown to contain HEV RNA, and phylogenetic analysis demonstrated that these strains were closely related to human strains (*8*).

Another suspected zoonotic source of HEV genotype 3 infection is the domestic pig (*12*). Hepatitis E cases have been linked to eating uncooked pork-liver sausage in southeastern France (*13*). We found that a high proportion (44%) of pig-liver sausages purchased in Toulouse contained HEV RNA. These air-dried sausages are popular in Midi-Pyrénées and are usually eaten raw. Their infectivity is unknown, but cell-culture experiments have demonstrated that high virus loads correlate with high infectivity (*14*).

In addition to direct foodborne transmission, the growing boar population in Midi-Pyrénées and the spreading of pig manure on land may pose indirect risks through fecal contamination of soil and watercourses. These 2 factors and the 2 foodborne sources of HEV might explain the high HEV antibody prevalence in Midi-Pyrénées and the statistical association of HEV seropositivity with rural residence and hunting, but they cannot explain the association with cat contact. HEV RNA has not yet been detected in domestic cats.

The high percentage of donors with HEV antibodies in our area contrasts with the low recorded incidence of autochthonous hepatitis E in France and other industrialized countries (*1*,*15*). Even though we documented dozens of hepatitis E cases in Midi-Pyrénées during the past decade, the remarkably high seroprevalence in this area suggests that most infections must be subclinical or unrecognized. However, in susceptible persons, such as organ transplant recipients and patients with chronic liver disease, the consequences of HEV infection are grave and raise public health, as well as food and environmental safety, concerns.

HEV is highly endemic to the Midi-Pyrénées region in southwestern France. We showed that seroprevalence increases with age and is associated with rural residence, hunting, and exposure to cats. These associations became apparent only when we used a sensitive assay to detect HEV IgG. The reasons for the high HEV prevalence in this population are uncertain but may be due, at least partially, to the culinary culture of the local community. Thorough cooking of game meat and pork products would help minimize the risk for HEV infection and could form part of a public health initiative in this area.

Acknowledgments

We thank Wantai Biological Pharmacy Enterprise, Beijing, China, for providing the anti-HEV IgG kits at no cost.

The Royal College of Physicians (London) supporting this work by the award of the Dame Sheila Sherlock Travelling Fellowship. Agence Nationale de la Recherche France, grant PNRA HEVZOONEPI, provided financial support on HEV research.

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Human Cardioviruses, Meningitis, and Sudden Infant Death Syndrome in Children

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Cardioviruses cause myocarditis and encephalomyelitis in rodents; human cardioviruses have not been ascribed to any disease. We screened 6,854 cerebrospinal fluid and 10 myocardium specimens from children and adults. A genotype 2 cardiovirus was detected from a child who died of sudden infant death syndrome, and 2 untypeable cardioviruses were detected from 2 children with meningitis.

The cardioviruses (family *Picornaviridae*, genus *Cardiovirus*) are pathogens of rodents and include a murine encephalomyocarditis virus and Theiler's virus and related strains (species *Theilovirus*), the latter serving as laboratory models of the pathogenesis of multiple sclerosis in mice (*1*). The existence of specific human cardioviruses was suspected in the 1960s in conjunction with a rare infectious neurodegenerative disease known as Vilyuisk encephalitis (*2*,*3*). Recently, human cardioviruses (hCVs) were identified in archived diagnostic cell culture supernatants (*4*) and in clinical samples from children with diarrhea or respiratory infection (*5*,*6*). Up to 8 different putative hCV types have since been characterized in human feces (*7*).

Despite the remarkable pathogenicity of rodent cardioviruses, specific disease associations of hCV could not be made. An initial clinical study yielded no evidence of hCV in cerebrospinal fluid (CSF) of 400 patients with aseptic meningitis, encephalitis, or multiple sclerosis (*8*). To evaluate the pathogenetic potential of these emerging viruses, we investigated 6,854 CSF specimens from adults and children with neurologic disease and 10 myocardium specimens from infants who had died of sudden infant death syndrome (SIDS).

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DOI: http://dx.doi.org/10.3201/eid1712.111037

The Study

CSF specimens were collected from 3 cohorts. The first cohort comprised 2,562 specimens sent during 1998–2008 to the Institute of Virology, University of Bonn Medical Center (UBMC), Bonn, Germany, for routine investigation of meningoencephalitis (333 from the Department of Pediatrics and 2,229 from other departments). The second cohort comprised 3,960 specimens collected during 1982–2008 at the UBMC children's hospital from children with cancer and neurologic complications during chemotherapy. The third cohort comprised 348 specimens from hospitalized children with clinical meningitis or encephalitis in which no etiologic agent had been found; the specimens were sent for virologic investigation to the Institute for Hygiene and the Environment in Hamburg, ≈400 km from UBMC, during 2006–2008. Myocardium specimens were collected during 2010 at the UBMC Institute for Forensic Medicine from 10 epidemiologically unlinked children who died of SIDS.

Viral RNA was purified from clinical specimens by using the Viral RNA Mini and RNeasy Mini kits (QIAGEN, Hilden, Germany). Detection of hCV RNA was done in pools of 2–10 specimens by using quantitative real-time reverse transcription PCR (RT-PCR) and nested RT-PCR specific for the viral 5′ untranslated region (5′-UTR), as described (*6*). Amplification of further hCV genomic regions from individual positive specimens was conducted by using ≈20 sets of different nested RT-PCRs (primers available on request from C.D.).

In 2 of 681 CSF specimens (n = 333 and n = 348 from cohorts 1 and 3, respectively) from children with meningitis (online Appendix Table, wwwnc.cdc.gov/EID/article/17/12/11-1037-TA1.htm), hCV RNA was detected at low concentrations (1.14×10^4 and 9.63×10^2 copies/mL). In 1 of these patients, hCV was also detectable in feces (9.50×10^2 copies/g). In 1 of 10 myocardium specimens, hCV was detected by nested RT-PCR, and results of quantitative real-time RT-PCR were negative. Underquantification because of nucleotide mismatches below oligonucleotide binding sites and contamination of nested RT-PCR was excluded by sequence comparison (up to 5% nt divergence from other hCV strains, including the positive control). Serum and liver specimens from the patient who died of SIDS were negative according to real-time RT-PCR. No histopathologic alterations could be observed in myocardial tissue from this same patient.

To evaluate whether detected hCV strains differed from previously described genotypes, amplification and nucleotide sequencing of additional genomic

regions was attempted. In a case of meningoencephalitis (specimen 07/03981), we sequenced a 1,297-nt fragment comprising the near complete 5′-UTR and the first 489 nt of the structural protein gene (leader, viral protein [VP] 4 domain, and upstream VP2 domain, GenBank accession no. JN209931). Despite repeated trials, further sequence fragments could be amplified neither from the specimen from this patient nor from that from the second patient with meningoencephalitis that showed very low virus concentrations (specimen VI1607). From the specimen from the SIDS patient (specimen 347/10), amplification of the complete structural genome and partial nonstructural genome was successful (5,333 nt, GenBank accession no. JN209932). This virus belonged to hCV genotype 2 in the VP1 genomic region (i.e., the region used for the designation of genotypes) (Figure, panel A). The CSF specimen 07/03981 was also phylogenetically related to genotype 2 viruses in the 5′-UTR and Leader-VP2 genomic regions (Figure, panels B and C). On the basis of the 5′-UTR sequences, the closest known relative to both viruses was D/VI2229, obtained in Germany in 2004 (nucleotide percentage distance 4.7% for the SIDS specimen and 0.9% for the CSF specimen). In the structural protein gene fragment, the closest relative of both viruses was a strain obtained in the Netherlands in 2008 (Nijmegen2008, nucleotide distance 13.9% for the SIDS specimen and 3.5% for the CSF specimen). This suggested geographic rather than phylogenetic clustering of viruses detected within and beyond the respiratory and enteric tracts. However, formal and final virus typing is pending because VP1 regions could not be sequenced from 2 viruses.

Absence of other detectable pathogens in 1 of the meningoencephalitis case-patients (07/03981) made causation by hCV plausible (online Appendix Table). For the second case (VI1607), an enterovirus was co-detected by real-time RT-PCR in CSF and feces. Serotyping from feces classified this virus as echovirus type 30, known to cause aseptic meningitis. For the specimen from the child who died of SIDS, a rhinovirus was co-detected at low concentrations (real-time RT-PCR threshold cycle value >40), most compatible with shedding after previous respiratory infection (*9*).

Conclusions

The detection of hCVs in body compartments beyond the respiratory and enteric tracts is novel and suggests a role of these viruses in organ-related disease. A low detection rate in CSF does not contradict a general potential of these viruses to cause meningoencephalitis, as exemplified by enteroviruses for which lack of detection in CSF despite clear association with disease is not uncommon (*10*). Considering links between the related *Theilovirus* and demyelinating disease in laboratory models (*1*), long-term

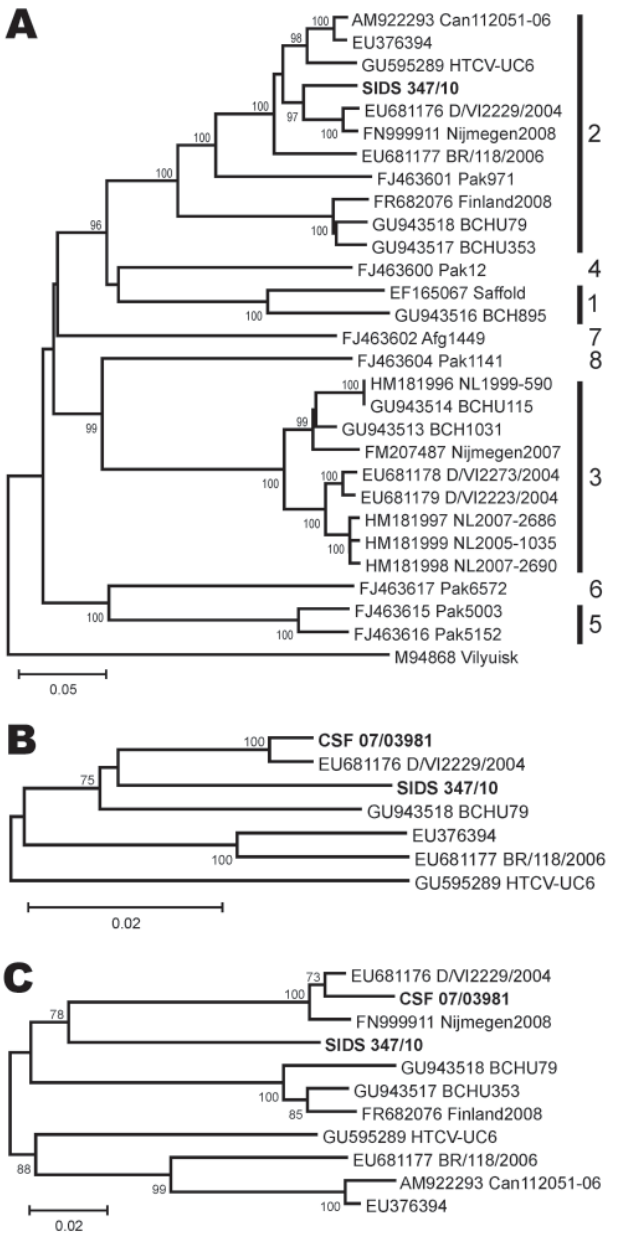

Figure. Human cardiovirus phylogeny including novel viruses from myocardial tissue and cerebrospinal fluid. A) The 798-nt complete viral protein (VP) 1 phylogeny, with genotypes indicated to the right. Vilyuisk virus was used as an outgroup. B) The 802-nt partial 5′ untranslated region phylogeny of genotype 2 human cardioviruses. C) The 489-nt complete leader, complete VP4 and partial VP2 phylogeny of genotype 2 human cardioviruses. Neighbor-joining phylogenies were calculated with MEGA5 (www.megasoftware.net) by using a percentage nucleotide distance substitution model with complete deletion of gaps and 1,000 bootstrap reiterations for confidence testing. Only bootstrap values >70% are shown at node points. Scale bars indicate percentage nucleotide distance. Novel viruses from this study (sudden infant death syndrome [SIDS] 347/10 and cerebrospinal fluid [CSF] 07/03981) are shown in **boldface**. Reference viruses are given with GenBank accession number and strain name (when available).

outcomes of patients with hCV infection of the central nervous system should be followed up. Such longitudinal studies should include sufficient numbers of patients because natural infections with *Theilovirus* in rodents are common and will less frequently result in multiple sclerosis–like disease than in laboratory models (*1*). The rarity of hCV detection in our study suggests the assembly of such cohorts to be a difficult and lengthy task that could benefit greatly from international coordination.

Despite the absence of histopathologic alterations, the detection of hCV in a child who died of SIDS is remarkable because the related encephalomyocarditis virus constitutes a prototypic model for myocarditis in mammals (*11*). Again, the high human seroprevalence against hCV (*12*) will complicate epidemiologic studies, yet investigations of links between hCV and SIDS are highly justified because diarrhea is an acknowledged risk factor for SIDS (*13*).

A limitation of our study is that the VP1 genomic region of the viruses detected in CSF could not be obtained. In analogy to enteroviruses and parechoviruses, certain genotypes may be associated with distinct disease profiles, like polioviruses with encephalitis or parechovirus 3 with meningitis (*14*). Although we were able to classify the virus detected in the child who died of SIDS as a common genotype 2, the partial hCV sequence from a patient with meningitis did not permit typing because hCVs, as all picornaviruses, recombine frequently (*15*). We thus cannot exclude that the viruses detected in the meningitis cases may have acquired distinct features in their capsid protein or elsewhere that might influence pathogenicity.

Acknowledgments

We thank Victor Max Corman, Ulrike Reber, Silke Vollbach, Dirk Böker, Doris Schiffer-Ngampolo, Stefanie Kramme, Heide Hilbig-Hanl, Heidi Kocken, Ulrike Krause, Gundula Mueseler, and Ewa Voß for technical assistance.

This study was funded by the EU FP7 projects EMPERIE (contract no. 223498) and EVA (contract no. 228292).

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Seroprevalence of Alkhurma and Other Hemorrhagic Fever Viruses, Saudi Arabia

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A 2009 deployment of military units from several Saudi Arabian provinces to Jazan Province, Saudi Arabia, enabled us to evaluate exposure to Alkhurma, Crimean-Congo, dengue, and Rift Valley hemorrhagic fever viruses. Seroprevalence to all viruses was low; however, Alkhurma virus seroprevalence was higher (1.3%) and less geographically restricted than previously thought.

Jazan is a Red Sea port city on Saudi Arabia's southern border with Yemen and the capital of Jazan Province. The region serves as an east–west portal from sub-Saharan Africa at Djibouti and a south–north route across the Yemeni frontier. It is a heavily traveled corridor for humans and animals entering Saudi Arabia, particularly during the annual Hajj pilgrimage. Malaria is endemic, and arbovirus infections are well described, most notably a 2000–2001 Rift Valley fever (RVF) outbreak on the Saudi Arabia–Yemen frontier. More than 880 confirmed RVF cases were reported. This outbreak was also notable for a case-fatality rate of 14% (*1*). Sporadic infections caused by Crimean-Congo hemorrhagic fever virus (CCHFV) occur as well. The virus is endemic across a wide geographic range, from the Middle East to Africa and central Asia (*2*). Dengue virus (DENV), a pathogen well known for large outbreaks and global circulation, also causes seasonal outbreaks in Saudi Arabia's western provinces; most outbreaks occur farther north in the urban centers of Jeddah and Makkah (*3*).

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DOI: http://dx.doi.org/10.3201/eid1712.110658

In contrast, Alkhurma hemorrhagic fever is an emerging infectious disease that has been described mostly in Saudi Arabia. The responsible virus, first isolated in Jeddah by A.M. Zaki, has since been characterized as a distinct lineage of Kyasanur Forest disease virus, a tick-borne member of the family *Flaviviridae* (*4*–*6*). Epidemiologic studies of Alkhurma hemorrhagic fever virus (AHFV) have focused on Jeddah and Makkah, where outbreaks were first described; most were characterized by high rates of illness and death (*6*). Recent studies in Najran Province extended the spectrum of disease to include subclinical infection, which was far more frequent than severe disease (*7*,*8*). A recent report suggests wider geographic range for AHFV, with infections identified in 2 Italian tourists after they traveled to Egypt (*9*).

The Study

In November 2009, Saudi military forces previously stationed in other parts of the country were deployed to Jazan Province. This situation enabled us to look at baseline arbovirus seroprevalence in a group of new arrivals, stratified by province of origin. During May 8–12, 2010, we enrolled 1,026 soldiers in 5 Jazan administrative units near the border with Yemen in a study to evaluate serologic reactivity to AHFV, CCHFV, DENV, and RVFV. After receiving consent and assigning numeric identifiers to anonymize data, we used questionnaires to record Jazan arrival dates, home province, previous administrative residence, health histories, vector exposures, and other risk factors. Answers were reviewed onsite, and a 5-mL blood sample was collected. Serum samples from each soldier were labeled, archived, frozen, stored at –80°C, and transferred to the Ministry of Health central laboratory in Riyadh for testing. Questionnaire and testing data were entered in Epi Info software (wwwn.cdc.gov/epiinfo) and then transferred to SPSS version 19.0 (IBM, Somers, NY, USA) for analysis.

A total of 197 (19%) enrolled soldiers reported symptomatic illness during deployment, 49 (25%) of whom were hospitalized. Reported signs and symptoms were fever (n = 81), rash (n = 50), and musculoskeletal complaints (n = 128). A diagnosis of malaria was recorded for 27 febrile soldiers and dengue fever for 1. Illnesses of the remaining soldiers were undiagnosed. As expected given the number of malaria cases, reported arthropod exposures favored mosquitoes over ticks, with 875 (85%) soldiers reporting mosquito contact compared with 153 (15%) reporting tick encounters. Thirty-seven (3%) soldiers reported contact with livestock carcasses, blood, or body fluids.

Serologic testing was completed for 1,024 soldiers; initial screening by IgG to each of the 4 viruses was followed by IgM testing of all IgG-reactive samples. Dengue antibodies were tested by using PanBio ELISA IgG

(E-DEN02G; Inverness Medical Innovations, Sinnamon Park, Queensland, Australia) and IgM (E-DEN01M; Inverness Medical Innovations) following manufacturer recommendations and protocols. IgG and IgM testing for AHFV, CCHV, and RVFV was done with Centers for Disease Control and Prevention (Atlanta, GA, USA) reagents and protocols by using cell culture–derived antigens (*7*,*10*,*11*). Briefly, the ELISA antigens used to coat plates (for IgG) or detect captured IgM were produced by infecting Vero E6 cells with respective reference virus strains or by using uninfected cells for control. Each sample was tested at 4 dilutions (100, 400, 1,600, and 6,400). IgG reactivity/IgM nonreactivity was considered evidence of past infection; concurrent IgG/IgM reactivity was interpreted as infection within the previous 6 months. IgG-seropositive persons without histories of illness were considered to have had subclinical or very mild infection.

Forty reactive serum samples were identified, for a combined seroprevalence of 3.9 cases/100 soldiers tested: RVF (n = 20), AHFV (n = 13), CCHV (n = 6), and DENV (n = 1) (Table). One soldier who had a positive test result for IgG and IgM to RVFV had a rash but no history of fever. No soldiers with AHFV, CCHFV, or DENV IgM were identified. We did not observe cross-reactivity of antibodies often seen with flaviviruses, and no person had positive test results against >1 antigen.

Analysis of the province of origin provided AHFV epidemiologic information (Figure). Two hundred sixty-eight (26%) soldiers were transferred to Jazan from Tabouk. This northern district accounted for most seropositive soldiers (8/13), 6 of whom might have resided in or visited a previously known AHFV-endemic region before transfer. Three AHFV-seropositive soldiers arrived from the Eastern Region; none had a previous residence or travel history elsewhere. In contrast, most RVFV-seropositive soldiers (18/20) and all 6 CCHV-seropositive soldiers had previously worked and resided in Jazan or other previously RVFV-affected region. The only RVFV IgM-seropositive soldier listed a home residence and previous workplace in an AHFV-endemic region.

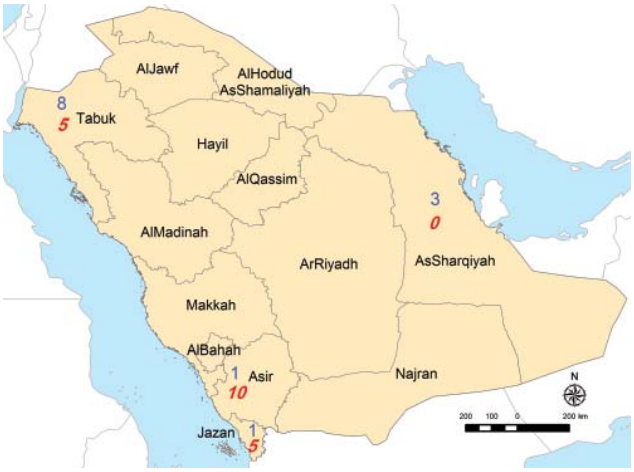

Figure. Numbers of soldiers with seropositive test results distributed according to Saudi Arabian province before transfer to Jazan, 2009. Blue (n = 13), seropositive for Alkhurma hemorrhagic fever virus; red (n = 20), seropositive for Rift Valley fever virus. Map courtesy of Al Zahrani.

Conclusions

Although the full geographic distribution and severity of AHFV infection is still being characterized, data from this study imply a wider range of endemicity than previously reported. For instance, most seropositive persons came from Tabouk and Eastern Directorates. This finding is not surprising knowing the large geographic distribution of the suspected tick vectors, *Ornithodoros savignyi* and *Hyalomma dromedarii* (*12*,*13*); however, neither hemorrhagic fever nor AHFV infection has been reported from either region. Nor did any AHFV-seropositive soldier disclose a history of severe illness, consistent with a 2006–2009 Narjan outbreak study that found seropositive status to be highly correlated with mild or asymptomatic infection. Although additional studies are required to further characterize Alkhurma's natural history, case-fatality rates of 25%–30% reported after earlier outbreaks in Jeddah and Makkah appear to be overestimates (*14*).

In contrast, all CCHFV-seropositive and most who RVFV-seropositive soldiers had resided in Jazan or an adjacent region previously endemic for those viruses. Apart from the reported episodes of CCHF surrounding the importation of infected livestock or ticks, the epidemiology and distribution of this virus in Saudi Arabia are unclear. Our results showed that it is circulating at least in the

Table. Serologic status of 1,024 soldiers evaluated for IgG and IgM ELISA reactivity against AHFV, CCHF, DENV, and RVFV antigens, Saudi Arabia, 2009*

Virus	No. (%) IgG reactive	No. IgM reactive	No. symptomatic	Seroprevalence/100 soldiers tested
RVFV	20 (2.0)	1	1 (rash)	1.95
AHFV	13 (1.3)	0	0	1.27
CCHF	6 (0.6)	0	0	0.58
DENV	1 (0.1)	0	0	0.1
Total	40 (3.9)	1	1	3.9

*AHFV, Alkhurma hemorrhagic fever virus; CCHF, Crimean-Congo hemorrhagic fever; DENV, dengue virus; RVFV, Rift Valley fever virus.

western part of the country. A 2% RVFV seroprevalence in our study, although not necessarily generalizable to the entire population, is not surprising given the large number of cases during the 2000–2001 outbreak (*1*,*15*). Low dengue seroprevalence contrasts with large recurring outbreaks in Jeddah and Makkah and may have implications for future prevention strategies.

Although IgM testing was not done on IgG-seronegative samples, the absence of compatible illness in surveyed soldiers suggests IgG was adequate for surveillance screening. Other potential limitations include unknown specificity of some tests used to survey these viruses and recall accuracy of travel histories or residences reported by the study's participants. Nonetheless, this study provides systematic evidence that Alkhurma, Crimean-Congo hemorrhagic fever, dengue, and Rift Valley fever viruses are endemic to western, if not all provinces of, Saudi Arabia. A better understanding of their ecology, natural history, and epidemiology is needed to assess the potential risks to public health.

Dr Memish is an infectious disease specialist at the Ministry of Health, Riyadh, Saudi Arabia. His research interests include emerging infectious diseases and their effects on public health, surveillance systems, and health policies.

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Knowledge of Avian Influenza (H5N1) among Poultry Workers, Hong Kong, China

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In 2009, a cross-sectional survey of 360 poultry workers in Hong Kong, China, showed that workers had inadequate levels of avian influenza (H5N1) risk knowledge, preventive behavior, and outbreak preparedness. The main barriers to preventive practices were low perceived benefits and interference with work. Poultry workers require occupation-specific health promotion.

In 1997, a zoonosis in humans caused by a highly lethal strain of avian influenza virus (H5N1) was reported in Hong Kong. Live-poultry markets were the source of this outbreak (*1*). As one of the world's most densely populated regions (16,000 persons/mile^2 [>6,300 persons/km^2]) (*2*), Hong Kong is a city at high risk for a large-scale outbreak of avian influenza caused by live poultry in large-volume wholesale markets and within neighborhood wet markets (open food stall markets).

Because members of the average household in Hong Kong shop in wet markets on a habitual basis, these markets are located in the most densely populated areas (Figure) and are commonly multistory complexes or in basement levels of shopping centers. Because poultry workers are a potential bridge population (*3*,*4*), the government has instigated voluntary avian influenza training since 2001 that reviews regulations for workplace disinfection, waste disposal, poultry storage, and personal hygiene measures (*5*,*6*).

Despite occupational risk for exposure to avian influenza (*7*,*8*), there have been few studies of poultry workers (*8*–*12*). Most studies were conducted in rural settings in developing countries (*9*–*12*), but findings cannot be readily extrapolated to cities such as Hong Kong because of differences in food-handling practices and occupational settings. Knowledge, perceptions, and work practices of live-poultry workers in Hong Kong have not been examined. Therefore, a survey of these workers is timely and warranted, given confirmed persistence of avian influenza in Asia. (*13*)

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DOI: http://dx.doi.org/10.3201/eid1712.110321

The Study

An anonymous, cross-sectional survey was conducted during June–November 2009. Interviewers approached 132 licensed live-poultry retail businesses in wet markets and 23 wholesale establishments. The final sample was 360 poultry workers (194 retailers and 166 wholesalers; response rate 68.1%).

Respondents were asked about their demographics, past month's work and preventive behavior, and avian influenza–related knowledge on the basis of a World Health Organization factsheet (*14*). We asked perception questions based on the Health Belief Model and the likelihood of adopting certain behavior patterns in the event of a local bird-to-bird or bird-to-human outbreak of avian influenza.

Summative scores were computed for avian influenza–related knowledge, current preventive behavior patterns, outbreak preparedness, and various perception domains. Higher scores reflected more beneficial levels of each domain. Unconditional multilevel regression indicated no evidence of clustering effect by poultry market. Standard multivariable linear regression was conducted by using SAS version 9.1.3 (SAS Institute, Cary, NC, USA) with knowledge, practice, and preparedness scores as outcomes and potential predictors showing $p<0.25$ in unadjusted analyses as input variables. Distribution of standardized residuals and their association with predicted values were examined to assess model assumptions.

Most (208, 60.1%) respondents were men 35–54 years of age, of whom 192 (55.3%) had worked a mean of 16.1 years in the poultry industry. Respondents showed low mean summative scores for knowledge of avian influenza (online Appendix Table 1, wwwnc.cdc.gov/EID/article/17/12/11-0321-TA1.htm). Nearly two thirds (232, 64.1%) of poultry workers reported that avian influenza virus (H5N1) infects wild birds, but fewer workers reported that this virus could infect live poultry (212, 60.1%), domesticated birds (159, 44.8%), or humans (178, 50.0%).

A total of 242 (69.1%) workers reported that consuming undercooked poultry could transmit the virus, and 210 (59.7%) knew that infection could result from touching bird feces. For other transmission routes, awareness was lower, ranging from 14.0% (48) for eating undercooked eggs to 29.1% (102) for slaughtering poultry.

Ninety-six (27.4%) workers were unsure whether avian influenza virus (H5N1) infection had occurred in humans in Hong Kong, 198 (58%) incorrectly believed that nearly everyone survives this infection, and 110 (32.8%)

incorrectly believed that a human vaccine for avian influenza was available. Most (208, 89.9%) respondents were familiar with influenza-like symptoms of avian influenza virus (H5N1) infection such as fever, but fewer workers were aware of respiratory and gastrointestinal symptoms of virus infection.

The Internet and other sources (e.g., health talks) of information about avian influenza were strong independent predictors of greater knowledge. However, training did not result in higher knowledge levels.

Poultry workers reported low-to-moderate levels of compliance with hand hygiene and other preventive measures (ranging from 7.3% [36] using eye protection to 65.2% [245] using handwashing with soap after slaughtering poultry). Working in the poultry industry ≥10 years, lower perceived barriers to preventive behavior, and retail poultry work were independent predictors of higher preventive behavior scores.

With regard to avian influenza–related perceptions, lack of training (277, 83.4%) and the view that compliance with all infection regulations is difficult during peak hours (218, 64.9%) were the most frequently cited barriers to adoption of preventive behavior. A total of 154 (46.4%) workers believed that face masks reduced business, and 153 (46.1%) believed that vaccination was expensive.

Low anxiety about illness was reported by 242 (76.6%) respondents. In the event of a local outbreak, workers expressed various levels of acceptance for precautionary actions, ranging from 15.8% (56) for reducing work hours to 82.4% (290) for seeking medical care for influenza-like symptoms. Ninety-six (27.4%) respondents anticipated taking oseltamivir. Greater perceived benefit of preventive behavior was the strongest independent predictor of higher preparedness scores (online Appendix Table 2, wwwnc.cdc.gov/EID/article/17/12/11-0321-TA2.htm).

Conclusions

Similar to other regions (*8*–*11*), poultry workers in Hong Kong showed low risk perceptions for avian influenza, inadequate knowledge, and a wide range of compliance with preventive measures. Because training (*6*) was not associated with overall preventive behavior or preparedness, there may be an unmet need for occupation-specific health information.

Higher levels of knowledge demonstrated by workers who accessed health information sources (e.g., Internet) that provide detailed information suggest that comprehensive, occupation-relevant information should be more widely accessible. However, occupational practices of animal workers might not be amenable to change solely on the basis of improvements in knowledge. Only 129 (42.1%) respondents reported that poultry workers could realistically adhere to all government guidelines (*6*). Interference with work, high cost, and reduction of business were repeatedly cited as impediments to the adoption of preventive behavior. Even in the event of local outbreaks of avian influenza, most workers were not amenable to actions having adverse economic effects such as reducing work hours. Animal workers are thereby unlikely to widely adopt preventive behavior if these measures conflict with their economic interests.

Despite the ongoing government regulations regarding avian influenza in Hong Kong (*6*), a complete ban on live poultry is unrealistic because of the culturally entrenched demand for fresh poultry. Increasing knowledge and risk perceptions while simultaneously reducing occupational barriers to preventive behavior thereby continues to be the cornerstone of effective zoonotic infection control among animal workers.

Implications of these findings extend to other poultry-borne pathogens, such as *Campylobacter* spp. and

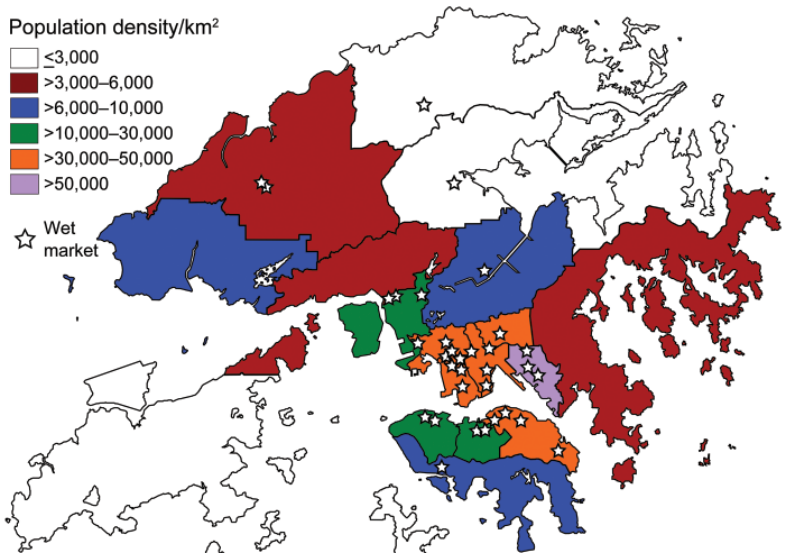

Figure. Location of live poultry wet markets (open food stall markets) in relation to population density, Hong Kong, China, June–November, 2009.

Salmonella spp., which share common preventive measures. Close adherence to workplace measures will likely reduce outbreak risk for other poultry-borne diseases. Therefore, a framework for greater integration of risk management strategies and worker education of these poultry-borne infections tailored to the local context is worthwhile and cost-effective.

In the spirit of the One Health Commission, which calls for an integrated, interdisciplinary approach to human–veterinary–environmental health challenges (*15*), the fight against global pandemics, such as those of avian influenza virus (H5N1), necessitates greater dialogue and collaborative leadership between governments and livestock industries. Development of realistic occupational safety measures is an ongoing challenge for national governments.

Acknowledgments

We thank the poultry workers for participating in the study and Terry Wong for assisting with preliminary data collection.

This study was supported by the Hong Kong Research Fund for the Control of Infectious Diseases.

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Risk for Human African Trypanosomiasis, Central Africa, 2000–2009

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Comprehensive georeference records for human African trypanosomiasis in Cameroon, Central African Republic, Chad, Congo, Equatorial Guinea, and Gabon were combined with human population layers to estimate a kernel-smoothed relative risk function. Five risk categories were mapped, and ≈3.5 million persons were estimated to be at risk for this disease.

The most recent continental estimates of persons at risk for human African trypanosomiasis (HAT), also known as sleeping sickness, were published by the World Health Organization in 1998 (*1*). These estimates were provided on a country-by-country basis, and they were largely based on educated guesses and rough estimations of experts. Since that time, major progress has been made in HAT control and surveillance (*2*). Data collection and reporting have also substantially improved and increasingly include an explicit and accurate mapping component (*3*–*6*). The magnitude of the recent advances in HAT control and surveillance is such that up-to-date estimates of the number and distribution of persons at risk are urgently needed. The purpose of this study was to develop a method to estimate and map the risk for HAT in central Africa.

The Study

The study area in central Africa comprised Cameroon, Central African Republic, Chad, Congo, Equatorial Guinea, and Gabon. The Gambian form of sleeping sickness caused by *Trypanosoma brucei gambiense* is endemic to these 6 countries.

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DOI: http://dx.doi.org/10.3201/eid1712.110921

Reported cases for 2000–2009 were obtained from the Atlas of HAT (*7*). This Atlas provided mapped data at the village level for 15,083 (94.2%) of 16,005 cases of HAT reported in the study region. Average mapping accuracy for these cases was ≈800 m (*7*). For the remaining 5.8% of cases, village-level information was unavailable, but the focus of geographic origin was known. Therefore, these cases were distributed among the disease-endemic villages of their focus by using proportional allocation, whereby proportionally more unmapped cases were attributed to mapped villages that reported more cases during the study period.

Human population distribution was obtained from LandScan databases (www.ornl.gov/sci/landscan). LandScan provides global grids in which census counts are allocated to grid nodes by probability coefficients (*8*,*9*). Probability coefficients were based on land cover, elevation, slope, roads, and populated areas/points. LandScan spatial resolution is <1 km at the equator, and population layers are updated yearly. To delineate risk areas, an average of all LandScan population layers during 2000–2009 was used. Subsequently, LandScan 2009 data were combined with the risk map to provide estimates of persons at risk at the end of the study period.

Both input layers (i.e., HAT cases and LandScan human population) can be regarded as spatial point processes, thus amenable to spatial smoothing (*10*). Smoothing results in intensity surfaces where the intensity of a point process is the mean number of events per unit area (*11*). For this study, the intensity of HAT cases and human population was estimated by using a quadratic kernel function (*12*). Intensity surfaces were generated by using the same search radius (*13*), which was set at 30 km.

Before spatial smoothing, the number of HAT cases reported in 2000–2009 was divided by 10 to yield the average number of cases per year. All LandScan layers from 2000 through 2009 were also averaged and subsequently converted from grid (raster format) to points (vector format) to enable spatial smoothing. No attempt was made to account for edge effects of smoothing (*11*), but edge effects were not expected to matter unduly because our final objective was to estimate the ratio of 2 intensities (*14*).

Spatial smoothing resulted in 2 surfaces, D(x,y) and P(x,y), which represented average annual estimates of disease intensity and population intensity, respectively. A relative risk function R(x,y) could subsequently be estimated as the ratio D(x,y)/P(x,y). Thresholds were applied to the function R(x,y) to distinguish 5 categories of risk ranging from very high to very low (Table 1). When R(x,y) was <1 HAT case/million persons/year, risk was considered marginal.

[1]These authors contributed equally to this article.

Table 1. Thresholds for definition of risk for human African trypanosomiasis, central Africa, 2000–2009

Category	No. cases/inhabitants/y
Very high	$\geq 1/10^2$
High	$<1/10^2$ to $\geq 1/10^3$
Moderate	$<1/10^3$ to $\geq 1/10^4$
Low	$<1/10^4$ to $\geq 1/10^5$
Very low	$<1/10^5$ to $\geq 1/10^6$

The results of the analysis are shown in the Figure, in which risk areas are mapped, and in Table 2, which summarizes the number of persons at risk, the extent of areas at risk, and the corresponding number of cases. In the 6 countries studied, ≈3.5 million persons are estimated to be at risk for contracting HAT (8.9% of the total population), distributed over an area of 224,000 km^2 (7.5% of the total land area).

Very high–risk areas comprise the most active foci in the Central African Republic (Batangafo, Obo, Mboki, and Zemio) and in Congo (Mpouya and Ngabé). These zones are located mainly in rural areas in which human population density is low, but they also include a few small towns. Areas included in the high-risk category are in foci in Bodo (Chad); Maitikoulou and Djemah (Central African Republic); Noya (Gabon); Kogo (Equatorial Guinea); and Loukoléla, Mossaka, Ignié, and Loudima (Congo). High-risk zones are also located around very high–risk areas. The moderate-risk category includes foci in Nola-Bilolo and Lobaye Prefecture (Central African Republic), Bipindi and Campo (Cameroon), Kango and Port Gentil (Gabon), Mbini (Equatorial Guinea), and areas in Bouenza and Gamboma (Congo). Low-risk areas were found mainly at the periphery of zones to which HAT is highly endemic, but they also include a few isolated foci with low levels of transmission, such as Mamfé, Fontem, and Doumé (Cameroon). Very low–risk zones represent the extreme periphery of active foci, but they also include isolated rural foci, such as Mbandjock (Cameroon) and Libreville (Gabon), one of the largest urban agglomerations in the region.

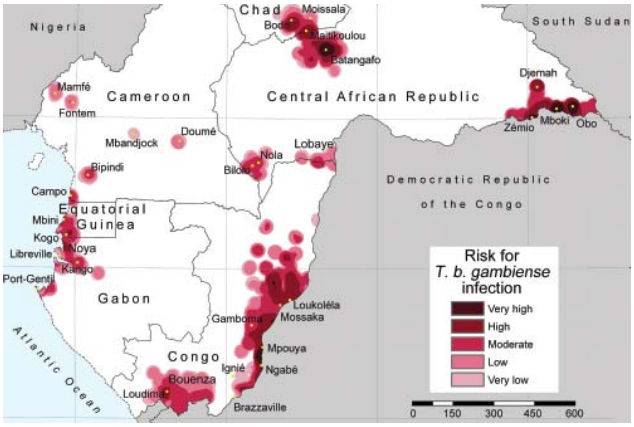

Figure. Lambert azimuthal equal-area projection (www.quadibloc.com/maps/maz0204.htm) of risk for infection with *Trypanosoma brucei gambiense*, central Africa, 2000–2009.

Conclusions

The methods reported provide an evidence-based approach to mapping the risk for HAT and estimating at-risk population. The use of different risk categories enables severity of the disease to be ranked.

We did not attempt to model underascertainment and underreporting, which are known to affect a neglected disease such as HAT. However, recent progress in the fields of active and passive surveillance (*3*) and comprehensive and systematic collection and mapping of HAT data over a 10-year period (*7*) substantially contributed to the robustness of the presented risk estimates. At the same time, we highlight that further improvements in consistency and coverage of HAT case detection and reporting are needed and require long-term efforts.

Because of the novel approach used in this study, it is unwarranted to make comparisons with previous figures of at-risk population, especially if the goal is to explore trends. Conversely, use of global human population layers and regular updating of the Atlas of HAT will enable future trends to be captured and the method to be applied to all countries to which HAT is endemic.

The type of risk maps presented will help target the most appropriate, site-specific strategies for HAT control and surveillance, such as the optimal frequency of active screening activities (*15*). These maps will enable limited

Table 2. Estimated risk for infection with *Trypanosoma brucei gambiense* in 6 countries, central Africa, 2000–2009*

Country	Risk category					Total
	Very high	High	Moderate	Low	Very low	
Cameroon	0 (0); 0	0 (0); 0	28 (22); 97	236 (80); 87	366 (71); 1	630 (172); 185
Central African Rep	29 (59); 6,075	33 (123); 1,193	115 (212); 287	133 (140); 28	65 (78); 0	374 (612); 7,583
Chad	0 (0); 0	109 (33); 2,884	110 (34); 76	120 (34); 19	119 (36); 1	458 (137); 2,980
Congo	3 (16); 1,250	105 (205); 2,266	479 (367); 1,169	443 (337); 68	138 (164); 3	1,168 (1,089); 4,756
Equatorial Guinea	0 (0); 0	2 (4); 42	27 (37); 130	8 (16); 1	4 (8); 0	40 (65); 173
Gabon	0 (0); 0	2 (6); 161	21 (58); 116	19 (69); 24	762 (36); 27	804 (168); 328
Total	32 (74); 7,325	251 (372); 6,546	780 (730); 1,875	959 (675); 227	1,453 (392); 32	3,475 (2,244); 16,005

*Values are no. persons x 10^3 at risk (area in km^2 × 10^2 at risk); no. cases of human African trypanosomiasis. Risk categories are defined in Table 1. Rep, Republic.

resources available to be allocated rationally and where they are needed most.

This study was conducted in a collaboration between the World Health Organization and the Food and Agriculture Organization of the United Nations within the Programme Against African Trypanosomosis.

The boundaries and names shown and the designations used on the maps presented in this paper do not imply the expression of any opinion whatsoever on the part of the World Health Organization or the Food and Agriculture Organization of the United Nations concerning the legal status of any country, territory, city, or area, or of its authorities, or concerning the delimitation of its frontiers or boundaries.

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Animal Diseases Caused by Orbiviruses, Algeria

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Antibodies against bluetongue virus were detected in cattle, sheep, goats, and camels in Algeria in 2008. Antibodies against epizootic hemorrhagic disease virus were detected in cattle, but antibodies against African horse sickness virus were not detected in horses and mules. Epizootic hemorrhagic disease in northern Africa poses a major risk for the European Union.

The genus *Orbivirus* contains several viruses such as bluetongue virus (BTV; 26 serotypes), epizootic hemorrhagic disease virus (EHDV; 8 serotypes), and African horse sickness virus (AHSV; 9 serotypes). These viruses cause serious diseases in domestic and wild animals. Several orbiviruses have been reported in Algeria. Bluetongue disease caused by BTV serotype 2 (BTV-2) was detected in 2000 (*1*); serotype 1 was detected in 2006 (*2*). BTV-1 was detected again in 2008, and new outbreaks were detected in 2009 and 2010 (*2*). Epizootic hemorrhagic disease (EHD) caused by EHDV serotype 6 (EHDV-6), was reported in Morocco in 2004 and 2006 (*3*). AHSV serotype 9 (AHSV-9) was detected in Morocco in 1965 (*4*).

These orbiviruses were frequently reported to have originated in sub-Saharan Africa (*3*,*5*–*7*). In many cases, outbreaks of orbivirus diseases in Algeria were followed by incursions of these viruses into southern Europe, most likely through passive wind-borne transmission of infected vectors (*8*,*9*). The aim of this study was to identify 3 particular orbiviruses (BTV, EHDV, and AHSV) in Algeria and determine their geographic distribution.

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DOI: http://dx.doi.org/10.3201/eid1712.110928

The Study

Algeria contains 19.6 million sheep, 3.8 million goats, 1.6 million cattle, ≈230,000 horses, and 290,000 camels. This country is divided into 48 provinces (wilayas). For reasons of animal health, transportation of animals is not allowed between southern and northern Algeria. Vaccination against bluetongue disease, EHD, and African horse sickness in Algeria is forbidden by law because vaccinated animals cannot be differentiated from naturally infected animals.

Sampling was conducted during August–September 2008. Cattle, sheep, goats, and camels were sampled in the BTV survey, cattle were sampled in the EHDV survey, and horses and mules were sampled in the AHSV survey. To avoid detection of antibodies from previous outbreaks, only livestock 6–12 months of age were sampled. For detection of EHDV and BTV, the epidemiologic unit was the herd.

Sample size was calculated to enable detection of ≥2% of infected cattle farms at a 95% confidence level (149 herds) and a within-herd prevalence ≥30% (9 animals/herd). In addition to cattle for detection of BTV, 359 samples were obtained from 65 sheep flocks, 71 samples from 27 goat herds, and 92 samples from 26 camel herds. For detection of AHSV, the epidemiologic unit was the animal, and sample size was calculated for detection of ≥2% of infected horses and mules at a 95% confidence level (149 animals).

IgG against BTV was detected by using a competitive ELISA (Pourquier Bluetongue Competitive ELISA; Pourquier Laboratory, Montpellier, France). To detect BTV genotypes, a real-time reverse transcription PCR (RT-PCR) (TaqVet BTV-FCO-all genotypes rRT-PCR; LSI Vet, Lissieu, France) was performed. Positive samples were tested by using RT-PCR kits for BTV-1, 2, 4, 6, 9, 11, and 16 (Taqvet BTV European BTV Typing; LSI Vet). A competitive ELISA provided by the Institute of Animal Health (Pirbright, UK) was performed to detect IgG against EHDV according to the protocol of Thevasagayam et al. (*10*). IgG against AHSV was detected by using a blocking ELISA (Ingezim AHSV compact plus 14.AHS.K.3; Ingenasa Laboratory, Madrid, Spain). Tests were performed according to manufacturer's instructions.

We detected an overall BTV seroprevalence of 24%. BTV seroprevalence differed among wilayas (Table; Figure) but was higher in northern wilayas and Ghardaia in central Algeria. In contrast with official 2008 data in which only 6 outbreaks were reported, our results indicated that BTV was widespread in 2008.

BTV seroprevalence differed between species (Table): 29% in cattle, 14% in sheep, and 21% in goats. In addition, a high seroprevalence (21%) was found in camels. In a recent study, BTV was isolated from the blood of 3

Table. Seropositivity for BTV and EHDV in livestock in Algeria, 2008*

Location†	No. positive/no. tested				
	BTV, cattle	BTV, sheep	BTV, goats	BTV, camels	EHDV, cattle
Adrar	0/31	0/19	0/6	0/27	6/29
Aïn Témouchent (1)	13/54	1/16	1/11	NT	1/54
Algiers (2)	14/41	0/18	NT	NT	0/41
Annaba (3)	8/28	1/17	NT	NT	0/28
Béjaïa (4)	6/40	0/21	NT	NT	0/40
Blida (5)	13/44	4/25	NT	NT	1/44
Boumerdès (6)	12/37	3/15	NT	NT	1/37
Chlef (7)	12/22	0/21	NT	NT	1/22
Djelfa	5/49	2/13	0/5	NT	15/49
El Tarf (8)	54/54	9/17	NT	NT	3/54
Ghardaia	17/22	6/15	5/12	7/7	5/22
Jijel (9)	4/27	0/22	NT	NT	0/27
Mostaganem (10)	58/72	NT	NT	NT	11/72
Naama (11)	0/55	2/23	0/4	10/35	9/56
Oran (12)	9/53	7/18	NT	NT	11/53
Skikda (14)	3/13	3/11	NT	NT	1/13
Souk Ahras (15)	8/18	0/9	NT	NT	1/18
Tebessa (16)	2/26	6/17	4/11	NT	6/26
Tindouf		6/15	5/13	2/23	
Tipasa (16)	0/48	0/20	NT	NT	0/48
Tizi Ouzou (17)	0/64	1/9	NT	NT	0/62
Tlemcen (18)	9/54	1/18	0/9	NT	3/54
Total	247/852	52/359	15/71	19/92	75/849

*BTV, bluetongue viruses; EHDV, epizootic hemorrhagic disease virus; NT, not tested.
†Values in parentheses indicate provinces (wilayas) shown in the Figure.

experimentally infected camels, which indicated that this animal might play a role in BTV transmission (*11*). Given that camels are frequently moved across desert areas in Algeria, they could potentially transport BTV over long distances, enabling viruses to cross the Sahara Desert.

Of ELISA-positive samples, 335 samples (250 from cattle, 51 from sheep, 15 from goats, and 19 from camels) obtained in 20 wilayas throughout Algeria were tested by BTV RT-PCR. BTV RNA, which indicates recent infection, was detected in 37 of samples (34 from cattle and 3 from sheep), most of which had been obtained in northeastern Algeria. The serotype identified was BTV-1.

Antibodies against EHDV were detected in 9% of the cattle tested. EHDV seroprevalence was detected in 15 of 21 wilayas sampled (Table), although EHD was not officially reported in 2008. This finding might be explained by often inconclusive clinical diagnoses and the fact that definitive diagnoses of this disease require specific laboratory tests (*3*). Given that only animals 6–12 months of age were sampled, seropositivity indicated circulation of EHDV over the previous year; the last reported outbreak in Algeria was in September 2006. In addition, the EHD epidemic in 2006 affected only central Algeria. However, our results indicate that EHD was widespread in 2008. EHDV seroprevalence seemed to be higher in central and southern Algeria (Table). None of 145 mules and 6 horses sampled in southwestern Algeria had antibodies against AHSV.

Conclusions

Our results indicated that BTV and EHDV were widespread in Algeria. Distribution of orbiviruses is determined by distribution of competent vectors, and entomologic surveys indicated that *Culicoides imicola* midges are abundant in northern and central Algeria (*12*), which is consistent with our results. Conversely, *C. imicola* midges were not present in southern desert regions, which would indicate that the livestock were infected elsewhere or that other *Culicoides* spp. might play a role in transmission. Moreover, although BTV seroprevalence was higher in northern and central wilayas, EHDV seroprevalence was higher in southern and central regions. This finding might be explained by the fact that different vector species can transmit EHDV and BTV (*3*). The temperature requirements for replication of these viruses and further transmission are also likely to differ (*3*). Furthermore, differences in distribution of these viruses might be influenced by exposure to viruses in previous years (*13*).

EHDV in northern Africa poses a major risk for the European Union because of likely wind-borne dispersal of infected vectors (*3*). In Europe, the presence of a known competent vector for EHDV (*C. imicola*), plus several suspected vectors (*14*), and the climatic conditions could be conducive to EHDV circulation (*3*). As reported in Israel in 2006, an EHDV epidemic can have a major economic effect through loss of milk production and increased animal

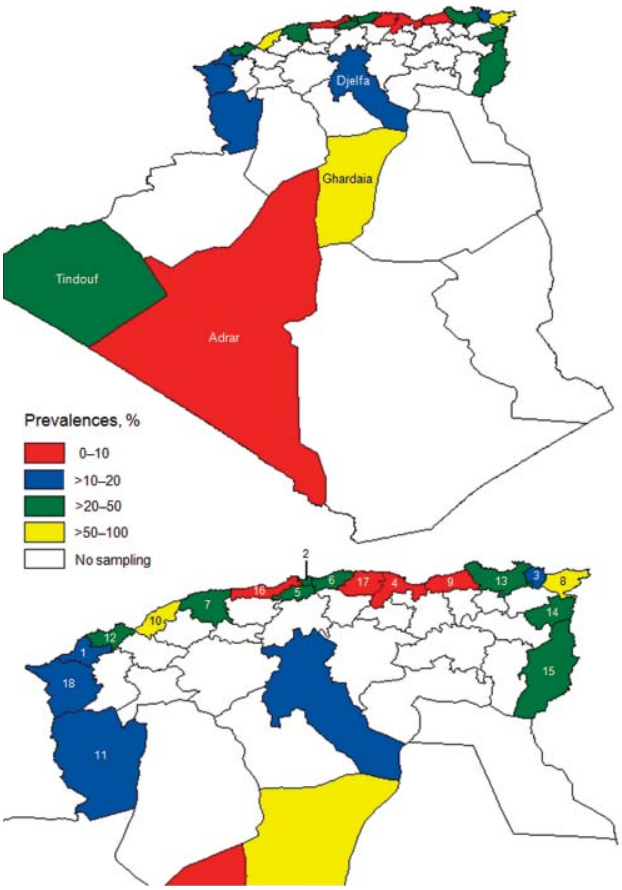

Figure. Seroprevalence of bluetongue virus in cattle, sheep, goats, and camels, by province (wilaya), Algeria, 2008. A) Northern Algeria; B) entire country. 1, Aïn Témouchent; 2, Algiers; 3, Annaba; 4, Béjaïa; 5, Blida; 6, Boumerdès; 7, Chlef; 8, El Tarf; 9, Jijel; 10, Mostaganem; 11, Naama; 12, Oran; 13, Skikda; 14, Souk Ahras; 15, Tébessa; 16, Tipasa; 17, Tizi Ouzou; 18, Tlemcem.

deaths (*15*). If EHDV were introduced into the European Union, detection of infected animals would be hampered by a lack of diagnostic methods (*3*). EHD control would also be complicated by a lack of vaccines (*3*). In addition, the high prevalence of BTV in camels in Algeria and their potential role in BTV transmission warrants further investigation.

Acknowledgments

We thank the veterinarians for helping collect samples and Peter Biggins for providing an English revision of the manuscript.

This study was supported by the MedReoNet Surveillance Network of Reoviruses, Bluetongue and African Horse Sickness, in the Mediterranean basin and Europe, Sixth Framework Programme.

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Genogroup I and II Picobirnaviruses in Respiratory Tracts of Pigs

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Sequence-independent amplification and specific reverse transcription PCRs identified genogroup I and II picobirnaviruses in respiratory tracts of pigs. These data expand knowledge of picobirnavirus diversity and tropism. Genetic relationships between porcine respiratory and human enteric picobirnaviruses suggest cross-species transmission of picobirnaviruses between pigs and humans.

A thorough understanding of virus diversity in animals provides epidemiologic baseline information about potential pathogens and can lead to identification of emerging human pathogens. On the basis of relevance to reemerging viruses, such as Nipah virus and influenza A virus, pigs are a key risk host for emerging RNA virus–associated disease in humans in different areas (*1*–*3*). In an effort to identify unknown porcine viruses in the respiratory tracts of pigs, we performed sequence-independent amplification on partially purified viral nucleic acid from swab samples of respiratory tracts from pigs that were PCR negative for influenza A virus (*4*–*6*).

The Study

We analyzed 197 respiratory tract swab specimens from pigs obtained in slaughterhouses in Hong Kong, China, and Colombo, Sri Lanka. Large-scale molecular RNA virus screening, based on host nucleic acid depletion, sequence-independent amplification, and sequencing of partially purified viral RNA was performed on nucleic acids isolated from 10 respiratory tract swab samples from pigs (*4*–*6*). Most of the 893 analyzed sequences were of unclassified porcine genome origin, unclassified, or bacterial origin. Three clones showed picobirnaviruses, which are double-stranded RNA viruses with a segmented genome belonging to the family *Picobirnaviridae* (*7*).

Because picobirnaviruses have been identified only in fecal specimens, diagnostic PCRs for genogroup I and II picobirnaviruses specific for the RNA-dependent RNA polymerase (RdRp) gene (*6*,*8*,*9*) were performed on 60 respiratory tract swab specimens to determine whether picobirnaviruses are present in the porcine respiratory tract. Sixteen (26.6%) of 60 samples were confirmed by sequencing as positive for genogroup I picobirnaviruses, and 4 (6.5%) of 60 were confirmed by sequencing as positive for genogroup II picobirnaviruses. Three of 60 porcine swab samples that showed evidence of genogroup II picobirnaviruses were also positive for genogroup I picobirnavirus.

Subsequent sequence analysis suggested that the genogroup II pan-picobirnavirus PCR might be suboptimal for detecting the novel genogroup II swine picobirnaviruses. Thus, we designed a new genogroup II diagnostic PCR that used primers 5′-GACCGGTWTGGATGTTTCCGATG-3′ and 5′-GTATCTGTGCTGGSCGCAT-3′, AmpliTaq Gold DNA polymerase (Life Technologies, Carlsbad, CA, USA), and 2 mmol/L $MgCl_2$ (incubation at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 20 s), and screened an additional 137 porcine respiratory tract swab samples for genogroup II picobirnaviruses. Twenty-two (16.0%) samples were positive for genogroup II picobirnaviruses.

To determine genetic relationships between porcine respiratory genogroup I picobirnaviruses with reported genogroup I viruses detected in wastewater and human and porcine feces, a phylogenetic tree was constructed on the basis of a 165-nt fragment of the RdRP gene as described (*6*,*10*) (online Appendix Figure, wwwnc.cdc.gov/EID/article/17/12/11-0934-FA1.htm). Genogroup I picobirnavirus nucleotide sequences from 6 porcine respiratory samples showed 58%–80% similarity with each other and belonged to different phylogenetic clades.

PBVI/*Sus scrofa*/VS4400033/2010 and PBVI/*Sus scrofa*/VS4400051/2010 were most closely related to porcine enteric picobirnaviruses identified in Hungary in 2005. PBVI/*Sus scrofa*/VS4400034/2010, PBVI/*Sus scrofa*/VS4400039/2010, and PBVI/*Sus scrofa*/VS4400023/2010 clustered with picobirnaviruses identified in wastewater in the United States in 2007. PBVI/*Sus scrofa*/VS4400030/2010 was most closely related to an enteric human picobirnavirus identified in the Netherlands in 2007. The close relationship between human and porcine genogroup I picobirnaviruses can also be observed in other parts of the phylogenetic tree, e.g., in branches 23–25 and 70–71 (online Appendix Figure).

To determine genetic relationships between porcine respiratory genogroup II picobirnaviruses with reported

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DOI: http://dx.doi.org/10.3201/eid1712.110934

genogroup II viruses detected in human feces, a phylogenetic tree was constructed on the basis of a 339-nt fragment of the RdRP gene as described (*6*,*10*) (Figure). Porcine genogroup II picobirnavirus partial RdRP nucleotide sequences from samples VS4400017, VS4400028, VS4400041, and VS4400049 showed 69%–96% similarity with each other (Table). Genogroup II picobirnaviruses PBVII/*Sus scrofa*/VS4400017/2010 and PBVII/*Sus scrofa*/VS4400049/2010 grouped in the same phylogenetic clade as human picobirnavirus genogroup II strains PBVII/*Homo sapiens*/USA-4-GA-91/1991 (*9*) and PBVII/*Homo sapiens*/NLD-142–3/2007 (*6*) and displayed only 2%–5% sequence diversity with each other, suggesting they could be considered the same virus (Figure; Table).

Genogroup II picobirnaviruses PBVII/*Sus scrofa*/VS4400028/2010 and PBVII/*Sus scrofa*/VS4400041/2010 could be grouped with human picobirnavirus genogroup II strain PBVII/*Homo sapiens*/IND-GPBV6G2P/2007 from India but displayed 20%–30% sequence diversity with each other (Figure; Table). Close genetic relationships could also be observed between genogroup II picobirnaviruses from pigs and humans.

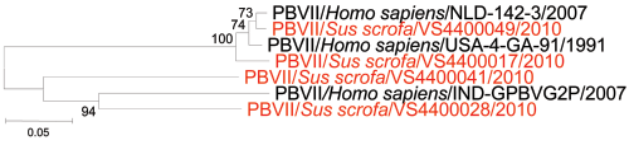

Figure. Phylogenetic analysis of genogroup II picobirnaviruses. Neighbor-joining (Jukes-Cantor model) phylogenetic tree of an ≈339-bp fragment (reference strain 4-GA-91 (*9*) of the picobirnavirus genogroup II RNA-dependent RNA polymerase gene from known human picobirnaviruses and newly characterized porcine picobirnaviruses in this study (JN176312–315, shown in red). Significant bootstrap values are shown. Nomenclature of depicted viruses is according to recent proposals (*7*). PBVII/*Homo sapiens*/USA-4-GA-91/1991 (AF246940) (*9*); PBVII/*Homo sapiens*/NLD-VS142–3/2007 (GU968925) (*6*); and PBVII/*Homo sapiens*/IND-GPBV6G2P/2007 (AB526257). Scale bar indicates nucleotide substitutions per site.

Conclusions

Our results indicated that picobirnaviruses can be commonly found in the respiratory tract of pigs from different locations and identified genogroup II picobirnaviruses in animals. Picobirnaviruses have been regarded as enteric viruses because all described cases were associated with virus shed in feces. Picobirnaviruses have been detected in human patients with and without gastroenteritis and are found in patients co-infected with enteric pathogens such as rotaviruses, caliciviruses, and astroviruses (*8*,*9*). Prevalence studies of picobirnaviruses in immunocompromised patients suggest that picobirnaviruses might be opportunistic enteric pathogens (*11*,*12*). Picobirnaviruses in the respiratory tracts of pigs suggest that picobirnaviruses might be not only potential enteric pathogens but also respiratory pathogens. The pigs used in our study showed no evidence of overt respiratory or other disease at the time of sampling. Whether these viruses contribute to disease early in life remains unclear.

Genogroup II porcine picobirnaviruses detected in this study were highly diverse. Phylogenetic analysis of porcine and human genogroup II picobirnaviruses indicated that ≥2 phylogenetic clades of genogroup II picobirnaviruses co-exist in these populations. Similar observations were found regarding the high genetic diversity of porcine respiratory genogroup I picobirnaviruses. Genetic relationships between porcine respiratory picobirnaviruses and picobirnaviruses from wastewater in the United States and human feces were observed. These results suggest that there were multiple cross-species transmissions of picobirnavirus strains between swine and humans (*8–10*,*13–15*).

Because molecular characterization of picobirnaviruses is limited, mostly to partial RdRP sequences, and only 1 complete genome has been determined, the zoonotic potential of picobirnaviruses awaits further characterization of full-length genomes. Attempts to obtain full-length genomes in this study were undertaken by using next-generation sequencing platforms but were unsuccessful. However, the extent of sequence variation along the 165-bp fragments of the RdRP gene of genogroup I picobirnaviruses for which an entire RdRP gene sequence is available (strains 1-CHN-97, HY005102, and VS10, and GenBank accession nos. AB186898, AF246939, and

Table. Pairwise nucleotide sequence identity between genogroup II picobirnaviruses for a 339-bp fragment of the RdRP coding region*

Strain	4-GA-91 (AF246940)	VS142–3 (GU968925)	GPBV6G2P (AB526257)	VS4400017	VS4400028	VS4400041	VS4400049
4-GA-91 (AF246940)		97.1	68.8	95.3	70.5	69.6	97.9
VS142–3 (GU968925)			68.5	95.3	69.3	69.3	97.9
GPBV6G2P (AB526257)				67.9	79.5	75	68.2
VS4400017					69.9	69	95.6
VS4400028						74.7	69.9
VS4400041							68.8
VS4400049							

*Reference strain 4-GA-91, GenBank accession no. AF246940. Values are percentages. Blank spaces indicate 100% identity. RdRP, RNA-dependent RNA polymerase.

GU968924) show good correlation with overall sequence variation observed in the entire RdRP gene (*13*).

A thorough understanding of the diversity of viruses in animals, virus transmission routes, and virus tropism provides epidemiologic baseline information about potential pathogenic threats from animal reservoirs for human health. Detection of genogroup I and II picobirnaviruses in porcine respiratory tract swab samples is an example of the needed expansion of our knowledge of picobirnavirus diversity and also expands our knowledge of picobirnavirus tropism. To better understand the epidemiology of genogroup II picobirnaviruses in pigs and to define whether zoonotic or reverse-zoonotic transmissions occur, more intensive surveillance on this group of virus in pigs from other regions needs to be conducted. Whether genogroup I and/or II picobirnaviruses can also be detected in the human respiratory tract and whether they play a causal role in respiratory diseases remain to be determined. This study illustrates how novel molecular techniques can provide new understanding of viral ecology, evolution, and spread.

This study was supported by the European Community Seventh Framework Programme (FP7/2007-2013) under project European Management Platform for Emerging and Re-emerging Infectious Disease Entities (grant agreement no. 223498).

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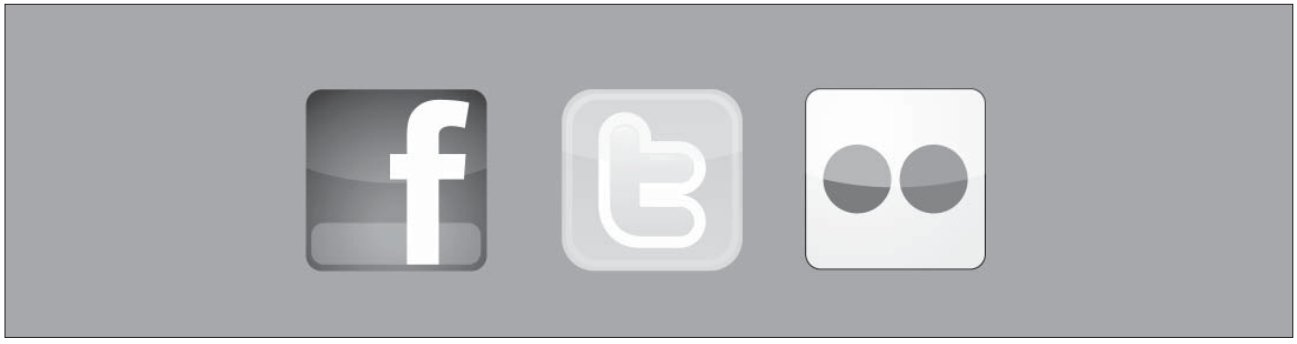

High Prevalence of Human Liver Infection by *Amphimerus* spp. Flukes, Ecuador

Manuel Calvopiña, William Cevallos, Hideo Kumazawa, and Joseph Eisenberg

Amphimerus spp. flukes are known to infect mammals, but human infections have not been confirmed. Microscopy of fecal samples from 297 persons from Ecuador revealed *Opisthorchiidae* eggs in 71 (24%) persons. Light microscopy of adult worms and scanning electron microscopy of eggs were compatible with descriptions of *Amphimerus* spp. This pathogen was only observed in communities that consumed undercooked fish.

The genus *Amphimerus* Barker 1911 infects mammals from the Americas, including Canada, the United States, Costa Rica, Panama, Colombia, Brazil, and Peru. Eleven species are reported (*1*–*7*). In Ecuador, a trematode resembling *Amphimerus* spp. but identified as *Opisthorchis guayaquilensis* has been reported (*8*,*9*).

Amphimerus spp. are parasitic liver flukes in the bile ducts of mammals, birds, and reptiles (*1*). Although these digenetic trematodes of the *Opisthorchiidae* family are closely related to the genera *Clonorchis* and *Opisthorchis*, there are morphologic differences. The vitellaria in adult *Amphimerus* spp. trematodes are distributed in 4 groups, 2 anterior and 2 posterior; the latter groups extend beyond the posterior testis; the ventral sucker is larger than the oral, and the testes are rounded or slightly lobulated. In contrast, the vitellaria in *Clonorchis* and *Opisthorchis* spp. trematodes exist only in front of the testes. Additionally, *Clonorchis* spp. trematodes have 2 large highly branched testes; testes in *Opisthorchis* spp. worms are always lobulated (*1*,*2*). The eggs of the flukes from these genera can be differentiated only by using scanning electron microscopy (SEM). Definitive diagnosis using light microscopy of flukes of the *Opisthorchiidae* family, therefore, is not possible unless the adult worm is collected and identified. Through isolation of adult worms and SEM of eggs, we found a high prevalence of human infection with a trematode of the genus *Amphimerus* in Ecuador.

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DOI: http://dx.doi.org/10.3201/eid1712.110373

The Study

In June 2009, during a routine fecal examination for the parent study, 4 samples tested positive for eggs of the *Opisthorchiidae* family in 3 indigenous Chachi communities along the Cayapas River in the northern coastal rainforest of Ecuador. In January 2010, a follow-up survey was conducted in the same 3 communities (total population 589); all villagers, whether symptomatic or not, were asked to provide a fecal sample. Specifically, a community meeting was held in each village, study objectives were explained, and villagers were asked for their voluntary participation. Flasks were distributed to all villagers and collected the next day in the school and by going house to house. The Chachis, the predominant group in these 3 communities, represent 13% of the 24,000 inhabitants in the region. Afro-Ecuadorians and mestizos also reside in this region (*10*,*11*).

A total of 297 (50.4%) community members 3–77 years of age provided samples. To each person providing a sample, a questionnaire was administered regarding types of food eaten and cooking practices. Samples were preserved in 10% formalin, transported to a laboratory in Quito, and stored at 4°C until examination by light microscopy. Eggs were concentrated by using the formalin-ether technique. In addition, 120 fecal samples from Afro-Ecuadorian and mestizo persons were examined. The villagers were informed of the study in their local Chapalache language by community health community workers. The ethical committee of the Central University approved this study.

Duodendoscopy was performed in 4 patients by a gastroenterology specialist to examine the biliary liquid; the microscopy of this liquid showed eggs identical to those found in their feces. These patients received praziquantel (75 mg/kg in 3 doses/3 d), and were purged with 10 mg of bisacodilo. Fecal samples were collected and examined for worms as previously described (*12*). Recovered worms were fixed with 10% formalin, stained with Diff-Quik fixative (Sysmex, Kobe, Japan), and identified by comparing their morphologic features to known adult *Clonorchis* and *Opisthorchis* spp. worms. Community health workers collected and examined the livers of 3 cats and 3 dogs from 1 of the 3 communities. All 6 livers had eggs and high numbers of adult parasites in the bile ducts. Adult parasites were stained, and microscopic studies showed them to be identical to those in the human specimens.

A total of 71 (24%) of the 297 fecal samples from the indigenous Chachi were positive for *Opisthorchiidae* eggs (Table). In contrast all 120 samples from Afro-Ecuadorian and mestizo persons were negative. Eggs

were yellow-brown and measured 28–33 μm ×12–15 μm (n = 20). The operculum and the shoulders, however, were not prominent as they are in *Clonorchis* and *Opisthorchis* eggs. Occasionally, a small knob, but most frequently a curved spine, was seen on the abopercular end. Although, by light microscopy, the shape and size of the eggs resembled that of the other liver flukes, the patterns of the eggshell surface were distinct when viewed with SEM (Figure 1). This observation is corroborated with published photographs (*3*).

After participants were treated with praziquantel, a total of 8 worms were recovered from 4 human participants and dozens from cat and dog livers; all were placed in saline. The worms were delicate, leaf-shaped, elongated, and red-pink and measured 8–13.6 mm long (average 10.2 mm) × 0.5–1.1 mm wide (n = 15). After a few minutes, the worms coiled in an S shape and became transparent or whitish. Once stained, the following features were observed: 1) the vitellaria divided into an anterior and posterior group with the posterior group extending the level of the posterior testis; 2) a ventral sucker larger than oral sucker; and 3) 2 rounded testes (Figure 2). On the basis of these morphologic characteristics of the adults and the SEM findings of the eggs, the parasite was identified as *Amphimerus* spp.

Conclusions

Our study demonstrates that the liver fluke of the genus *Amphimerus* can infect humans. We found a high prevalence (15.5%–34.1%) of infection with *Amphimerus* spp. trematodes in the surveyed communities (Table). Samples from the Afro-Ecuadorian and mestizo population were all negative for *Opisthorchiidae* eggs. *Amphimerus* spp. trematodes are believed to be transmitted, as are the other members of the *Opisthorchiidae* family, by ingestion of raw or undercooked fish (*2*). In our survey, most Chachis reported eating smoked fish caught in the rivers. Food sharing is more common among Chachi than Afro-Ecuadorians and mestizo families (*13*). Notably, the most remote village (120 km inland from the coast) had the highest prevalence. Our results suggest that *Amphimerus* spp. flukes are zoonotic pathogens of domestic animals living with humans.

Table. Prevalence of *Amphimerus* eggs in feces in 3 villages, Ecuador

Village	Total population	No. samples examined	No. (%) positive	Distance to the coast, km
1	116	82	28 (34.1)	120
2	248	86	23 (26.7)	91
3	253	129	20 (15.5)	85
Total	617	297	71 (23.9)	

Amphimeriasis should be considered an endemic liver fluke infection among residents of this Chachi population in Ecuador. Further studies are needed to determine the complete epidemiology and geographic distribution of infection in this region, as well as in other provinces of Ecuador where freshwater fish is eaten undercooked or where the same tropical ecology is found. For example, the Amazonian region has indigenous groups where other foodborne trematodiasis-like paragonimiasis are endemic (*14*). *Amphimerus* spp. flukes infecting domestic and wild animals have been reported from Ecuador's neighboring countries as well as from Central and North America. The existence of undiscovered foci of human infections is possible.

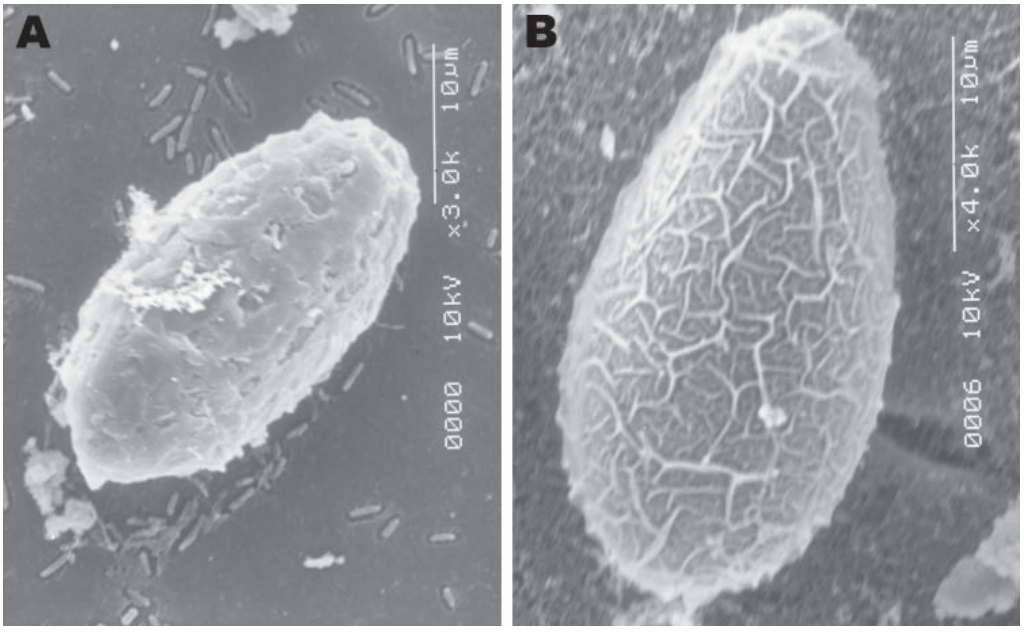

Figure 1. Scanning electron microscopy images of A) an egg of the Ecuadorian *Amphimerus* spp. trematode (original magnification ×3) obtained from a human and B) an egg of the Asian *Clonorchis sinensis* trematode (original magnification ×4). Although the size is similar, the pattern of the surface is different, thus differentiating the 2 genera.

Figure 2 . *Amphimerus* spp. adult trematode (10.1 mm) recovered from a human, Ecuador.

In 1971, Yamaguti (*1*) suggested that a parasite previously reported in Ecuador (*8*) as *O. guayaquilensis* might in fact be *Amphimerus* spp. Subsequently, publications referred to this parasite as *A. guayaquilensis* (*5*,*7*); however, the accuracy of this reclassification is unclear. Molecular analysis could help clarify the ambiguities in genus/species identification of *O. guayaquilensis* parasites and the conspecific species of *Amphimerus* (*15*).

We have much to learn about the pathology and epidemiology of *Amphimerus* spp. flukes. For example, nothing is known about the clinical and pathologic significance of infections with this parasite. Praziquantel eliminated the parasites in these patients, but whether the dose and treatment time were adequate are unknown. Additionally, little is known about epidemiologic factors responsible for the differences in the number of infections among the different population groups. Future studies can help determine the direct and indirect public health implications of this new foodborne zoonosis.

Acknowledgments

We thank the community health workers of Borbon and Rio Cayapas, Esmeraldas, for informing study participants, preparing and obtaining the consent, and translating to the local language in the communities surveyed. We also thank Jeyson Abarca for performing duodendoscopy and Ronald Guderian for revising the manuscript.

This study was supported by a grant from the US National Institute of Allergy and Infectious Disease, National Institutes of Health, grant no. RO1-AI050038.

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Aedes aegypti Mosquitoes Imported into the Netherlands, 2010

Julia E. Brown, Ernst-Jan Scholte, Marian Dik, Wietse Den Hartog, Jacob Beeuwkes, and Jeffrey R. Powell

During summer 2010, *Aedes aegypti* mosquitoes were discovered in the Netherlands. Using genetic markers, we tracked the origin of these mosquitoes to a tire shipment from Miami, Florida, USA. Surveillance of tire exports from the United States should be included as part of a comprehensive surveillance system.

During summer 2010, national surveillance activities detected *Aedes aegypti* mosquitoes in 2 tire yards in the Netherlands (*1*,*2*). *Ae. aegypti* mosquitoes are the principal worldwide vectors of dengue and yellow fever viruses, which cause a wide range of illnesses varying from asymptomatic to life threatening (*3*). Typically, these mosquitoes are found in tropical and subtropical regions throughout the world and had not been found in Europe since they were eliminated in the region shortly after World War II (*3*).

In the Netherlands, a tire shipment from southern Florida, USA, was identified as a potential source of *Ae. aegypti* mosquitoes (*1*,*2*). Tires were received from Miami, Florida, USA, at the 2 affected tire yards during the months before the discovery. Tire transportation has not been considered to play a large role in recent invasions of *Ae. aegypti* mosquitoes, as it has been for the Asian tiger mosquito, *Ae. albopictus* (*4*). However, several decades ago, tires from the United States were implicated as a source of *Ae. aegypti* mosquitoes transported to Central and South America after abandonment of the *Ae. aegypti* mosquito eradication program (*5*).

Effective vector control and prevention measures require knowledge of the origin of invasive mosquitoes and how they are transported. Therefore, we set out to determine the origin of the *Ae. aegypti* mosquitoes in the Netherlands by using a genetic approach.

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DOI: http://dx.doi.org/10.3201/eid1712.110992

The Study

Previous work in our laboratory validated a set of 12 microsatellite markers to distinguish between global populations of *Ae. aegypti* mosquitoes (*6*). We screened these markers in 8 mosquito specimens from the 2010 invasion in the Netherlands and compared their genotypes with those from 736 *Ae. aegypti* mosquito specimens from 15 reference populations around the world, including 4 Florida locations.

We analyzed 8 mosquitoes from 2 tire yards in the Netherlands, 2 mosquitoes from site 1 and 6 from site 2 (*2*). The samples consisted of individual legs preserved in 70% ethanol. These samples were compared with previously screened *Ae. aegypti* mosquito populations from 14 locations worldwide: Palm Beach County, Vaca Key, and Conch Key, Florida, USA; Houston, Texas, USA; Pijijiapan and Coatzacoalcos, Mexico; Dominica; Bolivar and Zulia, Venezuela; Rayong and Prachuabkhirikan, Thailand; Tahiti, French Polynesia; and Cairns and Townsville, Queensland, Australia. The number of mosquitoes analyzed per reference population is indicated in Brown et al. (*6*). We also included in the analyses 47 newly acquired *Ae. aegypti* mosquito samples from Miami. Collection methods are described elsewhere (*2*,*6*).

Genomic DNA was extracted from each mosquito by using DNeasy kits (QIAGEN, Valencia, CA, USA). The samples from the Netherlands and Miami were screened for variation at 12 microsatellite loci following published methods (*6*,*7*). Chord distances between each pair of populations were calculated in GENETIX (*8*) and used in 2 distance-based cluster analyses: a principal components analysis using PAST (*9*) and a neighbor-joining network using MEGA4 (*10*). The Bayesian clustering algorithm in the program STRUCTURE (*11*) was used to identify genetic clusters and assign individual mosquitoes to these clusters with no a priori information about sampling locations. To determine the best genetic match for the samples from the Netherlands, we conducted 5 independent runs for each assumed number of populations, K, 1–17. For all runs, we assumed an admixture model and correlated allele frequencies and used a burn-in value of 100,000 iterations followed by 500,000 replications. Results from STRUCTURE were visualized using DISTRUCT (*12*). A group assignment test was implemented in GENECLASS2 (*13*) to assign the mosquitoes in the Netherlands of unknown origin back to the reference populations with relative probabilities.

Population-level (Figure, panels A, B) and individual-level (Figure, panel C) analyses suggest that the *Ae. aegypti* mosquito samples from the Netherlands are in the same genetic group as populations from southern Florida. Among these Florida populations, the group assignment test (*13*) identified Miami as the likely source of the

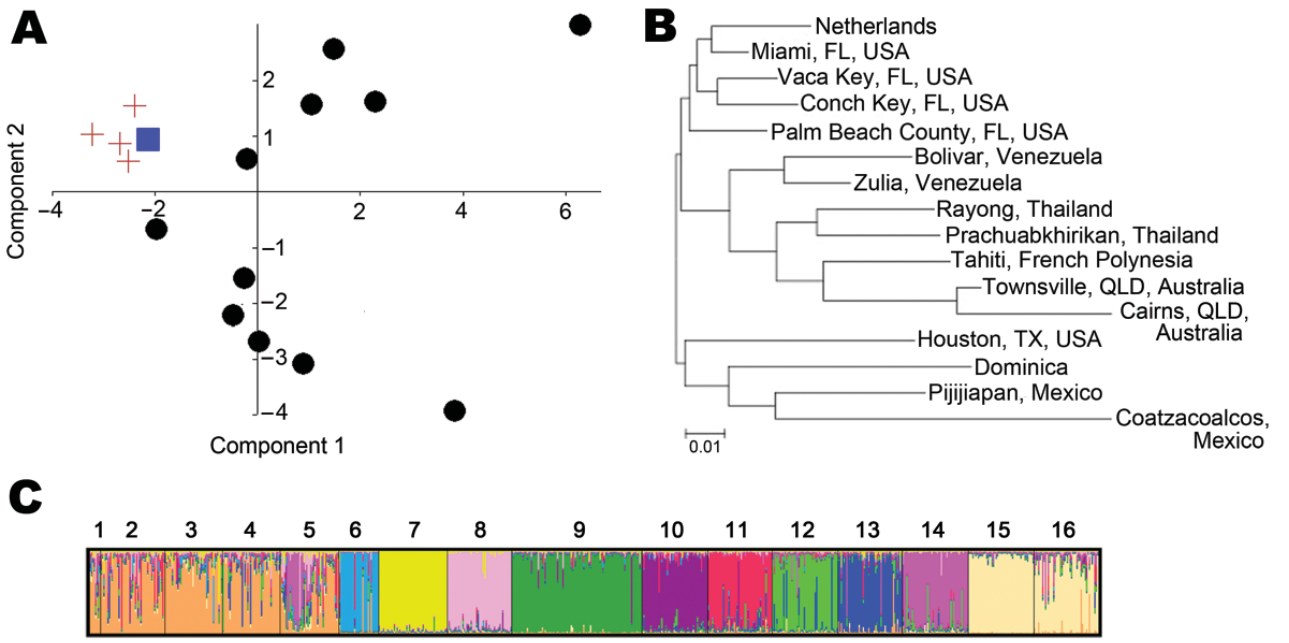

Figure. A) Principal components analysis based on pairwise population chord distances. The *Aedes aegypti* mosquito population in the Netherlands is represented by a blue square, the Florida, USA, populations by red crosses, and all other populations by black circles. B) Neighbor-joining network based on chord distances. QLD, Queensland. Scale bar indicates nucleotide substitutions per site. C) Individual mosquito–based Bayesian cluster analysis (K = 11) of the *Ae. aegypti* mosquito samples from the Netherlands and 15 reference populations. Populations are labeled as follows: 1, the Netherlands; 2, Miami, Florida, USA; 3, Vaca Key, Florida, USA; 4, Conch Key, Florida, USA; 5, Palm Beach County, Florida, USA; 6, Houston, Texas, USA; 7, Coatzacoalcos, Mexico; 8, Pijijiapan, Mexico; 9, Dominica; 10, Bolivar, Venezuela; 11, Zulia, Venezuela; 12, Rayong, Thailand; 13, Prachuabkhirikan, Thailand; 14, Tahiti, French Polynesia; 15, Cairns, Queensland, Australia; 16, Townsville, Queensland, Australia.

samples from the Netherlands, with a relative probability of 100% compared with the other 14 reference populations. The recorded import of tires from the Miami area to the sites in the Netherlands where *Ae. aegypti* mosquitoes were discovered strongly corroborates the results from our genetic data, clearly indicating introduction of *Ae. aegypti* mosquitoes from Miami.

Conclusions

Our findings suggest that 1 of the world's most dangerous vector arthropods entered Europe through a tire shipment from Miami. Although the importation of mosquitoes into the United States through the used tire trade has received considerable focus, our results indicate that equal caution should be exercised when tires are exported out of the southern United States, particularly into regions where *Ae. aegypti* mosquitoes are absent. Because vector exportation from the United States has now occurred multiple times (*5*,*14*), tires should be included as part of a comprehensive surveillance system to prevent future incidents.

Given the recent reemergence of dengue fever in Florida (*15*), we know that populations of *Ae. aegypti* mosquitoes from that region are fully capable of causing outbreaks of arboviral diseases. In the temperate climate of northern Europe, the epidemiologic risk is higher during the warm summer months, when viruses could be introduced to these new vector populations by travelers from tropical locations. This scenario would likely require close human–mosquito interactions at the site of the introductions. Overall, the risk is much greater in southern Europe, where the climate allows for year-round establishment of *Ae. aegypti* mosquito populations (*3*). Vector surveillance will prove crucial to prevent reinvasion of the region by this species of mosquitoes. In addition, cooperation between government scientists, policy makers, and companies involved in international trade is necessary domestically and internationally to determine the origins of exotic mosquito vector invasions, rather than fighting diseases as they occur.

Acknowledgments

We thank Vanessa Obas and Mario Porcelli for the Miami mosquito collections and Gisella Caccone for her suggestions regarding the manuscript.

This work was supported by National Institutes of Health (NIH) RO1 AI046018 (J.R.P.), NIH predoctoral Genetics training grant T32 GM007499 (J.E.B.), and the Yale Institute for Biospheric Studies, Center for Field Ecology pilot grant (J.E.B.).

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Fatal Outbreak of *Mycoplasma capricolum* Pneumonia in Endangered Markhors

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A pneumonia outbreak reduced the numbers of a wild population of endangered markhors (*Capra falconeri*) in Tajikistan in 2010. The infection was diagnosed by histologic examination and bacteriologic testing. *Mycoplasma capricolum* subsp. *capricolum* was the sole infectious agent detected. Cross-species transmission from domestic goats may have occurred.

Mycoplasma capricolum subsp. *capricolum* and *M. capricolum* subsp. *capripneumoniae* are closely related subspecies of the *M. mycoides* cluster (*1*). Whereas *M. capricolum* subsp. *capripneumoniae* is the etiologic agent of contagious caprine pleuropneumonia (CCPP), a severe and typically lethal respiratory disease, *M. capricolum* subsp. *capricolum* infection is usually not fatal and instead results in chronic inflammation in a variety of organs, including joints, udder, eyes, and lungs (*2*). *M. capricolum* subsp. *capricolum* infection occurs worldwide and appears widespread but has rarely been found in species of small ruminants other than domestic goats and, more occasionally, sheep (*2*,*3*). This lack of evidence may be partially because few studies have applied sensitive molecular techniques for its detection in nondomestic ruminants (*2*,*3*). Domestic goats can carry *M. capricolum* subsp. *capricolum* asymptomatically, notably in the ear canal (*4*), and pose an insidious risk for cross-species transmission with sympatric wild caprines (*2*,*3*).

The Study

The markhor (*Capra falconeri*) is an endangered wild goat in a continuous decline; the global population is <2,500 mature animals (*5*). In Tajikistan, <350 animals may survive in fragmented subpopulations in the remote Hazratishoh and Darvaz mountain ranges along the Afghanistan border (*6*). They live sedentarily over relatively small home ranges, moving <5 km per day (*7*). Throughout its range, the markhor has to forage in close proximity to domestic goats (*8*) and is therefore prone to infections of contagious agents transmitted by these animals.

During September 17–October 18, 2010, eleven markhors that displayed labored breathing and 64 markhors that had recently died were found in 5 localities (Table) in the district of Shuroabad, Khatlon Province, usually in close proximity to water sources (Figure 1, panel A). All but 4 carcasses were too scavenged for thorough examination in the field, and 1 dying adult female was sent on September 20 to the Republican Veterinary Laboratory in Dushanbe for necropsy. The most relevant necropsy findings noted in the field were the following: an abundant serous to mucopurulent nasal discharge; and internally, severe pneumonia associated with a variable level of pleural fluid. The female markhor examined in Dushanbe showed gray areas of consolidations in the apical and cardiac lobes of the right lung and yellow pleural fluid (Figure 1, panel B). The cut surface of the affected lobes revealed a fine granular texture, and mucopurulent exudate could be expressed from the bronchi. The joints, eyes, and udder were not affected. Regarding indications of CCPP, although gross lesions were limited to the thoracic cavity, fibrinous pleurisy (*9*) was not observed. The histopathologic findings were consistent with proliferative interstitial pneumonia associated with multifocal suppurations (Figure 1, panel C). Findings included diffuse thickening of the interlobular septa, alveolar epithelialization, and polymorphonuclear leukocytes in alveolar and bronchiolar spaces (Figure 1, panel D). Also, disseminated and abundant neutrophil infiltration of alveolar spaces was found that, when

Table. Geographic distribution of dead markhors (*Capra falconeri*) during outbreak of pneumonia, Tajikistan, 2010

Locality	UTM coordinates*	No. deaths†
Obidara	42N 584745 4163741	23‡
Shulashdara	42N 584415 4161151	7
Siyorish	42N 587145 4163989	13
Pamdara	42N 585515 4159600	8
Dudara	42N 589488 4163693	13
Total		64

*UTM, universal transverse mercator. Source: World Geodetic System 84.
†Minimum number.
‡13 adult males, 6 adult females, 4 juveniles (<1 year of age).

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DOI: http://dx.doi.org/10.3201/eid1712.110187

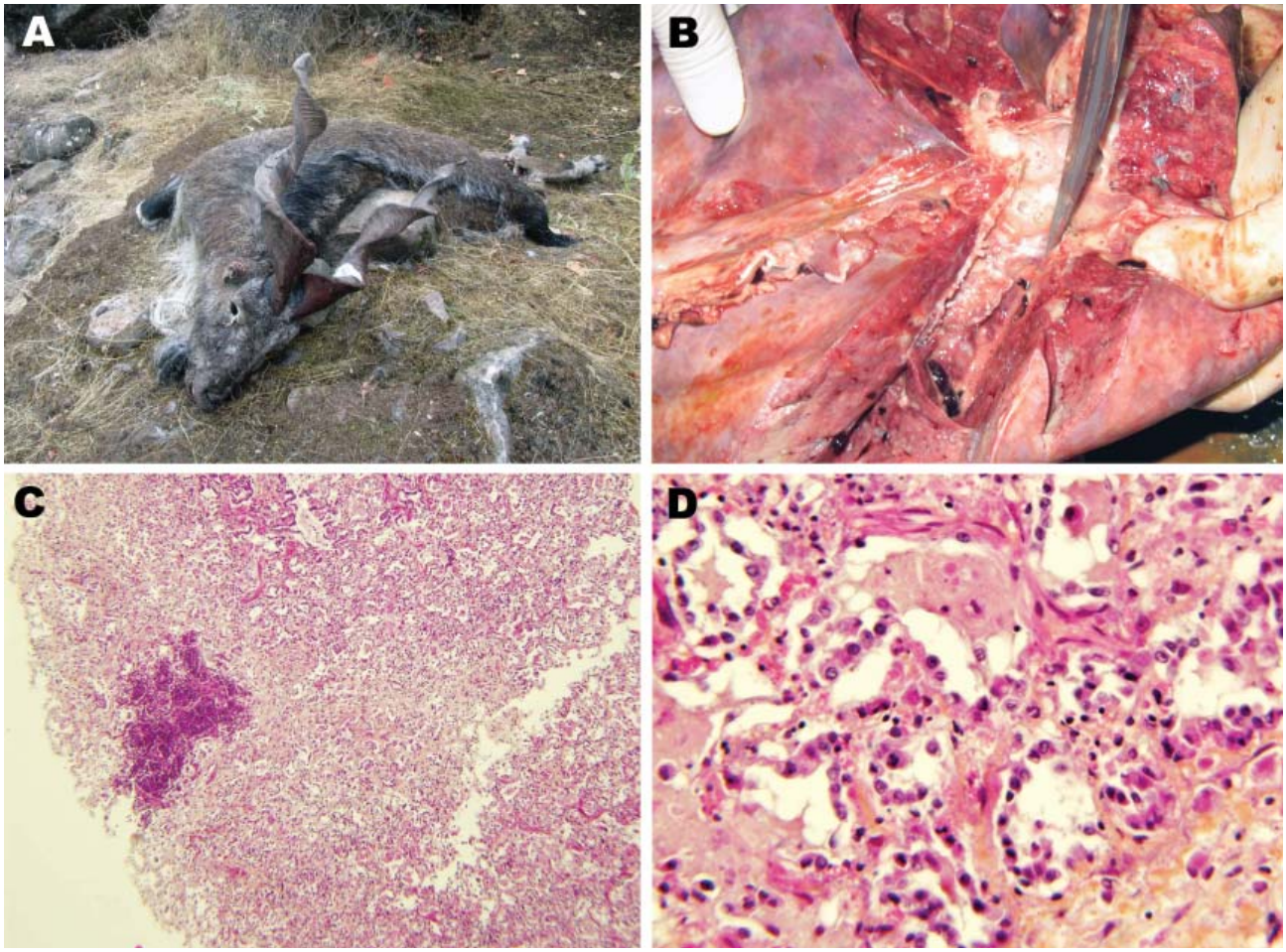

Figure 1. Pneumonia caused by *Mycoplasma capricolum* subsp. *capricolum* in markhors (*Capra falconeri*), Tajikistan, 2010. A) Adult male markhor found dead with signs of pneumonia and no indications of emaciation. B) Disseminated gray areas of consolidation in the cardiac lobe of the right lung with mucopurulent exudate in bronchi. C) Diffuse proliferative interstitial pneumonia associated with a lesion of suppuration (hematoxylin and eosin stain; original magnification ×40). D) Interstitial pneumonia showing fibrotic thickening of alveolar walls and epithelialization of pneumocytes (hematoxylin and eosin stain; original magnification ×250).

ruptured, created multifocal nodules. The left lung was congested.

No bacteria were isolated from pleural fluid that was obtained aseptically and inoculated onto 5% sheep blood agar. However, when a healthy 5-month-old domestic goat (*C. hircus*) was inoculated intratracheally with 5 mL of this pleural fluid, pneumonia developed within 8 days. To evaluate the possibility that the outbreak might have been caused by *M. capricolum* subsp. *capripneumoniae*, which was first identified in Tajikistan in 2009 (*10*), samples were sent to Centre de Coopération International en Recherche Agronomique pour le Developpement, Montpellier, France, a reference laboratory for CCPP for the World Organisation for Animal Health. Pleural fluid from the markhor was centrifuged at low speed (500 *g* for 10 min) to eliminate inflammatory cells, and DNA was extracted from the supernatant with the DNeasy blood and tissue kit (QIAGEN, Courtaboeuf, France), according to the manufacturer's instructions. Real-time PCR (*11*), specific for *M. capricolum* subsp. *capripneumoniae*, provided negative results, whereas a partial 16S rRNA gene sequence could be amplified and sequenced (*12*). BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the sequence corresponded to that of a mycoplasma from the *M. mycoides* cluster. Markhor pleural fluid and lung tissue from the domestic goat were then injected into modified Hayflick broth (*1*) and onto agar plates and incubated at 37°C in 5% CO_2. Typical *Mycoplasma* colonies appeared on solid medium after 1–2 days. A clone from the markhor culture, named 10074 (1.4), was used for PCR amplification and sequencing of *fusA*, *glpQ*, *gyrB*, *lepA*, and *rpoB* partial gene sequences as described (*1*). The *fusA* sequence was identical to a previously submitted sequence (GenBank accession no. EF071735). All other sequences were novel

and have been assigned new GenBank accession nos.: HQ882179 (*glpQ*), HQ882180 (*gyrB*), HQ882181 (*lepA*), HQ882178 (*rpoB*). The 5 protein-coding sequences were then concatenated and incorporated in the *M. mycoides* cluster phylogenetic tree (Figure 2). The strain clustered with *M. capricolum* subsp. *capricolum* and was therefore identified as belonging to this subspecies. The isolate from the inoculated domestic goat was undistinguishable from 10074 (1.4) by sequencing of the 5 housekeeping genes.

The pattern of inflammation in the right lung in the necropsied markhor was consistent with *M. capricolum* subsp. *capricolum* respiratory infection in domestic goats (*2*), and the presence of *M. capricolum* subsp. *capricolum* in pleural fluid, the lack of findings indicative of alternative etiologic agents, and the reproduction of the disease in the domestic goat with isolation of *M. capricolum* subsp. *capricolum* from the lungs support a causal relationship between *M. capricolum* subsp. *capricolum* and disease in markhors.

Conclusions

Although because of difficult field conditions, only 1 markhor could be thoroughly investigated, these findings support the hypothesis that *M. capricolum* subsp. *capricolum*, a newly recorded pathogen in free-ranging wild ruminants, may be responsible for the pneumonia epizootic observed in endangered markhors. This outbreak claimed ≈20% of the population remaining in Tajikistan, and more markhor deaths might have remained undetected.

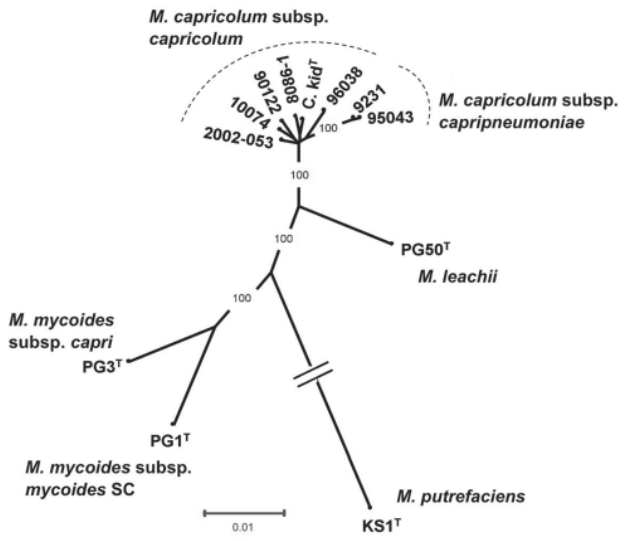

Figure 2. Phylogenetic tree of the *Mycoplasma mycoides* cluster including the isolate from markhor (*Capra falconeri*) 10074 (1.4), Tajikistan, 2010, together with available *M. capricolum* subsp. *capricolum* strains, as well as type strains corresponding to other species or subspecies from this cluster and *M. putrefaciens*, used as outgroup. The tree, derived from distance analysis of 5 concatenated protein-coding sequences (*fusA*, *glpQ*, *gyrB*, *lepA*, *rpoB*), was constructed by using the neighbor-joining algorithm. Bootstrap percentage values were calculated from 500 resamplings, and values >90% are indicated. Scale bar indicates distance equivalent to 1 substitution per 100 nt positions. Note that the branch corresponding to the outgroup has been shortened, as indicated by 2 parallel bars.

The source of *Mycoplasma* infection in markhors is unknown, but domestic goats, which have contact with markhors, particularly in the summer, might have been responsible for the emergence of *M. capricolum* subsp. *capricolum* in this wild species. In November 2008, a disease resembling CCPP affected domestic goats and, to a lesser extent, sheep in Shuroabad District (<40 km from the area where the dead markhors were found) with a case-fatality rate of 20%–30% (*13*). However, the origin of this outbreak could not be investigated. Although the clinicopathologic features of the disease in the markhors resembled CCPP, *M. capricolum* subsp. *capricolum* was identified as the most probable causative agent. In fact, several mycoplasmas have been associated with respiratory diseases in ruminants, including *M. capricolum* subsp. *capricolum* infection in young domestic goats (*2*,*14*). Further testing should evaluate the presence of sick animals or asymptomatic carriers of *M. capricolum* subsp. *capricolum* in livestock and wildlife in Shuroabad District.

Environmental and nutritional stressors may exacerbate the susceptibility of ruminants to *Mycoplasma* infections (*2*,*15*). The disease appeared at the end of summer, when markhors are forced by livestock and guard dogs to retreat to suboptimal habitats with poor forage (*8*). It was also the end of the dry season, when markhors may have contact with livestock at the few remaining water sources.

Findings from this study support the conclusion that the markhor is vulnerable to *M. capricolum* subsp. *capricolum*. In the generalized context of increasing encroachment of livestock into wild habitats, markhors and other wild caprines might be at risk for future mycoplasmosis outbreaks. This case emphasizes the need for continuous disease surveillance in domestic animals that have contact with valuable wildlife resources.

Acknowledgments

We thank L. Boulouha for performing the histopathologic examination; I. Ikromov and his staff who reported the outbreak, centralized reports of dead markhors, and captured the sick female markhor; and P. Zahler and 2 anonymous reviewers for their helpful comments.

The study was supported by the Deutsche Gesellschaft für Internationale Zusammenarbeit and the Wildlife Conservation Society.

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Characterization of African Swine Fever Virus Caucasus Isolate in European Wild Boars

Claudia Gabriel, Sandra Blome, Alexander Malogolovkin, Stanislav Parilov, Denis Kolbasov, Jens P. Teifke, and Martin Beer

Since 2007, African swine fever has spread from the Caucasus region. To learn more about the dynamics of the disease in wild boars (*Sus scrofa*), we conducted experiments by using European wild boars. We found high virulence of Caucasus isolates limited potential for establishment of endemicity.

African swine fever (ASF) is one of the most serious diseases affecting pigs (*1*). The causative agent, *African swine fever virus* (ASFV), is a complex DNA virus of the genus *Asfivirus* within the *Asfarviridae* family. Because of its ability to replicate in *Ornithodorus* ticks, ASFV can be classified as arthropod-borne virus (*2*). In domestic pigs, ASFV can cause a wide range of clinical signs, including hemorrhagic syndromes with high lethality. Little is known about ASF in European wild boars, although indications exist that the animals are highly susceptible (*3*).

In 2007, ASF affecting domestic pigs and wild boars was reported in the Caucasus region. The virus strain involved was related to isolates of genotype II, which are circulating in Mozambique, Madagascar, and Zambia (*4*). Especially in Russia, ASF recurs and shows a clear tendency to move northward (*5*). This unresolved situation increases the risk of introducing the virus into virus-free areas, and the involvement of wild boars raises special concerns. As seen with classical swine fever, the growing population of wild boars is problematic for animal disease control, particularly if the infection reaches endemicity (*6*). Therefore, knowledge about disease dynamics is vital for risk assessment and strategy design, particularly because no vaccine against ASF is available.

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DOI: http://dx.doi.org/10.3201/eid1712.110430

Therefore, animal experiments were carried out at the Friedrich-Loeffler-Institut (Greifswald–Insel Riems, Germany), and the National Research Institute for Veterinary Virology and Microbiology (NRIVVaMR, Pokrov, Russia). The aim was to define clinical signs, disease dynamics, and postmortem lesions in wild boars after intramuscular and oral infection with ASFV Caucasus isolates.

The Study

The study comprised 2 experimental parts: 1) oral infection conducted at the Friedrich-Loeffler-Institut and 2) intramuscular infection at NRIVVaMR. For oral infection, we used a 2008 isolate from Armenia. The experiment was conducted by using 6 wild boar piglets 9 weeks of age. Three domestic pigs were used as contact controls and were handled in the same manner as the wild boar piglets. The animals were kept under high-containment conditions. After acclimatization, the wild boars were infected orally with 2 mL of a spleen suspension containing 10^6 median tissue culture infectious dose ASFV/mL. Two days after infection, 3 domestic weaner pigs were added to the pen with the wild boar piglets. Starting from the day of infection, rectal temperature and clinical signs were recorded. Oral and fecal swabs were collected from the wild boars at 0, 1, 2, 3, 5, 6, and 7 days postinfection (dpi). In addition, blood samples were taken at 0, 2, 5, 6, and 7 dpi. Blood from the domestic pigs was sampled at 0, 6, 9, and 13 dpi. Necropsy was performed on all animals.

For real-time quantitative PCR (qPCR), viral DNA was extracted by using manual and automated extraction methods according to manufacturer instructions. Subsequently,

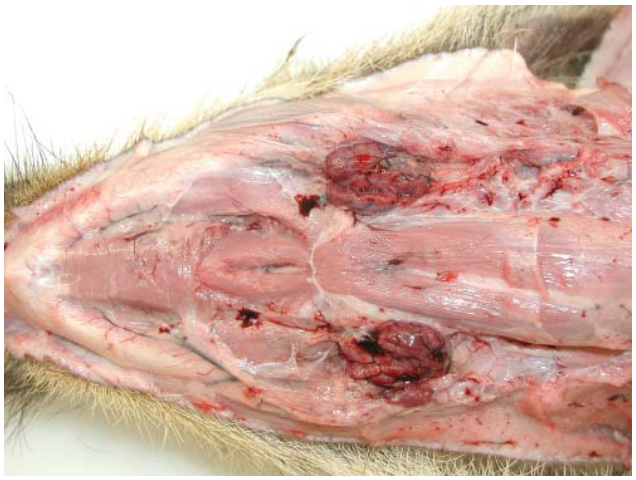

Figure 1. Ventral view of the head showing pathologic signs in a wild boar piglet after oral inoculation with 10^6 median tissue culture infectious dose of an African swine fever virus isolate from Armenia (experiment at the Friedrich-Loeffler-Institut). Note edematously enlarged and hemorrhagic mandibular lymph nodes. The animal died on day 7 postinfection.

qPCR was performed according to the protocol published by King et al. (*7*) with slight modifications by using an Mx3005P PCR Cycler (Stratagene, La Jolla, CA, USA).

For intramuscular infection, 4 wild boars 9 months of age were brought to the containment stables of the NRIVVaMR. One animal was inoculated intramuscularly with 1,000 hemadsorbing units 50% of a 2009 virus isolate from the Chechen Republic, which is identical to the isolate used in the oral trial in all genome fragments routinely sequenced. The remaining animals were housed together with the infected animal as contact controls.

Clinical signs of infection were recorded every day. Samples of visceral organs, skin, and hair were taken during necropsy and subjected to qPCR. Isolation of viral DNA was performed by using an in-house kit based on the modified method published by Boom et al. (*8*). The qPCR for ASFV detection was carried out according to the protocol published by King et al. (*7*) with a Rotorgene 6000 instrument (Corbett Research, Sydney, Queensland, Australia).

After oral infection, an acute fatal course of the disease developed in all wild boar piglets, and they died within 7 days. Apart from severe depression, slight diarrhea, and reduced feed intake, only high fever was observed starting

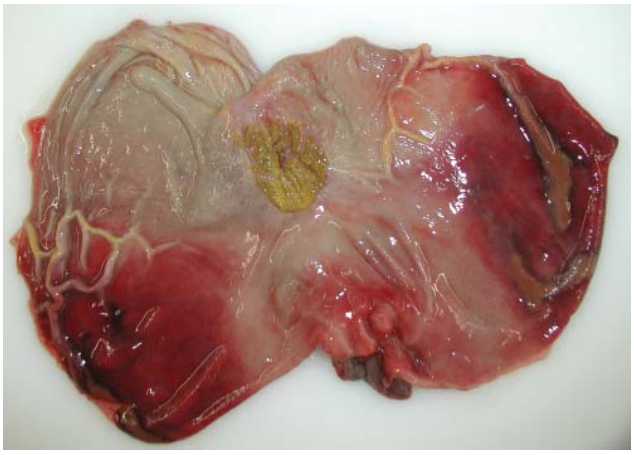

Figure 2. View of the mucosal surface of the dissected stomach showing representative gross lesions after oral inoculation of a wild boar with 10^6 median tissue culture infectious dose of an African swine fever virus isolate from Armenia (experiment at the Friedrich-Loeffler-Institut). The image illustrates acute gastritis; note diffuse mucosal hemorrhages affecting a large part of the mucosa. The animal died on day 7 postinfection.

Table 1. Real-time PCR results of blood and swab samples after oral infection in study of African swine fever virus in European wild boars*

Animal, sample source	Days postinfection of wild boar										
	0	1	2	3	5	6	7	9	13	17	20
Wild boar 1											
Blood	No C_t	ND	No C_t	ND	23						
Oropharyngeal swab	No C_t	No C_t	No C_t	No C_t	No C_t						
Fecal swab	No C_t	No C_t	No C_t	No C_t	No C_t						
Wild boar 2											
Blood	No C_t	ND	No C_t	ND	22	20	24				
Oropharyngeal swab	No C_t	No C_t	No C_t	No C_t	37	37	37				
Fecal swab	No C_t	No C_t	No C_t	No C_t	No C_t	38	No C_t				
Wild boar 3											
Blood	No C_t	ND	No C_t	ND	28	22	23				
Oropharyngeal swab	No C_t	No C_t	No C_t	No C_t	No C_t	38	34				
Fecal swab	No C_t	No C_t	No C_t	No C_t	37	34	33				
Wild boar 4											
Blood	No C_t	ND	No C_t	ND	25	26	26				
Oropharyngeal swab	No C_t	No C_t	No C_t	37	No C_t	34	37				
Fecal swab	No C_t	No C_t	No C_t	No C_t	30	29	33				
Wild boar 5											
Blood	No C_t	ND	39	ND	25	23					
Oropharyngeal swab	No C_t	No C_t	No C_t	No C_t	39	35					
Fecal swab	No C_t	No C_t	No C_t	No C_t	No C_t	29					
Wild boar 6											
Blood	No C_t	ND	No C_t	ND	23	24					
Oropharyngeal swab	No C_t	No C_t	No C_t	37	No C_t	34					
Fecal swab	No C_t	No C_t	No C_t	No C_t	35	32					
Domestic pig 1, blood	No C_t	ND	ND	ND	ND	39	ND	No C_t	21	20	
Domestic pig 2, blood	No C_t	ND	ND	ND	ND	No C_t	ND	No C_t	23	ND	
Domestic pig 3, blood	No C_t	ND	ND	ND	ND	No C_t	ND	No C_t	No C_t	ND	29†

*C_t, cycle threshold; ND, not done because of missing samples. Numbers indicate C_t values.
†Serum sample instead of whole blood sample was used.

3–4 dpi. During postmortem examinations, enlarged and hemorrhagic lymph nodes (Figure 1) and hemorrhagic gastritis (Figure 2) were observed. Acute fatal ASF developed in 2 of the domestic pigs 11–12 dpi of the wild boars. These animals died 1 week later showing severe but unspecific symptoms. One domestic pig became infected later. It only showed fever at 20 dpi and was euthanized on day 25. Infection of this animal was clearly linked to contact with blood from a moribund pen mate.

During the clinical phase of the disease, qPCR was positive for all blood samples with first positive results 2 dpi. Oropharyngeal and fecal swabs were positive mainly on days 6 and 7. An overview of the qPCR results is presented in Table 1.

On the third day after intramuscular inoculation, the infected wild boar showed depression, inappetence, and increased respiratory frequency. It died at 5 dpi showing hemorrhagic nasal discharge. The 3 contact animals showed similar symptoms at 8 dpi of the intramuscularly infected wild boar and died 2 days later. Postmortem examinations showed hemorrhages in multiple edematously enlarged lymph nodes, most prominent pulmonary hyperemia and alveolar edema, hyperplasia of the mesenteric lymph nodes, and acute gastritis with hemorrhages. Skin lesions were not present.

ASF genome was detected in the samples of visceral organs and lymph nodes of all animals. In samples of skin and kidneys, viral DNA was detected only in the infected animal. Results of qPCR are presented in the Table 2.

Conclusions

Knowledge about disease dynamics in domestic pigs and wild boars is a prerequisite for risk assessment and prevention strategy design. Unfortunately, wild boar data are scarce. To contribute to this information, animal trials were conducted for an experimental characterization of recent Caucasian ASFV isolates in wild boars.

We concluded that the Caucasian isolates are highly virulent in wild boars. Both oral and intramuscular infection resulted in 100% lethality.

PCR results showed that the ASFV genome is easily detected in blood and organ samples of diseased animals. Swab samples were positive in the clinical phase of infection but showed much lower genome loads. Shedding of ASFV through nasal discharge or feces, and thus overall contagiousness, seems to be limited.

Transmission to domestic pigs was delayed in comparison to transmission to wild boars. The most likely reason for this difference seems to be contact with blood. Although this factor could be observed most certainly for the contact wild boars, domestic pigs had only limited contact with blood.

On the basis of these data, it seems unlikely the Caucasian isolates have the potential to become endemic in European wild boar populations without a distinct change in virulence. So far no indications exist that the virulence of ASFV is changing in affected regions in Russia.

A risk factor for disease control could be the involvement of tick vectors. Until now, no indications exist that ticks are involved in ASFV outbreaks in the Caucasus region and Russia. Moreover, it has to be kept in mind that the wild boar's way of life does not facilitate contact with soft ticks. Nevertheless, this possibility was not examined during this study and needs further investigation.

Table 2. Real-time PCR results of organ samples taken after intramuscular infection in a study of African swine fever virus in European wild boars*

Wild boar	Lung	Heart	Spleen	Lymph nodes
1	19	19	18	19
2	20	20	19	20
3	21	21	20	20
4	20	21	19	20

*Cycle threshold values indicated.

Acknowledgments

We thank all animal caretakers and technicians involved in these studies. Our special thanks go to Raquel Portugal for virus titration and Katharina Brehm for helping us with sampling and sample processing.

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Novel Sylvatic Rabies Virus Variant in Endangered Golden Palm Civet, Sri Lanka

Takashi Matsumoto, Kamruddin Ahmed, Omala Wimalaratne, Susilakanthi Nanayakkara, Devika Perera, Dushantha Karunanayake, and Akira Nishizono

Information is scarce about sylvatic rabies virus in Asia and about rabies in palm civets. We report a novel sylvatic rabies virus variant detected in a golden palm civet in Sri Lanka. Evolutionary analysis suggests the virus diverged from canine rabies viruses in Sri Lanka in ≈1933 (range 1886–1963).

Rabies has been eliminated from domestic animals in industrialized countries, but sylvatic rabies remains an endemic disease. The ecology of rabies in wildlife populations and natural ecosystems is poorly understood (*1*), and, as a result, eliminating rabies from the wild is difficult. Little is known about sylvatic rabies in developing countries, where rabies takes its biggest toll on humans. Rabies is endemic to Sri Lanka and has been identified in different wild animals. However, all documented cases of rabies in wildlife in Sri Lanka have been considered a consequence of spillover from dogs. Rabies viruses circulating in this country are distinctly highly homogeneous (*2*,*3*).

Two species of palm civet are commonly found in Sri Lanka: the common palm civet, *Paradoxurus hermaphroditus*, which is widespread in southern Asia and Southeast Asia, and the golden palm civet, *P. zeylonensis*, which is indigenous to Sri Lanka. This species is closely related to the brown palm civet (*P. jerdoni*), which lives only in southern India (*4*). Moreover, 3 additional new species have been identified in Sri Lanka: the golden wet-zone palm civet (*P. aureus*), the golden dry-zone palm civet (*P. stenocephalus*), and the Sri Lankan brown palm civet (*P. montanus*) (*4*). Palm civets in Sri Lanka are, however, endangered because of hunting, parasitic diseases, and dwindling habitat. We report on a sylvatic rabies virus variant detected in a golden palm civet in Sri Lanka.

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DOI: http://dx.doi.org/10.3201/eid1712.110811

The Study

On a November morning in 2009, a "wild cat" appeared in the garden of a basic health clinic in Moneragala district, Uva Province, Sri Lanka. The animal, which showed aggressive behavior, was suspected to be rabid and was thus killed to prevent transmission of rabies virus to humans. The animal's head was packed in ice to avoid decomposition and sent to the Medical Research Institute (Colombo, Sri Lanka) for testing. We detected rabies virus in the animal's brain by using the fluorescent antibody test and extracted viral RNA and DNA by using Trizol (Invitrogen, Carlsbad, CA, USA). The rabies virus from this sample was designated as H-1413-09. The whole genome of the virus was sequenced directly from the sample as described (*5*).

To confirm the species of the rabid animal, we determined the nucleotide sequence of the mitochondrial cytochrome *b* (*cytb*) gene and performed a BLAST search (www.ncbi.nlm.nih.gov/blast) for similarity with other sequences. By aligning nucleotide sequences of the *cytb* gene of mitochondrial DNA of domestic cat, jungle cat, fishing cat, Asiatic golden cat, marbled pole cat, European pole cat, lynx, puma, leopard, African lion, tiger, jaguar, civet, and palm civet with ClustalW2 (www.ebi.ac.uk/clustalw), we designed primer Felis *cytb*-F, 5′-ATGACCAACATTCGAAAATCACACC-3′ (nt 1–25), and primer Felis *cytb*-R, 5′-CAATAAT GCCTGAGATGGGTATTAG-3′ (nt 1093–1,117). Using these primers, we performed PCR as follows: initial denaturation at 94°C for 2 min, followed by 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 35 cycles, followed by a final extension at 72°C for 5 min. PCR generated a 1,117-bp fragment from which a 1,004-nt sequence was determined. Analysis showed that the sequence has 100% identity with the partial (224-nt) sequence of the *cytb* gene of *P. zeylonensis* (GenBank accession no. FJ881681); this is the only sequence available for *P. zeylonensis*. The sequence also has 95% identity with *P. jerdoni* and 90%–92% identity with *P. hermaphroditus*.

We performed an evolutionary analysis by using the N gene. We inferred a maximum clade credibility phylogenetic tree by using the Bayesian Markov chain Monte Carlo method available in BEAST version 1.6.1 (*6*). The analysis used a relaxed (uncorrelated lognormal) molecular clock and GTR + Γ + I model of nucleotide substitution. We selected the model on the basis of Akaike Information Criterion by using jModelTest software (*7*). All chains were run for 9×10^7 generations and sampled

Table 1. Percentage identity shared between genes of 2 rabies virus strains from Sri Lanka, H-08-1320 and H-1413-09, by gene coding region*

Gene	H-1413-09		H-08-1320		% Identity	
	Coding region	Start codon, stop codon	Coding region	Start codon, stop codon	Nucleotide	Amino acid
N	71–1,423	ATG, TGA	71–1,423	ATG, TGA	99.1	97.1
P	1,514–2,404	ATG, TGA	1,514–2,404	ATG, TGA	99.1	97.5
M	2,496–3,104	ATG, TAA	2,497–3,105	ATG, TAA	98.0	96.9
G	3,317–4,891	ATG, TGA	3,318–4,892	ATG, TGA	98.7	97.4
L	5,407–11,793	ATG, TGA	5,408–11,794	ATG, TAA	99.0	97.0

*H-08-1320 is a human strain typical of canine rabies virus circulating in Sri Lanka; H-1413-09 is a novel sylvatic rabies virus variant from a golden palm civet in Sri Lanka.

every 3,000 steps. This procedure resulted in an effective sample size of >2,000 for all estimated parameters. The posterior densities were calculated with 10% burn-in and checked for convergence by using Tracer version 1.5 (http://beast.bio.ed.ac.uk/Main_Page). The mean rate of nucleotide substitution estimated for the N gene was 2.2×10^4 substitutions/site/year (95% highest posterior density [HPD] values $1.3–3.2 \times 10^4$ substitutions/site/year). This rate is in agreement with previous findings (*8*). Approximately 155.5 years ago (95% HPD 91.3–249.5 years)—that is, circa 1854 (95% HPD range 1760–1918)—rabies viruses from Sri Lanka and southern India diverged from their most recent common ancestor. Approximately 76.4 years ago (95% HPD 46.4–123.0 years)—that is, in ≈1933 (95% HPD range 1886–1963)—strain H-1413-09 diverged from canine rabies virus in Sri Lanka.

Compared with the genome sequence of rabies virus strain H-08-1320 from Sri Lanka, the genome sequence of strain H-1413-09 had a nucleotide deletion at residue 2,417 and an addition at nt 11,807. As a result, the start and stop signals of the mRNA and the start and stop positions of coding sequences of different genes were altered (Table 1). These altered start and stop codons advanced the coordinates of the intergenic signal of relevant intergenic regions. The P-M region and terminal sequence of strain H-08-1320 were 1 nt shorter (91 nt) and longer (136 nt), respectively. The nucleotide and amino acid identities between the coding regions of strains H-1413-09 and H-08-1320 are shown in Table 1. The substitutions detected in the deduced amino acid sequences of H-1413-09 were compared with the genomic sequence of H-08-1320 (Table 2). By using the MEGA5 Tamura-Nei model (www.megasoftware.net), we determined the genetic distance of the N gene to determine whether strain H-1413-09 is more diverse than other rabies viruses in Sri Lanka (Figure). The rate of variation among sites was modeled with a gamma distribution (shape parameter = 0.5). The genetic distances between strain H-1413-09 and other rabies viruses from Sri Lanka (0.027–0.036) were greater than the genetic distances among other rabies viruses (0.001–0.011). These

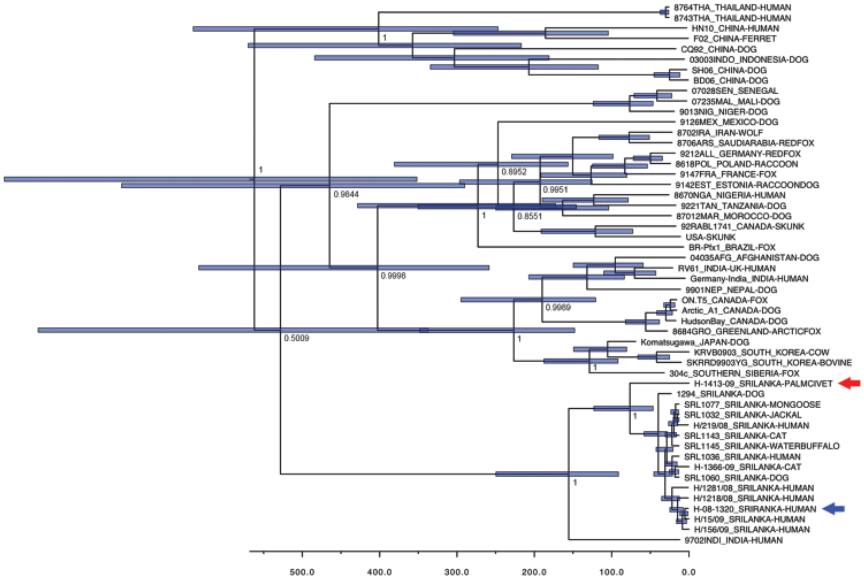

Figure. Bayesian maximum-credibility tree representing the genealogy of rabies virus as obtained by analyzing nucleotide sequences of full N gene sequences (1,350 nt). Nodes correspond to mean age at which lineages are separated from the most recent common ancestor; blue horizontal bars at nodes represent 95% highest posterior density of the most recent common ancestor. Numbers at main nodes represent posterior values. Horizontal axis at bottom represents time scales in years, beginning at 2010. Red arrow indicates strain H-1413-09; blue arrow indicates strain H-08-1320. Nucleotide sequence data for strains from Sri Lanka appear in nucleotide sequence databases of DNA DataBank of Japan, European Molecular Biology Laboratory, and GenBank with accession no. AB635373 (rabies virus strain H-1413-09), AB638767 (strain H-219-08), AB638768 (strain H-1218-08), AB638769 (strain H-1281-08), AB638770 (strain H-15-09), AB638771 (strain H-156–09), AB638772 (strain H-1366-09), and AB636165 (golden palm civet [*Paradoxurus zeylonensis*] strain H-1413-09).

results support our finding that strain H-1413-09 differs from other rabies viruses circulating in Sri Lanka.

Conclusions

Rabies virus probably survives favorably in the wild because it can infect a large spectrum of animals, thereby maximizing replication and dispersal opportunities (*9*). Most viruses replicate poorly when transferred to new hosts, but greater genetic variation assists in such species adaptation (*10*). Increased mutation in an RNA virus like rabies virus can give rise to variants with altered levels of fitness to persist and spread. A large number of substitutions were found in strain H-1413-09 compared with strain H-08-1320; these substitutions might represent changes that resulted from species adaptation. Phylogenetic analysis and comparative sequence data indicated that strain H-1413-09 is a variant rabies virus.

Palm civets are facing extinction in Sri Lanka because the species is losing its habitat, being hunted for its meat, and dying of parasitic diseases (www.sundaytimes.lk/090118/Plus/sundaytimesplus_01.html). Our study indicates that rabies might be another risk factor for extinction of these animals. Identification of a variant rabies virus in wildlife has serious implications for rabies control in Sri Lanka. Identification of such a virus would help provide epidemiologic data about the spread of rabies and its incursion into new geographic regions and would justify allocation of increased resources to help control rabies (*11*,*12*).

Several rabies virus variants associated with wildlife are known in the Americas and Africa (*1*,*13*–*15*), and this report identified classical sylvatic rabies in Asia. Whether *P. zeylonensis* is a reservoir of rabies virus or represents spillover from another animal deserves extensive investigation. The detection of rabies in wildlife indicates that much remains to be discovered in the tropical ecosystem of Sri Lanka. The circulation of a sylvatic variant rabies virus may be another hurdle in the rabies-control effort in Sri Lanka.

Table 2. Substitutions in genome sequence of rabies virus strain H-1413-09 from Sri Lanka, compared with genome sequence of strain H-08-1320*

Protein, amino acid substitution	Site/domain/region of protein†
N	
$Leu_{80} \rightarrow Phe_{80}$	
$Glu_{110} \rightarrow Asp_{110}$	
$Ile_{246} \rightarrow Val_{246}$	
$Ala_{372} \rightarrow Val_{372}$	Antigenic site I
P	
$Gln_{167} \rightarrow Arg_{167}$	N protein binding site in variable domain II
M	
$Ile_{16} \rightarrow Ala_{16}$	
$Pro_{19} \rightarrow Ser_{19}$	
$Ile_{55} \rightarrow Val_{55}$	
$Lys_{77} \rightarrow Arg_{77}$	
G	
$Val_{193} \rightarrow Ile_{193}$	
$Arg_{264} \rightarrow His_{264}$	
$Ile_{449} \rightarrow Thr_{449}$	Transmembrane region
$Thr_{459} \rightarrow Ile_{459}$	Transmembrane region
$Ala_{467} \rightarrow Thr_{467}$	Cytoplasmic domain
$Glu_{475} \rightarrow Gly_{457}$	Cytoplasmic domain
$Asn_{499} \rightarrow Ser_{499}$	Cytoplasmic domain
L	
$Ser_{26} \rightarrow Pro_{26}$	
$Ile_{49} \rightarrow Leu_{49}$	
$Cys_{137} \rightarrow Tyr_{137}$	
$Leu_{222} \rightarrow Ile_{222}$	
$Ser_{312} \rightarrow Gln_{312}$	Conserved domain I
$Glu_{313} \rightarrow Lys_{313}$	Conserved domain I
$Ser_{314} \rightarrow Ala_{314}$	Conserved domain I
$Arg_{315} \rightarrow Glu_{315}$	Conserved domain I
$Val_{317} \rightarrow Phe_{317}$	Conserved domain I
$Lys_{1056} \rightarrow Arg_{1056}$	Conserved domain IV
$Thr_{1137} \rightarrow Val_{1137}$	Conserved domain V
$Ala_{1520} \rightarrow Glu_{1520}$	
$Ile_{1555} \rightarrow Val_{1555}$	
$Leu_{1570} \rightarrow Met_{1570}$	
$Met_{1577} \rightarrow Leu_{1577}$	
$Lys_{1625} \rightarrow Arg_{1625}$	
$Asn_{1763} \rightarrow Asp_{1763}$	
$Arg_{1876} \rightarrow His_{1876}$	
$Asn_{2023} \rightarrow Asp_{1763}$	
$Gly_{2098} \rightarrow Arg_{2098}$	
$Leu_{2113} \rightarrow Phe_{2113}$	

*H-08-1320 is a human strain typical of canine rabies virus circulating in Sri Lanka; H-1413-09 is a novel sylvatic rabies virus variant from a golden palm civet in Sri Lanka.
†Blank spaces indicate no site/domain/region has been identified in that portion of the protein.

This study was supported in part by a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Sciences (grant no. 20406026) and by the Research Fund at the Discretion of the President, Oita University (grant no. 610000-N5010).

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Rickettsia parkeri in *Amblyomma maculatum* Ticks, North Carolina, USA, 2009–2010

Andrea S. Varela-Stokes, Christopher D. Paddock, Barry Engber, and Marcee Toliver

We detected *Rickettsia parkeri* in 20%−33% of *Amblyomma maculatum* ticks sampled in North Carolina. Results highlight the high frequencies of *R. parkeri*–infected ticks in the state with the highest annual incidence of Rocky Mountain spotted fever. Epidemiologic studies are needed to definitively link *R. parkeri* to cases of spotted fever rickettsiosis.

North Carolina historically reports some of the highest annual case counts of Rocky Mountain spotted fever (RMSF) and has accounted for >20% of total cases reported in the United States during the past 30 years (*1*–*4*). However, a species-specific diagnosis directly implicating infection with *Rickettsia rickettsii* is obtained for <10% of reported US cases. In 2010, the Centers for Disease Control and Prevention and Council of State and Territorial Epidemiologists modified the RMSF case designation to spotted fever rickettsiosis, acknowledging the complex epidemiology of tick-borne rickettsioses (*5*). Currently, *R. parkeri* is the only other tick-borne spotted fever group *Rickettsia* (SFGR) species known to cause disease in the southeastern United States, with >30 recognized cases from at least 9 states, including North Carolina (*6*). *R. parkeri* is detected in 20%−43% of *Amblyomma maculatum* ticks from the southeast, far greater than the recognized occurrence of *R. rickettsii* in any other tick species (*6*–*8*). We surveyed *A. maculatum* ticks collected from North Carolina for evidence of *R. parkeri* infection to assess the possibility that SFGR other than *R. rickettsii* result in cases categorized as RMSF in this state.

The Study

During May–September 2009 and 2010, adult ticks were collected by the Public Health Pest Management Section of the North Carolina Department of Environment and Natural Resources. Large numbers of *A. maculatum* ticks submitted by the general public through a tick-attachment project (www.deh.enr.state.nc.us/phpm/ticks_projects.htm) prompted further investigation in 3 counties in 2010. A total of 234 *A. maculatum* ticks were collected from 27 counties primarily distributed in the coastal plain and piedmont regions of North Carolina (Figure). These included 36 (2009) and 27 (2010) from the attachment project, all of which were removed from humans, except 6 (2009) that were removed from domestic dogs. The remaining 34 (2009) and 137 (2010) specimens were collected by dragging/flagging. Thirteen archived adult *A. maculatum* ticks collected during 1982–2008 were additionally tested, of which 6 were removed from humans. Nine adult *Dermacentor variabilis* ticks from the attachment project and 45 collected at sites with *A. maculatum* ticks were also tested. All ticks were identified by using standard taxonomic keys and were stored in 95% ethanol.

DNA was extracted by using the QIAmp DNA Mini Kit (QIAGEN, Valencia, CA, USA) for ticks collected in 2009 and Illustra Tissue and Cells genomicPrep Mini Spin Kit (GE Healthcare, Piscataway, NJ, USA) for ticks collected in 2010 and all archived *A. maculatum* and *D. variabilis*. All samples were tested by a PCR targeting a rickettsial outer membrane protein A (*ompA*) gene fragment. *D. variabilis* ticks were tested by using a broad-range SFGR-nested PCR (*9*). *A. maculatum* ticks were tested by using primers specific for *R. parkeri* and *Candidatus* Rickettsia andeanae. *R. parkeri*–specific primers were designed by aligning representative *ompA* gene sequences in GenBank for *R. parkeri*, *R. rickettsii*, *R. peacockii*, *R. amblyommii*, and *Candidatus* R. andeanae and identifying nonconserved regions among sequences. Primers RpompAF (5′-AATGCAGCATTTAGTGATGATGTTAA-3′) and RpompAR (5′-TCCTCCATTTATATTGCCTG-3′) were chosen. Final reagent concentrations were 300 nmol/L for each primer, 1.25 units GoTaq (Promega, Madison, WI, USA), 1.5 mmol/L $MgCl_2$, and 200 nmol/L each dNTP. Thermal cycler conditions were as follows: 94°C (2 min); 40 cycles of 94°C (30 s), 54°C (60 s), and 72°C (90 s); and a final extension of 72°C (5 min) to amplify the 447-bp fragment. We confirmed that *R. parkeri*–specific primers would not amplify *R. amblyommii* by testing 6 *A. americanum* ticks infected with *R. amblyommii* (determined by sequencing 17-kDa antigen gene amplicon) because this species has been detected in *A. maculatum* ticks (*10*). To detect *Candidatus* R. andeanae, primers Rx-190-F and Rx-190-R were used in a conventional PCR (*6*). All rickettsial PCRs included a positive control of DNA from cultured *R. parkeri* (Tate's Hell strain) or a previously confirmed *Candidatus* R. andeanae–infected *A. maculatum* tick, and water controls. DNA extractions, PCRs, and electrophoresis were performed in separate rooms or designated labora-

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DOI: http://dx.doi.org/10.3201/eid1712.110789

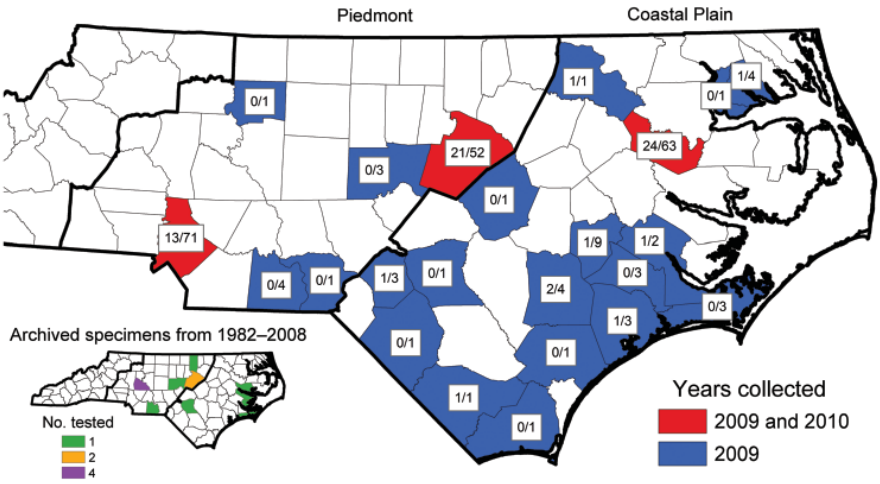

Figure. Distribution of *Rickettsia parkeri*–infected *Amblyomma maculatum* ticks collected in North Carolina, USA, during 2009-2010 and in archived specimens (inset). Numbers indicate total number positive for *R. parkeri* by PCR/total number tested in that county.

tory areas. DNA extractions from archived *A. maculatum* ticks were tested by PCR of a tick mitochondrial 16S rRNA gene amplicon (*11*) to ensure amplifiable DNA. Selected PCR products were submitted to Eurofins MWG Operon (Huntsville, AL, USA). Consensus sequences determined by ClustalX2 alignment for each sample were compared with sequences in GenBank for identification by using a BLAST search (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

An additional 21 *A. maculatum* ticks from Mecklenburg County (2010) were processed to isolate *R. parkeri* as described (*6*). The identity of each isolate was confirmed by *ompA* PCR and sequence analysis.

DNA extracts from 8 female and 6 male ticks (20%) of 70 *A. maculatum* ticks collected in 2009 tested positive for *R. parkeri* (Table). Of these, 6 were collected by dragging or flagging, 2 were unattached on domestic dogs, 5 were crawling or attached on persons, and 1 was on a vehicle. Sequences from 3 *R. parkeri*–positive extracts from 3 different counties were 100% identical to *R. parkeri*; next closest in identity (98%) were *R. sibirica* and *R. africae*. In 2010, 54/164 (27 females; 27 males) (33%) *A. maculatum* specimens tested positive for *R. parkeri*. Sequences from 17 positive samples (3 from Wake County, 6 from Mecklenburg County, 8 from Martin County) were 100% identical to *R. parkeri*. Ten of the 2010 *R. parkeri*–positive ticks were attached to persons; clinical symptoms for these persons were not assessed.

Candidatus R. andeanae was detected in 9 tick extracts. Sequences of all 2010 positive ticks were 100% identical to GenBank *ompA* sequences for *Candidatus* R. andeanae. One male *Candidatus* R. andeanae–positive tick from Martin County was co-infected with *R. parkeri*, which was confirmed by sequencing. No archived *A. maculatum* ticks were positive by PCR for *Candidatus* R. andeanae or *R. parkeri*. However, 8 archived samples showed faintly staining bands for mitochondrial 16S rRNA gene amplicons, suggesting loss of DNA integrity in these older samples. Three isolates of *R. parkeri*, designated NC-3, NC-8, and NC-15, were obtained in Vero E6 cell cultures. No SFGR was detected by PCR in any *D. variabilis* ticks.

Conclusions

Until recently, *A. maculatum* was considered an incidental tick species in North Carolina (*12*,*13*); however, we identified an overall prevalence of *R. parkeri* in 29% of *A. maculatum* ticks from multiple sites in North Carolina considered endemic for RMSF. These data, coupled with the frequency of *R. parkeri*–positive ticks removed from humans, suggest that *A. maculatum* ticks are well established in North Carolina and that *R. parkeri* causes at least some cases of spotted fever rickettsiosis in this state. We examined a small number of *D. variabilis* ticks; however, none were infected with *R. rickettsii*, consistent with previous surveys of this tick for SFGR in North Carolina and other states (*8*,*14*,*15*). More extensive surveys of *D. variabilis* may be warranted to better determine the relative contribution of this tick to spotted fever rickettsiosis in North Carolina. The pathogenicity and clinical significance of *Candidatus* R. andeanae are unknown; however, this rickettsia is detected in *A. maculatum* ticks less frequently than *R. parkeri* and thus far has not been directly associated with human illness (*6*). *Candidatus* R. andeanae has been detected in singly infected *A. maculatum* ticks from Virginia (*7*), Florida, Mississippi, and Georgia (*9*) but to our knowledge has not been found in ticks co-infected with other rickettsiae. Further studies that causally link *R. parkeri* in *A. maculatum* ticks with human disease in North Carolina are necessary to incriminate it as a causative agent in this state.

Acknowledgments

We thank Edward C. Swab and staff at Axiom Environmental, Inc., who collected specimens during the course of their workday and without whom the presence and impact of *A. maculatum* ticks in North Carolina would not have been noted. In addition,

Table. *Rickettsia* species detected by PCR in adult *Amblyomma maculatum* ticks collected in North Carolina, USA, 2009−2010

Year, county	No. adult ticks tested (no. found on person or domestic animal)	% Positive	
		R. parkeri	*Candidatus* Rickettsia andeanae
2009	70	14 (20)	1 (1)
Anson	4 (0)	0	0
Brunswick	1 (0)	0	0
Carteret	3 (67)	0	0
Chatham	3 (0)	0	0
Chowan	1 (0)	0	0
Columbus	1 (0)	1	0
Craven	2 (50)	1* (50)	0
Cumberland	1 (0)	0	0
Duplin	4 (25)	2 (50)	0
Forsyth	1 (100)	0	0
Halifax	1 (0)	1 (100)	0
Hoke	3 (0)	1 (33)	0
Johnston	1 (0)	0	0
Jones	3 (67)	1 (33)	0
Lenoir	9 (100)	1 (11)	0
Martin	6 (100)	2 (33)	1* (17)
Mecklenburg	1 (0)	0	0
Onslow	3 (33†)	1† (33)	0
Pender	1 (0)	0	0
Perquimans	4 (100)	1* (25)	0
Richmond	1 (100)	0	0
Robeson	1 (0)	0	0
Wake	15 (47)	2‡ (13)	0
2010	164	54 (33)	8 (5)
Martin	57 (46)	22§ (39)	3 (5)
Mecklenburg	70 (3)	13*¶ (17)	5 (7)
Wake	37 (3)	19 (51)	0

*One specimen on person.
†Specimen was found on vehicle.
‡Specimens were removed from domestic animals.
§Nine specimens were removed from individual persons.
¶Thirteen specimens were processed for culture isolation; 3 isolates obtained.

we are grateful for the assistance of Rob McHenry and Christa Rogers for contributing specimens and facilitating our collections at Cowan's Ford Wildlife Refuge. Finally, we thank Erle Chenney and Whitney Smith for their contributions to the molecular analysis of ticks.

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Japanese Encephalitis Virus Genotype Replacement, Taiwan, 2009–2010

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Genotype I of Japanese encephalitis virus first appeared in Taiwan in 2008. Phylogenetic analysis of 37 viruses from pig farms in 2009–2010 classified these viruses into 2 unique subclusters of genotype I viruses and suggested multiple introductions and swift replacement of genotype III by genotype I virus in Taiwan.

Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, is a common cause of viral encephalitis in southern and eastern Asia. Pigs are a readily available virus amplifying host, and *Culex tritaeniorhynchus* mosquitoes, the primary transmission vector of JEV, breed predominantly in rice paddies (*1*). Molecular epidemiologic studies have found that the introduction of JEV into subtropical regions from tropical regions, possibly associated with the Pacific flyway of spring migratory birds, may contribute to annual JEV epidemics and epizootics in subtropical regions (*2*).

Phylogenetic reconstruction, based on capsid and precursor membrane or envelope (E) structural protein genes of JEV, supports an Indonesian origin and further classifies JEV into 6 genotypes (*3*,*4*). The genotype III (GIII) virus is most widely distributed in the temperate zone and is the genotype most frequently associated with JEV outbreaks and epidemics in eastern and Southeast Asian countries (*4*). Genotype I (GI) JEV, which originated in Indonesia and circulated in Thailand and Cambodia during the 1970s, appeared in South Korea and Japan during the 1990s. The replacement of GIII by GI was swift, completed in just a few years in Japan, South Korea, Thailand, and Vietnam (*2*,*5*–*7*). The introduction of GI JEV into Japan may have followed a south-to-north or west-to-east route and has been reported to have come from Southeast Asia and mainland People's Republic of China (*8*). However, events leading to replacement, including introduction, transition dynamics, and interplay between the 2 virus genotypes, are still unclear. The only 2 remaining GI-free island countries, the Philippines and Taiwan, provide a location where the transmission dynamics of these 2 JEV genotypes can be elucidated. GI JEV was first detected in northern Taiwan during the winter of 2008 by the Centers for Disease Control, Taiwan (CDC-Taiwan) (*9*); however, GIII JEV remained the dominant virus in Taiwan that year.

The Study

To understand early events leading to the replacement of GIII viruses by GI viruses in JEV-endemic areas, we conducted virologic surveillance using mosquitoes and pig serum specimens collected from pig farms in multiple counties in Taiwan during the 2009 and 2010 transmission seasons. Eight pig farms were selected as sites for virologic surveillance (Figure): 3 in the central counties of Taichung and Changhua; 4 in the southern counties of Yulin, Chiayi, and Tainan; and 1 in the eastern county of Hualien. Mosquito pools and swine serum specimens were collected every other week during the JEV transmission season from March 2009 through October 2010.

The mosquito and swine specimens were subjected to viral RNA detection by using a multiplex reverse transcription PCR (RT-PCR). A QIAamp Viral RNA kit (QIAGEN, Hilden, Germany) was used to extract viral RNA from pooled ground mosquitoes or from plasma specimens following the manufacturer's protocol. Three primer pairs were used in RT-PCR to differentiate GI and GIII JEV (Table). Amplifying and sequence primers were used as was done in our previous study (*10*) to obtain E gene region and the full genomic sequence. The DNA fragment of the correct size was excised, purified with a Viogene gel extraction kit (VIOGENE, Sunnyvale, CA, USA), and sequenced directly by using a Prism automated DNA sequencing kit (Applied Biosystems, Foster City, CA, USA).

A total of 62,266 mosquitoes were collected at multiple sites in Taiwan from March 2009 through October 2010. The most common species was *Cx. tritaeniorhynchus* (n = 59,386). Of the 787 mosquito pools collected, 37 pools were JEV-positive by multiplex RT-PCR, and all positive pools contained *Cx. tritaeniorhynchus* mosquitoes. The JEV-positive mosquitoes were collected from central (Taichung and Changhua Counties), southern (Yulin, Chiayi, and Tainan Counties), and eastern (Hualin County)

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DOI: http://dx.doi.org/10.3201/eid1712.110914

[1]These authors contributed equally to this article.

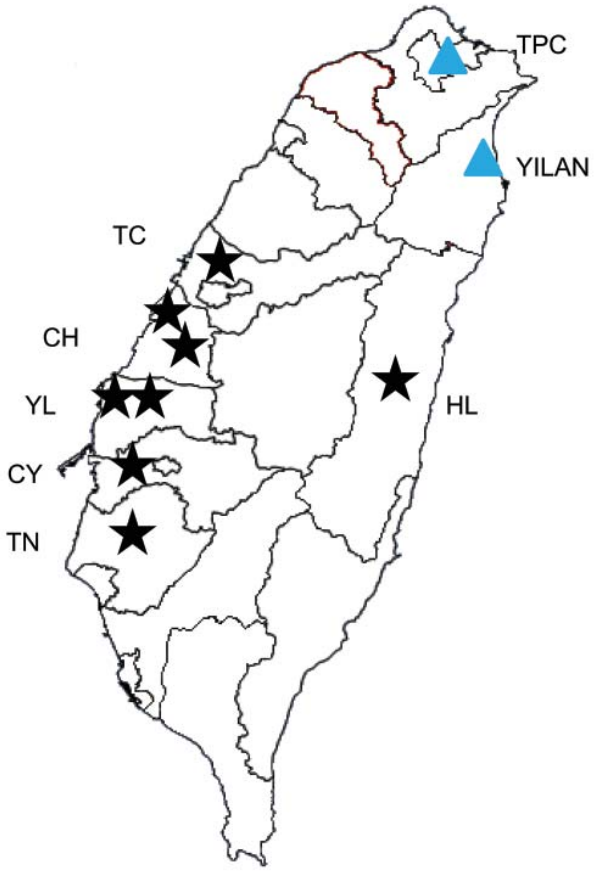

Figure. Location of pig farms from which mosquitoes were collected for phylogenetic analysis of Japanese encephalitis virus (JEV) isolates, Taiwan, 2009–2010. Black stars indicate location of pig farms. Blue triangles indicate locations at which genotype I JEV was first detected by the Taiwan Centers for Disease Control (*9*). CH, Changhua County; CY, Chiayi County; HL, Hualien County; TC, Taichung County; TN, Tainan County; TPC, Taipei City; YL, Yulin County.

Taiwan (Figure). Unfortunately, JEV was not detected from pig serum specimens, but seroconversion, defined by plaque-reduction neutralizing assay at the 50% reduction titer of ≥10, was evident among these samples after JEV detection in mosquitoes (data not shown).

Full-length JEV sequences, including TC2009–1, TC2009–1–3, and YL2009–4 (GenBank accession nos. JF499788–JF499790), were obtained from 3 positive mosquito pools, and a neighbor-joining phylogenetic tree confirmed all 3 viruses belong to GI (data not shown). Additionally, the E gene was sequenced from 37 JEV-positive mosquito pools (GenBank accession nos. JF499791–JF499827). The full-length E gene sequence of these isolates was more closely related to the GI K94P05 strain than to the GIII Nakayama strain; sequence identities ranged from 97.6% to 98.6%, compared with 87.4% to 88.1%, respectively. The E gene tree supports that all 37 isolates detected in this study belong to GI (online Appendix Figure, wwwnc.cdc.gov/EID/article/17/12/11-0914-FA1.htm). However, of JEV detected by CDC-Taiwan in 2008, only TPC0806c and YILAN0806f were classified as GI; all others belonged to GIII (*9*).

GI JEV can be classified into various subclusters based on phylogeny (*8*). In this study, 21 of the 37 viruses could be classified as subcluster I and the other 16 as subcluster II. The TPC0806c and YILAN0806f strains, reported by CDC-Taiwan, belong to subcluster II (online Appendix Figure) (*9*). This result indicates that the GI JEV introduced into Taiwan originated from multiple sources.

To understand the origin of the GI JEV introduced into Taiwan, we selected the K91P55 strain as the root virus and constructed a minimum-spanning tree by using BioNumerics software version 5.00 (Applied Maths; Austin, TX, USA). Among the subcluster I JEV isolated in Taiwan, the most pronounced genetic linkages appeared between viruses isolated in Taichung in 2009 and in Japan in 2007. The Taichung isolates (TC2009-2, -5, -6, -8, -9, and -11) were most closely linked to a group of viruses (JaNAr06, 14, 15, and 17) isolated from mosquitoes collected in Nagasaki, Japan, in 2007. Among subcluster II JEV in Taiwan, the TC2009-3 Taichung isolate and the TPC0806c Taipei City isolate were most closely related to JaNAr32-04, which was isolated from mosquitoes collected in Nagasaki, Japan, in 2004. In summary, the JEV GI isolates from Taiwan, most closely related to GI viruses isolated from Nagasaki, Japan, were introduced at least twice into central and once into northern Taiwan.

Conclusions

GI JEV first appeared in Taiwan in 2008 when GIII viruses were still the dominant circulating genotype in the region. We conclude, on the basis of molecular evidence, that the dominant JEV genotype in Taiwan has switched from III to I. Also, our study suggests that 1) the genotype

Table. The 3 primer pairs used in reverse transcription PCR to differentiate genotypes I and III Japanese encephalitis virus, Taiwan, 2009–2010*

Specificity/ name	Sequence, 5′ → 3′	Location†
All JEV		
JE1F	TGTGTGAACTTCTTGGCTTAGTAT	12–35
JE1R	CARCATCTGTTYTCWCCTTTTGA	559–581
GI JEV only		
GIJEVF	CAGTCGCGAGTTTAAACGAC	2000–2019
GIJEVR	CATTCAGTTCGTCCCGCACA	2688–2707
GIII JEV only		
GIIIJEVF	GGATGCTTGGCAGTAACAAC	904–923
GIIIJEVR	AAGTCCACATCCGTTGCC	1281–1298

*JEV, Japanese encephalitis virus; GI, genotype I; GIII, genotype III.
†According to JEV strain T1P1 complete genome (GenBank accession no. AF254453).

replacement may have been accomplished within 1 year; 2) the JEV ecology remains unchanged, as evidenced by the involvement of *Cx. tritaeniorhynchus* mosquitoes and of swine in maintaining and circulating GI virus; 3) the introduction of GI JEV strains occurred multiple times, resulting in the detection of subcluster I and II viruses; and 4) the GI JEVs isolated in Taiwan were most closely related to GI viruses isolated from Nagasaki, Japan. Current human and swine vaccines are derived from GIII viruses. Thus, the systemic evaluation of the cross-neutralizing activity of vaccinated human, as well as swine, serum specimens should be used to estimate the protective efficacy of GIII-based vaccine.

Acknowledgments

We thank Yuan-Ching Huang for helping us collect swine serum and Nicole Trainor and Jaimie Robinson for assistance in preparing this article.

This study was funded by grants from the National Science Council, Taiwan (NSC 96-2313-B-005-023-MY3), and the Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture, Taiwan.

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Altitude-dependent *Bartonella quintana* Genotype C in Head Lice, Ethiopia

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To determine the presence of *Bartonella quintana* in head and body lice from persons in different locations in Ethiopia, we used molecular methods. *B. quintana* was found in 19 (7%) genotype C head lice and in 76 (18%) genotype A body lice. *B. quintana* in head lice was positively linked to altitude (p = 0.014).

Head (*Pediculus humanus capitis* de Geer) and body lice (*Pediculus humanus humanus* Linnaeus) have been parasites of humans for thousands of years (*1*). Genetic studies based on mitochondrial DNA (mtDNA) have found 3 phylotypes of *P. humanus* (*2*,*3*). Clade A is the most common genotype with worldwide distribution and is found among both head and body lice. Clade B comprises only head lice and has been found in South America, Europe, and Australia, whereas clade C comprises only head lice from Ethiopia and Nepal (*2*,*4*,*5*). Only body lice have been implicated as vectors of *Bartonella quintana,* which causes trench fever, bacillary angiomatosis, endocarditis, chronic bacteremia, and chronic lymphadenopathy (*6*). However, *B. quintana* has been identified in head lice from homeless children in Nepal (*7*), in head lice from homeless adults in San Francisco, California, USA (*8*), and recently in head lice nits from a homeless man in Marseille, France (*9*). The objective of our study was to use molecular methods to determine the presence of *B. quintana* infection in head and body lice collected from patients in different locations in Ethiopia, a country where epidemiologic and clinical studies of zoonoses are scarce and a widespread louse infestation exists (*6*). Moreover, we assessed whether a phylogenetic difference existed between head and body lice collected from the same patient.

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DOI: http://dx.doi.org/10.3201/eid1712.110453

The Study

After obtaining ethical approval from Jimma University Ethics Review Board, we collected head and body lice from persons at locations at different altitudes in Ethiopia. Lice were transferred to Marseille in sterile tubes at room temperature. Each louse was rinsed twice in sterile water for 15 minutes, and then total genomic DNA was extracted from each louse by using a QIAamp Tissue kit (QIAGEN, Hilden, Germany) as described by the manufacturer. Samples were screened by using a quantitative real-time PCR that targeted a portion of the *Bartonella* 16S-23S intergenic spacer region (*10*) and a specific *B. quintana* gene, *fabF3*, encoding 3-oxoacyl-(acyl-carrier-protein) (*9*). Negative controls (DNA from noninfected lice and sterile water) and positive controls (DNA from *B. elizabethae*) were included in each assay. From 50 randomly selected persons, each of whom had at least 3 body lice and 3 head lice; we selected 1 head louse and 1 body louse from each person for amplification and sequencing of the mitochondrial gene cytochrome b as described (*11*). For phylogenetic analysis, we used MEGA3.1 software (www.megasoftware.net). For data comparison, we used Epi Info version 6.0 software (Centers for Disease Control and Prevention, Atlanta, GA, USA). A p value <0.05 was considered significant.

Overall, we tested 271 head and 424 body adult lice collected from 134 persons (109 women). Head and body lice co-infection existed on nearly all persons with a range of louse infestation of ≈5 to 20 lice per person. On 1 person, >100 lice were found. All but 2 persons had only black head lice; those 2 persons each had 1 gray louse among their populations of black head lice. All body lice were gray-transparent. All black head lice belonged to genotype C, and all gray lice belonged to genotype A (GenBank accession nos. JF694384–JF694442). Head and body lice grouped in 2 different clusters (online Appendix Figure, wwwnc.cdc.gov/EID/article/17/12/11-0453-FA1.htm).

B. quintana was identified in 19 (7%) head lice collected from 9 (6.7%) patients and in 76 (18%) body lice collected from 17 (12.7%) patients (Table). Positive and negative controls showed expected results in all tests. No difference between male and female patients was observed in infestation with *B. quintana* (p = 0.36). No patients had both head and body lice infected with *B. quintana.* Significantly more body lice than head lice were infected with *B. quintana* (p<0.001). Significantly more body lice were infected with *B. quintana* in Dembi and in Tum than in the other locations (p = 0.001 and p = 0.02, respectively). *B. quintana* in body lice was not significantly linked to altitude (p = 0.3 with the Kruskal-Wallis test).

Only in the locations with the highest altitudes (Gibarku, altitude 2,395 m, and Tikemit Eshet, altitude 2,121 m) did we find *B. quintana* in head lice (Figure).

Table. *Bartonella quintana* in body and head lice from 7 different locations in Ethiopia

Location (altitude)	No. persons tested	No. head lice tested/no. persons	No. body lice tested/no. persons	No. (%) head lice with *B. quintana*	No. (%) body lice with *B. quintana*	No. (%) persons with head lice	No. (%) persons with body lice
Mizan Teferi (1,451 m)	35	66/29	53/32	0	0	0	0
Agaro (1,560 m)	15	19/15	25/15	0	2 (8)	0	1 (7)
Dembi (1,679 m)	19	34/15	187/19	0	55 (29)	0	11 (57)
Tum (1,830 m)	18	54/18	52/15	0	15 (28)	0	4 (26)
Balt (2,050 m)	12	19/12	44/12	0	4 (9)	0	1 (8)
Tikemit Eshet (2,121 m)	18	32/15	20/12	6 (18)	0	3 (20)	0
Gibarku (2,395 m)	17	47/17	43/17	13 (27)	0	6 (35)	0
Total	134	271/121	424/122	19 (7)	76 (18)	9 (7)	17 (14)

At these higher altitudes, we did not find *B. quintana* in body lice. As a result, in Gibarku and in Tikemit Eshet, we found significantly more head lice that were infected with *B. quintana* than in the other locations ($p = 0.0003$ and $p = 0.002$, respectively). The presence of *B. quintana* in head lice was positively and significantly linked to altitude ($p = 0.014$ with the Kruskal-Wallis test).

Conclusions

Our results confirm previous studies that show that genotype C head lice are prevalent in Ethiopia. Genotype C head lice cohabited with genotype A body lice, but we did not find an infestation of genotype A head lice in the persons studied. Only 2 persons each had 1 gray louse (genotype A) among many black head lice (genotype C). As a result, dual transmission cycles of lice appear to be occurring, and genotype C head lice may be inhibiting outbreaks of genotype A head lice.

We found that 7% of the head lice and 18% of the body lice from persons from Ethiopia were infected with *B. quintana*. For a long period, *B. quintana* was found only in body lice (*12*). It was first identified in head lice from homeless children in Nepal, where genotype C head lice exist (*4*,*7*). Additionally, *B. quintana* was recently found in lice from 138 homeless persons in the San Francisco area; 25% of head lice–infested persons had lice pools infected with *B. quintana* (*8*). However, we were unable to determine whether the study in Nepal reported *B. quintana* infection of genotype C or genotype A head lice (*7*) or whether the study in San Francisco reported infection of genotype A or genotype B head lice (*8*).

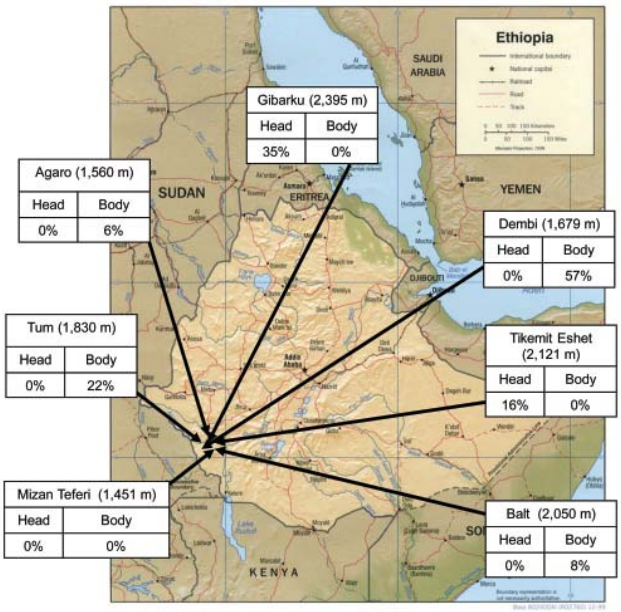

Figure. Percentage of humans with body or head lice infected with *Bartonella quintana* at different altitudes, Ethiopia.

All persons with head lice infected with *B. quintana* were from locations with higher altitudes (≥2,121 m), whereas at these altitudes, no body lice were infected with *B. quintana*. The permanent foci of body lice occur in regions subject to cold weather and in poverty-stricken areas such as Ethiopia. Body lice were found in 39% of Ethiopian migrants, and head lice were found in 65% (*13*), whereas 67% of schoolchildren from 3 different cities in Ethiopia harbored body lice (*14*). We do not have current hypotheses to explain the links from *B. quintana* to head lice at high altitudes or the mutual exclusion of *B. quintana* in head or body lice in the same person.

In conclusion, we identified *B. quintana* in genotype C head lice from Ethiopia only at altitudes ≥2,121 m and only in patients without infected body lice. However, further epidemiologic studies with head lice collected from more patients and from different countries should be performed to determine whether this altitude dependence of *B. quintana* in head lice exists only in Ethiopia or is a general phenomenon.

Dr Angelakis is a clinician and researcher at the Unité des Rickettsies in Marseille. His research interests are zoonotic pathogens.

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etymologia

Q Fever

[ku fe'vər]

From Q for query, because the disease was an illness of unknown etiology. In 1937, Australian researcher Edward Derrick reported a disease that affected workers in slaughterhouses. *Coxiella burnetii*, the bacteria that causes the disease, was found shortly after Derrick's investigation began. However, many aspects of the disease continue to puzzle researchers, making the name Q fever as appropriate today as it was 74 years ago.

Sources: Cooke RA. Q fever. Was Edward Derrick's contribution undervalued? Med J Aust. 2008;189:660–2; Derrick EH. "Q" fever, a new fever entity: clinical features, diagnosis, and laboratory investigation. Med J Aust. 1937;2:281–99; Dorland's illustrated medical dictionary. 31st ed. Philadelphia: Saunders; 2007; Mackerras IM. Australian Academy of Sciences biographical memoirs of deceased members: Edward Holbrook Derrick 1898–1976 [cited 2011 Sep 9]. http://www.asap.unimelb.edu.au/bsparcs/aasmemoirs/derrick.htm#mac

Edited by Nancy Männikkö

http://dx.doi.org/10.3201/eid1712.ET1712

Proximity to Goat Farms and *Coxiella burnetii* Seroprevalence among Pregnant Women

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During 2007–2009, we tested serum samples from 2,004 pregnant women living in an area of high Q fever incidence in the Netherlands. Results confirmed that presence of antibodies against *Coxiella burnetii* is related to proximity to infected dairy goat farms. Pregnant women and patients with certain cardiovascular conditions should avoid these farms.

Dairy goat farms were implicated in the large Q fever epidemic (>3,500 human cases) in the Netherlands during 2007–2009 (*1*,*2*). However, most human infections remain asymptomatic or appear as a self-limiting febrile illness and are therefore not reported. Seroprevalence studies are needed to discover the true infection pressure in the population. We aimed to establish whether the presence of antibodies to *Coxiella burnetii*, the etiologic agent of Q fever, is associated with physical proximity to infected small ruminant (dairy sheep and goat) farms.

The Study

Serum samples from pregnant women were obtained from laboratories located in the high-incidence Q fever area in the province of Noord-Brabant. The samples had been collected during June 20, 2007–May 26, 2009, during a screening program for syphilis, hepatitis B, and HIV infection, which was routinely offered to all pregnant women. Serum samples were analyzed by using an immunofluorescence assay (Focus Diagnostics, Cypress, CA, USA) for detecting IgG and IgM against phases I and II of *C. burnetii* infection in a single dilution of 1:64. An IgG II titer ≥64 was considered indicative of past infection. Possible recent infection was defined as IgM II ≥64 combined with either an IgG II or IgM I titer ≥64.

We included 10 adjacent municipalities (Figure 1) that had an incidence of Q fever notifications over the study period of 0.5–19.6 per 1,000 population and with clear seasonal peaks (Figure 2). Median age of the 2,004 women in the study was 30 years (interquartile range 27–33 years) with no significant differences between the municipalities. Seroprevalence for past infection was 9.0% (181/2,004), ranging from 0% to 21% between the 10 municipalities (Table 1). In 57 (31%) of 181 women, IgM II titer was ≥64. Only 2 women had an IgM II titer ≥64 and IgM I titer ≥64 without an IgG II titer ≥64. Seroprevalence for possible recent infection was therefore 2.9% (59/2,004), with a range of 0%–10% between the 10 municipalities.

For each pregnant woman, we calculated the distance from her home address to the closest farms in the following 3 categories: 1) dairy goat or dairy sheep farm

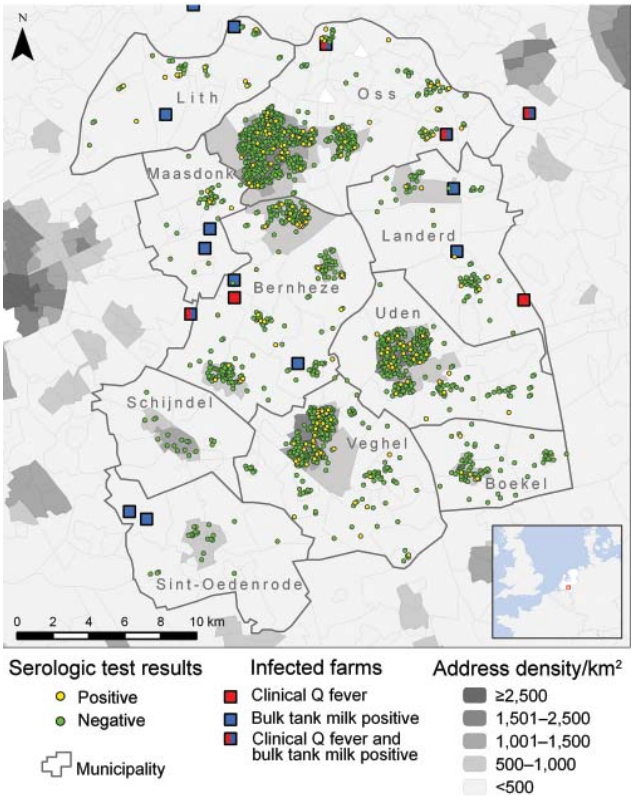

Figure 1. Location of the 10 municipalities studied in southern area of the Netherlands, 2007–2009, with residence and serologic results for 2,004 pregnant women, sites of small ruminant farms with infected animals, and address density. Three of the 20 farms included in the analysis are not visible.

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DOI: http://dx.doi.org/10.3201/eid1712.110738

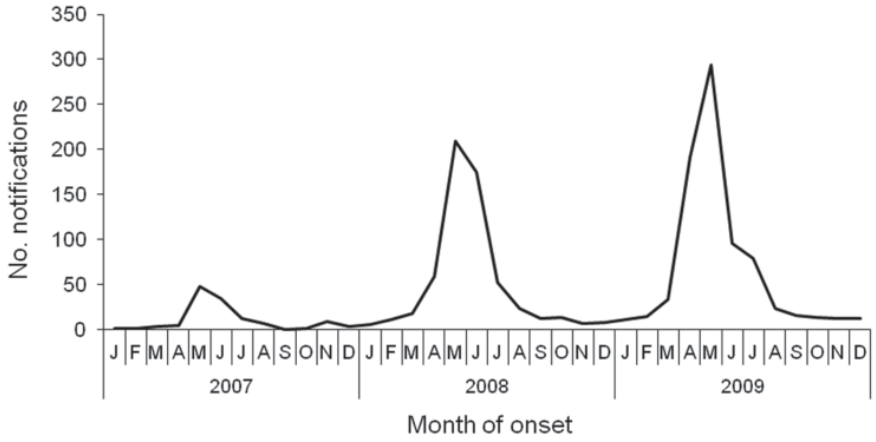

Figure 2. Q fever notifications by month of onset of illness in 10 municipalities in southern area of the Netherlands, 2007–2009.

where clinical Q fever (i.e., abortion waves) occurred during 2005–2009 (data from Animal Health Service) (8 farms [all goat farms] were identified in this way, of which 6 were located within or just outside of the study area) (Figure 1); 2) dairy goat or dairy sheep farms at which bulk tank milk tested positive for *C. burnetii* antibodies in the mandatory bulk tank milk monitoring program in 2009 (data from Food and Consumer Product Safety Authority; 12 goat farms were identified); and 3) a farm with >100 goats or sheep, irrespective of the infection status and production type (milk or meat) of the farm (data from the Ministry of Agriculture, Nature and Food Quality). Details of category 1 and category 2 farms are provided in the online Appendix Table (wwwnc.cdc.gov/EID/article/17/12/11-0738-TA1.htm).

Univariate regression analysis showed that pregnant women living <2 km from a farm that had experienced clinical Q fever had a higher risk of testing positive for antibodies to *C. burnetii* than those living ≥5 km away (odds ratio 2.63, 95% confidence interval 1.33–5.20 for IgG II titer ≥64 and odds ratio 6.58, 95% confidence interval 2.78–15.55 for possible recent infection). The increased risk for farms that had positive test results during monitoring of bulk tank milk was not significant. No increased risk was found for women who lived close to any farm with >100 animals. However, 98% of the population in the study area live within 5 km of such farms. In multivariate logistic regression analyses, taking into account address density of the neighborhood and other relevant variables, living <2 km from a farm with clinical Q fever remained a strong risk factor (Table 2).

Conclusions

The presence of antibodies against *C. burnetii*, especially levels suggesting recent infection, is associated with living near a farm with infected dairy goats. This finding applied only to farms where animals had clinical Q fever and not for farms where tests of bulk tank milk were positive. A study in 2008 showed that persons who lived near (<2 km) an infected dairy goat farm had a much higher risk for Q fever than did persons who lived further away (>5 km) (*3*). However, these results were based on notified cases, i.e., patients with clinical signs. Because the link to goat farming has received substantial attention in the public media, persons living close to goat farms may have sought medical care for suspected Q fever more rapidly than those

Table 1. Antibodies to *Coxiella burnetii* in pregnant women in 10 municipalities in Noord-Brabant Province, the Netherlands, June 2007–May 2009

Municipality	Total population	No. pregnant women tested	Serologic profile, no. (%) women	
			IgG II titer ≥64	Possible recent infection*
Lith	6,667	42	9 (21.4)	4 (9.5)
Oss	77,097	702	57 (8.1)	22 (3.1)
Maasdonk	11,260	38	5 (13.2)	0
Bernheze	29,615	291	30 (10.3)	15 (5.2)
Landerd	14,805	72	6 (8.3)	4 (5.6)
Uden	40,360	380	32 (8.4)	8 (2.1)
Veghel	37,125	355	37 (10.4)	6 (1.7)
Boekel	9,692	84	5 (6.0)	0
Schijndel	22,889	18	0	0
Sint-Oedenrode	17,427	22	0	0
Total	266,937	2,004	181 (9.0)	59 (2.9)

*IgM titer to phase II antigen ≥64 combined with either an IgG II or IgM I titer ≥64.

Table 2. Multivariate logistic regression models for *Coxiella burnetii* IgG II seropositivity and serologic indication for possible recent infection based on house location of 2,004 pregnant women, the Netherlands, 2007–2009*

Variable	OR (95% CI)	
	IgG II titer ≥64	Possible recent infection†
Distance to nearest farm with clinical Q fever, km		
<2.0	2.38 (1.19–4.73)	6.68 (2.53–17.64)
2.0–4.9	1.12 (0.74–1.71)	2.82 (1.34–5.92)
≥5	Reference	Reference
No. infected farms within 5 km		
≥1		0.92 (0.46–1.85)
0		Reference
Total no. locations with sheep or goats within 5 km		
≥140	1.29 (0.95–1.76)	
<140	Reference	
Address density of neighborhood, addresses/km^2‡		
<500	1.85 (1.23–2.78)	1.32 (0.65–2.70)
≥500	Reference	Reference

*OR, odds ratio; CI, confidence interval. Blank cells indicate variable not included.
†IgM antibody titer to phase II antigen ≥64 combined with either an IgG II or IgM I titer ≥64.
‡Neighborhoods were categorized as not urban if the address density was <500 addresses/km^2 and urban if ≥500/km^2.

who lived distantly from goat farms. Our population-based serologic study had a control group of seronegative women and identified asymptomatic infections. Combined, both studies provide evidence that living near dairy goat farms that experience abortion waves increases the risk in humans for symptomatic and asymptomatic *C. burnetii* infection.

A limitation of this study is that the exact infectious periods for each of the 20 farms in the study are unknown. Mandatory systematic monitoring of bulk tank milk only started in October 2009. However, for 17 of the 20 farms identified in the distance calculations in the present study, bulk tank milk testing results from 2008 were available from the records of the Animal Health Service. When an ELISA was performed, animals at 13 of the 17 farms tested positive for *C. burnetii* antibodies (online Appendix Table). Furthermore, for 15 of the 17 farms, bulk tank milk tested positive by PCR in 2008. The assumption that persons became infected where they lived, although infection might have occurred elsewhere, might have weakened the association between house location and infected farms because of nondifferential misclassification. We did not account for circumstances that may play a role in transmission from farm to humans, such as wind, vegetation patterns, and soil conditions around infected farms (*4*). We assume that the effect of the voluntary vaccination program of small ruminants in 2008 had only limited effects on the study. Culling of pregnant animals on infected farms did not affect the results of the study because that began in December 2009.

We found an overall prevalence of IgG II antibodies of 9.0%. In comparison, during 2006–2007, a seroprevalence of 2.4% was found in a nationwide seroprevalence survey in the Netherlands, just before the first major outbreak (*5*). The present Q fever epidemic peaked right after the last data were collected for the present study. In the second half of 2009, seroprevalence for blood donors in the high-incidence area was estimated at 12.2% (*6*). The findings of the different seroprevalence studies are consistent with the view that Q fever newly emerged in the Netherlands, peaking in 2009, and that a high infection pressure has resulted in increased seroprevalence in the general population, including in pregnant women.

Whether infection during pregnancy is associated with adverse pregnancy outcomes remains uncertain. The international literature suggests this conclusion, but an analysis based on 1,174 of the 2,004 women included in the present study showed no evidence of adverse pregnancy outcome among women with antibodies to *C. burnetii* (*7–9*). Despite uncertainties surrounding the clinical significance of asymptomatic seropositivity, this study supports the recommendation that pregnant women and persons at risk for chronic Q fever, such as patients with certain cardiovascular conditions, should avoid visiting infected farms.

Acknowledgments

We thank Marianne van der Sande and Roel Coutinho for their comments on the manuscript.

This study was funded from a budget made available by the Ministry of Health, Welfare and Sport to the Center for Infectious Disease Control (project nos. V/210402 and S/210206).

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The Life and Death of *Anaplasma*

Setu Vora

This is the dark saga of *Anaplasma phagocytophilum*
That lurks inside white-footed mice and white-tailed deer.
Ferried by blood-thirsty ticks in the sticks,
This *Anaplasma* soul is passed on to its bodily incarnations—
Larva to nymph to tick.
A new generation of infected vampires is born.
Our love for the outdoors encroaches on tick territory.
A tick bite injects *Anaplasma* storming defender neutrophils,
Using MSP2 hooks to scale the walls.
Zombie neutrophils forget to defend–the aliens multiply into a morula.
Natural killer cells spew IFN-γ to inflame the fire
That reaches a feverish frenzy of cell death.
Now whether the man lives or dies, it is the end of the road for *Anaplasma*.
It has reached *Moksha* liberation from the cycles of death and rebirth.

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DOI: http://dx.doi.org/10.3201/eid1712.AD1712

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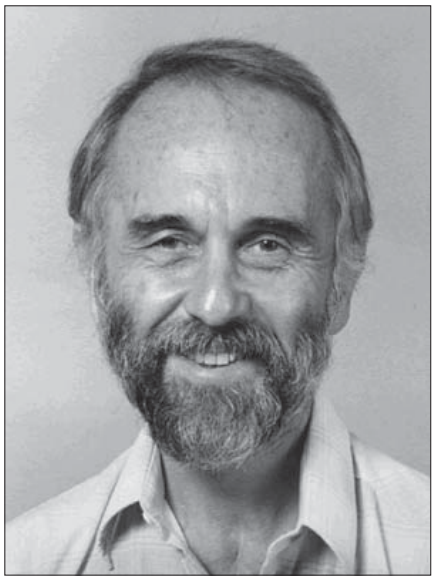
Photo Quiz

Who is this man?



Here is a clue: He founded the discipline of veterinary epidemiology and the concept of One Medicine.

Is he:

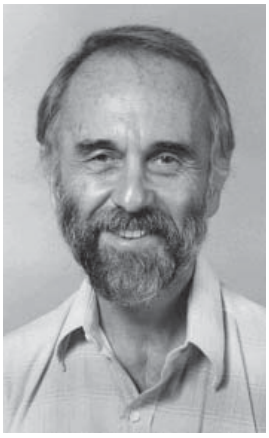
A) Martin Kaplan

B) John McFadyean

C) Karl F. Meyer

D) Calvin W. Schwabe

Decide first. Then turn the page.



Calvin W. Schwabe

Myron G. Schultz and Peter Schantz

This is a photograph of Calvin W. Schwabe (1927–2006). In a career that spanned 52 years, Schwabe made valuable contributions to a broad array of subjects, including epidemiology, zoonotic diseases, interactions of veterinary and human medicine, public health practice, livestock health in pastoral societies, ancient origins of human and veterinary medicine, and the philosophy of science. Calvin Schwabe is best known as one of the two 20th century founders of the discipline of veterinary epidemiology and the concept of One Medicine (now commonly referred to as One Health). The other founder of veterinary epidemiology and the One Medicine concept is James H. Steele, who worked at the Communicable Diseases Center (now Centers for Disease Control and Prevention) in Atlanta, Georgia.

Calvin W. Schwabe was born on March 15, 1927, in Newark, New Jersey. He graduated in 1948 with a BS in biology from Virginia Polytechnic Institute. In 1950, he received an MS in Zoology from the University of Hawaii. In 1954, Schwabe was awarded a DVM (with highest honors) from Auburn University, and in 1955, he received an MPH in tropical public health from Harvard University. His ScD in parasitology–tropical public health was awarded by Harvard in 1956.

From 1956 until 1966, Dr. Schwabe was a member of the medical and public health faculties of the American University of Beirut. There he developed a notable research program on hydatid disease and other parasitic zoonoses and founded a joint Department of Tropical Health within the medical and public health faculties and a Department of Epidemiology and Biostatistics within the School of Public Health. Beginning in 1960, he served as a consultant to the World Health Organization, developing a collaborative global program on hydatid disease research and control. During 1964–1966, he directed several parasitic disease programs at the World Health Organization headquarters in Geneva, Switzerland.

In 1966, Schwabe established the Department of Epidemiology and Preventive Medicine at the University of California, Davis, School of Veterinary Medicine, the first of its kind in the world at a veterinary school. He also established the Master of Preventive Veterinary Medicine degree program that teaches the principles and strategies of population-based medicine, disease surveillance, and prevention and control of diseases of food animals and zoonotic diseases that affect humans and animals. Today, his graduate students populate leadership positions in public health and research in many universities and government agencies.

In his masterwork, Veterinary Medicine and Human Health, first published in 1969, as well as in his many other writings, Schwabe promoted the concept of One Medicine, that is, stronger links and collaborations between the fields of human and veterinary medicine. Schwabe believed that the critical needs of humanity include combating diseases, ensuring sufficient and safe food, improving environmental quality, and creating a society in which humane values prevail. He saw the world as an ecosystem of interdependent civilizations and cultures in which human progress is inexorably linked with the co-evolution of the animal kingdom. Schwabe coined the term One Medicine, a concept previously expressed by Rudolph Virchow and William Osler, to focus attention on the commonality of human and veterinary health interests. One Medicine is the science of health and disease in which differences between humans and animals are not considered. Schwabe pointed out that most infectious diseases of humans have an animal origin. Schwabe also recognized the essential role of veterinary medicine in advancing medical research. Because human medical progress had been so dependent on veterinary studies, he urged schools of veterinary medicine to assume a much greater role in the training of scientists to conduct research to improve human health. He outlined specific reforms in the curricula of schools and colleges of

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DOI: http://dx.doi.org/10.3201/eid1712.110484

veterinary medicine that would provide for the education of medical investigators.

Disciples of Schwabe and Steele have taken the One Medicine concept and advanced it into the One Health Initiative. The One Health Initiative is a movement that seeks to forge greater collaboration between the health disciplines, following the paths created by Schwabe and Steele. For example, at the University of California, Davis, School of Veterinary Medicine, where Schwabe taught for 25 years, the Calvin Schwabe One Health Project seeks to produce a new generation of veterinarians who will be ready to lead collaborative, multidisciplinary efforts to improve global health. With expertise in biohazard events, food and water safety, vector-borne diseases, established and emerging zoonotic diseases, herd health, foreign animal risks, and public health issues such as antimicrobial drug resistance, graduates of the program will be positioned as true One Health advocates and practitioners of the future.

In his book Cattle, Priests, and Progress in Medicine, Schwabe shows the link between animals and humans in medical progress by recounting highlights in the history of medicine since ancient times. He describes the early history of humans in terms of animal cultures, focusing on the ancient Nile Valley, and points to similarities in medical knowledge between present-day so-called cattle societies in northeastern Africa and the ancient people of the Nile. He discusses the comparative healers of ancient Egypt, the founders of Greek medicine, the Arabic contribution, and the beginnings of modern medicine. Schwabe shows that over the centuries many of the most notable breakthroughs in improving human health have been closely associated with observations and experiments on animals other than humans.

In Unmentionable Cuisine, Schwabe looks at food prejudices or why we eat what we eat, why we reject certain food sources as unpalatable, and the nutritional value of all food sources. He also introduces his readers to a vast number of authentic recipes, collected from all over the world, including recipes for preparing bugs, cow eyes, fertilized duck eggs, dog stew, and armadillo and turkey testicles, that are staples in some cultures but abhorrent to persons in other cultures.

Cal Schwabe was a man of great charm and intellect. He had many colleagues and admirers throughout the world. He belonged to the small class of people who are givers rather than takers—fountains rather than wells. Cal Schwabe was a Renaissance man, not merely because of his wide-ranging scholarly interests, but because he created a real renaissance in 20th century public health by showing the way to the unity of veterinary and human medical sciences.

Dr Schultz is a senior medical officer in the Global Disease Detection Operations Center at the Centers for Disease Control and Prevention (CDC). Formerly, he was director, Parasitic Diseases Division, and worked in the Epidemiology Program Office at CDC. He has published articles on medical history in numerous medical journals and writes the text for CDC's annual contest, Great Moments in Public Health.

Dr Schantz is an adjunct professor, Department of Global Health, Rollins School of Public Health, Emory University, Atlanta. From 1974–2008 he served as an epidemiologist and veterinary officer in the Division of Parasitic Diseases, CDC. Dr Schantz is the author or co-author of more than 375 articles and book chapters.

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Q Fever in Woolsorters, Belgium

To the Editor: Recent outbreaks of Q fever in the Netherlands and the United Kingdom raised public awareness about this ubiquitous bacterial disease known for decades to circulate worldwide (*1*–*4*). The disease, which ranges from a self-recovering influenza-like illness to pneumonia and severe meningoencephalitis, myocarditis, or endocarditis, is usually transmitted from animals to humans by airborne particles derived from contaminated feces and birth products. Clinical symptoms develop in only ≈40% of infected humans (*1*). In ≈1%–2% of these persons, symptoms evolve toward the chronic form of the disease, which can be life-threatening (*5*).

Q fever seroprevalence in the general population in Europe ranges from 2.4% to >30% in some countries in the Mediterranean region (*5*). Despite improved awareness during the past 3 years and geographic proximity with the Netherlands, few human cases were reported in Belgium (14, 27, and 33 cases in 2007, 2008, and 2009, respectively) (*6*). A retrospective survey of blood donors in the Netherlands showed a seroprevalence of 2.4% before the start of outbreaks (*7*). In France, the prevalence of the disease in the Nord-Pas-de-Calais region bordering Belgium is low and accounts for only 0.5% of Q fever cases in France (*8*). Seroprevalence of the general population in Belgium, although unknown, is thus probably comparable with that in neighboring countries in absence of outbreaks and is not expected to exceed 5%.

We report a serologic, epidemiologic, and microbiological Q fever survey conducted in a scouring factory that processed wool and goat hair products in Belgium. No acute Q fever episodes were previously reported by the factory workers. Data on clinical symptoms and risk factors were obtained in face-to-face interviews, and associations with seropositivity were explored by using regression analysis. Airborne dust collected inside the factory during goat hair processing (*9*) contained 10^2–10^3 genome equivalents of *Coxiella burnetii*, the Q fever agent, per liter of air as estimated by real-time PCR (Laboratoire Service International, Lissieu, France) (online Appendix Table, wwwnc.cdc.gov/EID/article/17/12/10-1786-TA1.htm). Sheep wool processing generated less dust and resulted in a *C. burnetii* air load that never reached 10 genome equivalents/L in our analyses. No information is available about the infectivity or viability of air-suspended *C. burnetii* in the studied environment.

Q fever serologic analysis was conducted by using an in-house ELISA for serum samples from 69 workers obtained annually during 2007–2009. Results of samples from the third year were confirmed in parallel by using an immunofluorescent assay (IFA) (Focus Diagnostics, Cypress, CA, USA) in the reference laboratory in Belgium and with follow-up samples in cases of noninterpretable or suspected serologic profiles. The 3-year cumulative seroprevalence was 50.7% (Table). This high value likely results from occupational exposure inside the factory. However, one cannot exclude that characteristics such as traveling abroad, farming, or living near farm animals might account for part of the seroprevalence. Nevertheless, such characteristics could not be associated with positive serologic results in our epidemiologic analysis.

The serologic status of 2 workers (T3 and T42) was compatible with ongoing chronic Q fever as assessed by IFA and ELISA (Table). Another IFA conducted in the reference laboratory in France confirmed a serologic status compatible with chronic Q fever for worker T3 and detected anti–phase I and II IgA in this worker. However, chronic status was not confirmed for worker T42 by this laboratory (online Appendix Table).

Profiles of other workers in the cohort were characterized by increased anti–phase II IgG, IgM, or both (9/69, 13%) over a 2-year period. These profiles suggest relapse and may result from continuous exposure to the Q fever agent, which led to reinfection or repeated stimulation of the immune response. Molecular testing did not detect *C. burnetii* DNA in any blood sample, and clinical examinations did not detect endocarditis in worker T3 as analyzed by positron emission tomography and transthoracic and transesophagian echocardiography. However, infection of tissues other

Table. Serologic results for Q fever in woolsorters, Belgium, 2007–2009*

Serologic status	ELISA† (years 1–3)	IFA‡ (year 3)	Confirmed§ (years 1–3)
No. negative	29	27	34
No. nonspecifically reactive	NA	9	NA
No. with past infection	31	31	26
No. with recent or active infection	7	NA	8
No. with chronic infection	2	2	1
% Seroreactive	57.9	47.8	50.7

*IFA, immunofluorescent assay; NA, not applicable.
†Conducted on samples collected annually for 3 y. Serologic titer for chronic Q fever: phase I IgG ≥12,800 and ≥ phase II IgG; for recent or active infection, phase II IgG ≥1,600 and phase II IgM ≥800; for past infections, phase II IgG ≥1,600 and phase II IgM <800.
‡Conducted on paired samples in cases of suspected or noninterpretable initial results; conducted on single samples in all other cases. Serologic status was defined at year 3 according to the instructions of the test kit manufacturer (Focus Diagnostics, Cypress, CA, USA).
§Workers with test results above the threshold at least once over a 3-y period by ELISA and IFA. Serologic status was adjusted on the basis of the 3-y projection and retesting in the reference laboratory in France.

than the heart in this worker cannot be ruled out.

Our results indicate high seroprevalence of Q fever among workers at the scouring factory studied. Continuous exposure to the Q fever agent was the likely cause of atypical antibody responses evoking a chronic or relapsing disease in the absence of any clinical symptom. These results indicated the need to analyze paired serum samples and to rely on medical follow-up before establishing a definitive diagnosis.

Given the continuous occupational risk to which these workers are exposed, hiring of pregnant women or persons with underlying medical conditions, such as valvulopathy or immunologic depression, should be avoided. Moreover, annual serologic testing should be conducted on all exposed persons to detect any evolution toward the chronic form of the disease, which can be life-threatening. Although less dangerous than anthrax, Q fever is still a highly prevalent occupational disease that affects persons working with animal hairs in industrial environments and commonly referred to as woolsorters (*10*).

Acknowledgments

We thank the factory workers for participating in the study and S. Malbrecq for assistance with real-time PCRs.

This study was supported by the Occupational Medicine group Provikmo, the Veterinary and Agro-chemical Research Centre, the Slovak Ministry of Education (grant no. 2/0127/10), and the Slovak Academy of Sciences.

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DOI: http://dx.doi.org/10.3201/eid1712.101786

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Coxiella burnetii Infection in Roe Deer during Q Fever Epidemic, the Netherlands

To the Editor: A Q fever epidemic among humans started in the Netherlands in 2007 and peaked in 2009 (*1*). Epidemiologic evidence linked the epidemic to abortions and deliveries among *Coxiella burnetii*–infected dairy goats and dairy sheep (*1*,*2*). However, questions arose about whether *C. burnetii* infection in free-living wildlife might be another source of Q fever in humans. *C. burnetii* has a wide host range (*3*), but to our knowledge no studies had addressed its occurrence in nondomestic animals in the Netherlands (*4*).

The main objective of this study was to look for evidence of *C. burnetii* infection in carcasses of free-living roe deer (*Capreolus capreolus*) in the Netherlands, where *C. capreolus* is the most common species of wild ruminant. Additional objectives were to 1) analyze characteristics, location, and time of death of case-animals for

more information on the infection in roe deer and 2) determine the genotype of *C. burnetii* strains from roe deer and compare them with the genotype of strains from domestic animals and humans for evidence of spillover.

The sample consisted of 79 roe deer that were euthanized or found dead in 9 of the 12 provinces in the Netherlands during January 2008–May 2010. All animals had undergone postmortem examination, and tissue samples were frozen until testing. Tissues tested were lung (n = 46), spleen (n = 50), bone marrow (n = 50), liver (n = 74), and kidney (n = 75), as available. We extracted DNA by using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany). A duplex quantitative PCR targeting the IS*1111a* element was used with an internal control gene, as described (*2*). Tissues with cycle threshold (C_t) values <34 (1/case) were typed by using multilocus variable-number tandem-repeat analyses (MLVA) for 11 loci, as described (*2*,*5*); results were compared with known MLVA typing data from the Netherlands.

Of the 79 roe deer examined, 18 (23%) had positive PCR results for *C. burnetii* DNA in multiple (5/18, 28%) or single (13/18, 72%) tissues. The average C_t value was 36.30 (range 32.07–39.47). Among 29 roe deer for which all 5 tissues were tested, no single tissue was more frequently positive than others for *C. burnetii* (χ^2 = 1.07, df = 4, p = 0.9) or had lower C_t values (single factor analysis of variance, p = 0.58). These findings indicate that testing multiple tissues per individual enhances case detection.

No specific sex, age, or health effects were observed. Of 48 male deer, 10 (21%) had positive results, compared with 8 (27%) of 30 female deer (1 missing value; χ^2 = 0.35, df = 1, p = 0.55). Of 50 deer ≥1 year of age, 15 (30%) had positive results, compared with 2 (15%) of 13 deer <1 year of age (16 missing values; 2-tailed Fisher exact test, p = 0.49). Postmortem findings varied for *C. burnetii*–positive deer.

C. burnetii cases occurred in most provinces studied (6/9, 66%) and in all 3 study years. Significantly more *C. burnetii*–positive deer were observed in 2010 (13/30, 43%) than in 2008 (2/18, 11%) and 2009 (3/31, 10%) (χ^2 = 11.62, df = 2, p < 0.01). This finding might represent sample bias or indicate spatial or temporal clustering in 2010.

The *C. burnetii* genetic material found in roe deer may indicate past or ongoing infection (*6*). Although positive cases occurred in all seasons, those more likely to represent ongoing infection (multiple infected tissues and C_t values <36; n = 4) occurred in March, April, and June. Clinical Q fever in roe deer might occur more frequently in late gestation and around parturition, as in domestic ruminants (*7*,*8*). Furthermore, Q fever in wildlife might have its own sylvatic cycle (*4*,*9*). However, analogous to human cases in 2007–2010 (*1*), the pattern could also include spillover events from domestic livestock.

Tissues of 2 springtime case-animals had C_t values <34. MVLA typing of these strains yielded partial genotypes (Figure). Comparison with those of strains from domestic dairy animals or humans during 2007–2010 showed that these 2 strains from roe deer differed from the main goat- and sheep-derived strain involved in the Q fever epidemic (genotype CbNL01 [*2*]) and from other strains found (inconclusive for CbN108; Figure).

Our study confirmed that *C. burnetii* infection occurs in free-living roe deer in the Netherlands. *C. burnetii* DNA was detected in roe deer of both sexes and age groups with no particular health effect, and it was detected in animals in different provinces and in all years studied; the highest *C. burnetii* DNA loads occurred in spring and early summer. Detection of genetic material by PCR does not always imply viable infective bacteria (*6*). However, because the infectious dose of *C. burnetii* is

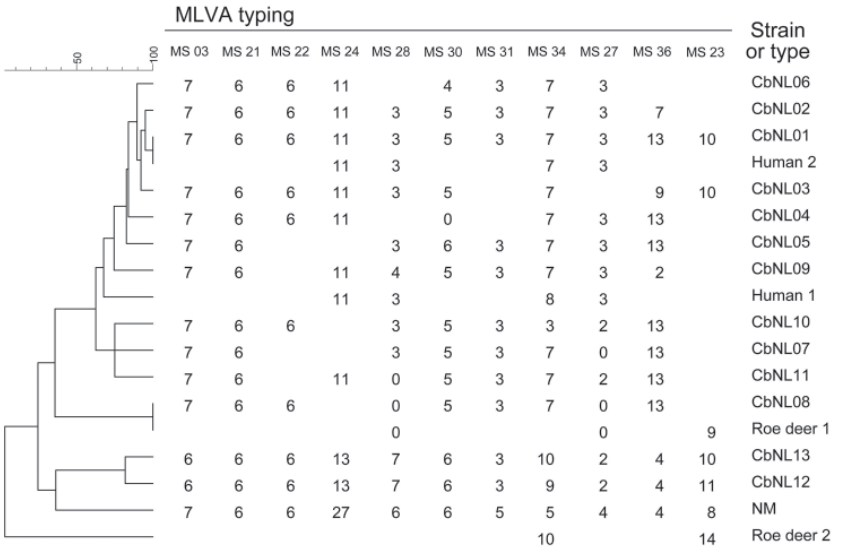

Figure. Phylogenetic tree with genotypes of *Coxiella burnetii* from goat, human, and roe deer samples from the Netherlands. Genotypes were determined on the basis of 11 multilocus variable-number tandem-repeat analyses (MLVA). The number of repeats per locus is shown; open spots indicate missing values. Roe deer 1 was an adult female found dead on March 30, 2010, in Friesland Province. Roe deer 2 was a young female deer involved in a traffic accident on April 6, 2010, in Utrecht Province. The goat and human samples have been described (*2*). Scale bar indicates genetic relatedness. Human 1, QKP 1; Human 2, QKP 2; NM, Nine Mile reference strain; MS, MiniSatellite.

low (*10*), our findings support the use of preventive hygiene measures (*4*) to minimize zoonotic risk when handling roe deer. The 2 MLVA-typed strains provided no evidence for spillover of the predominant strain involved in the Q fever epidemic in the Netherlands. More studies are required to adequately understand Q fever cycles in wildlife and their relationship with Q fever in domestic animals and humans.

Acknowledgments

We thank Natashja Beusekom-Buijs, Zorica Zivkovic, Ruby Wagensveld-van den Dikkenberg, Louis van den Boom, and Rob Buijs for logistical support and Albert de Boer for making the phylogenetic tree.

This study received financial support from the Dutch Ministry of Economic Affairs, Agriculture and Innovation; the Dutch Ministry of Health, Welfare and Sport; and the Faculty of Veterinary Medicine, Utrecht University.

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DOI: http://dx.doi.org/10.3201/eid1712.110580

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Ranavirosis in Invasive Bullfrogs, Belgium

To the Editor: Massive global declines in amphibians have been attributed to various causes, including infectious diseases such as chytridiomycosis and ranavirosis. Chytridiomycosis and ranaviral disease are international notifiable diseases because they have been listed by the World Organisation for Animal Health in its Animal Health Code.

Ranavirosis is caused by icosahedral cytoplasmic DNA viruses that belong to the family *Iridoviridae*, in particular by 4 species of *Ranavirus*: Frog Virus 3 (FV3), Bohle iridovirus, *Ambystoma tigrinum* virus, and a possible species *Rana catesbeiana* virus Z. In Europe, FV3 has been identified in several outbreaks of ranavirosis, characterized by mass deaths, notably in green frogs (*Pelophylax* sp.) in Denmark, Croatia, and the Netherlands (*1*,*2*); *Rana temporaria* and *Bufo bufo* in the United Kingdom (*3*,*4*); and *Alytes obstetricans* and *Ichthyosaura alpestris* in Spain (*5*). The invasive exotic bullfrog (*Lithobates catesbeianus*) has been introduced in several European countries and has established large breeding populations in France, Italy, Germany, Greece, and Belgium (*6*).

In addition to their direct effect on native amphibians through competition and predation, bullfrogs are thought to be carriers of chytridiomycosis (*7*,*8*) and, possibly, ranaviruses. Although mass deaths of *L. catesbeianus* tadpoles has been reported in aquaculture facilities, *L. catesbeianus* tadpoles are generally considered a subclinical reservoir of ranaviruses in the United States (*9*).

To assess the role of bullfrogs as carriers of ranaviruses in Europe, we collected 400 clinically healthy tadpoles of *L. catesbeianus* from 3 invasive bullfrog populations at Hoogstraten, Belgium (51°47′N,

4°75′E) during May–June 2010. All larvae were euthanized as part of an invasive species eradication project and stored at –20°C until further use. At necropsy, liver tissues were collected, and DNA was extracted by using the Genomic DNA Mini Kit (BIOLINE, London, UK). PCR to detect ranavirus was performed as described by Mao et al. (*10*).

Three samples showed positive results with this PCR. These samples were sequenced by using primers M4 and M5 described by Mao et al. (*10*) and blasted in GenBank. A 100% homology with the common midwife toad (*A. obstetricans*) ranavirus partial major capsid protein gene (GenBank accession no. FM213466.1) was found (*5*). Despite the low prevalence of *Ranavirus* infection (0.75%) in the bullfrog tadpoles examined, this study shows that invasive bullfrogs, a known reservoir of chytridiomycosis, are also a likely carrier of ranaviral disease in Europe.

This study was partly performed in the framework of European Union Interreg IVA project IVA-VLANED-2.31 "Invasieve exoten in Vlaanderen en Zuid-Nederland–INVEXO."

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DOI: http://dx.doi.org/10.3201/eid1712.110236

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Rift Valley and West Nile Virus Antibodies in Camels, North Africa

To the Editor: Different arboviral diseases have expanded their geographic range in recent times. Of them, Rift Valley fever, West Nile fever, and African horse sickness are of particular concern. They are endemic to sub-Saharan Africa but occasionally spread beyond this area. Trade and transport of animals and animal products, along with wildlife movements, are considered the driving factors in the spread of these pathogens.

In wide regions of Africa, 1-humped camels (*Camelus dromedarius*) are valuable livestock appreciated as a meat source and as a means for transportation of goods. Camels are susceptible to infection by Rift Valley fever virus (RVFV), West Nile virus (WNV), and African horse sickness virus (AHSV), although their epidemiologic role in these diseases is uncertain (*1*–*3*). Movements of camels across the Sahara Desert could carry these pathogens to northern Africa. To test this hypothesis, we conducted a serologic survey in 1-humped camels intercepted at different points by the Moroccan Veterinary Services in 2009. The camels were coming from the southeastern part of the Sahara Desert going to the northwest.

Serum samples were obtained in Smara-Laayoune, Dakhla, and Tata (Table). Most samples (71 of 100 total samples) were from male camels. Samples were also grouped by age of the camels (Table). RVFV antibodies were detected by using a competitive ELISA (*4*), and samples yielding positive ELISA results were confirmed by virus-neutralization test. WNV-specific antibodies were detected by ELISA (*5*), and positive results were confirmed by virus-

neutralization test. AHSV-specific antibodies were detected by using the ELISA prescribed by the World Organisation for Animal Health.

Fifteen of 100 samples were positive for RVFV-specific antibodies by competitive ELISA, all of which were confirmed by virus-neutralization test, with neutralization titers ranging from 40 to 1,280 (geometric mean titer = 229). With regard to WNV antibodies, the ELISA detected 44 positive samples and 1 doubtful sample, of which 29 were confirmed as positive by virus-neutralization test (virus-neutralization test titers ranging from 10 to 640; geometric mean titer = 20). As for AHSV antibodies, none of the samples was positive by ELISA. Prevalence data were analyzed by generalized linear model with locality (Dakhla or Smara), sex, and age as fixed factors. No differences by origin or sex were found in prevalence for WNV ($p>0.14$) but antibodies were more prevalent in camels >3 years of age ($\chi^2 = 14.04$, 3 df, $p = 0.003$). No differences in prevalence of RVFV antibodies were found by sex ($p = 0.29$), but prevalence was higher in Smara ($\chi^2 = 3.74$, $p = 0.05$) and among camels ≥6 years of age ($\chi^2 = 8.37$, df = 3, $p = 0.04$) (Table). We also examined the co-occurrence of antibodies to RVFV and WNV. Of 15 RVFV-positive samples, 12 were also positive for WNV antibodies, and 12 of 29 WNV-positive samples were also positive for RVFV ($\chi^2 = 8.37$, df = 1, $p < 0.05$).

Antibodies to 2 zoonotic arboviruses, i.e., RVFV and WNV, were present in camels moving to the northwestern part of the Sahara Desert, and antibodies to AHSV were absent in the populations examined. Despite the higher percentage of seropositivity for WNV than for RVFV, the epidemiologic consequence of RVFV-specific antibodies in this population could be higher than that for WNV antibodies. Camels can act as reservoir hosts for RVFV (*6*) but are unlikely to do so for WNV, which cycles between mosquitoes and wild birds with mammals usually being dead-end hosts. High prevalence of antibodies to RVFV in camels has been described in different sub-Saharan and Sahelian countries (*7–9*). Camels have been involved in the spread of disease in some instances (*10*). Immunity to RVFV indicates previous infection. Our results showed that seroprevalence of RVFV was higher among older than younger camels, indicating that contact could have occurred some years ago. Nevertheless, these populations should be monitored for RVFV and other arboviroses because these are known to reemerge under certain circumstances in locations where they have occurred in the past.

The results of this study support that camels moving across the Sahara have contact with RVFV and WNV, and frequently the same animals have been infected by both agents. In a particularly dry environment such as the desert, particular attention should be paid to singular wet areas such as oases. The presence of water in these areas results in an abundance of competent mosquitoes and hosts, which in turn makes these viruses likely to cycle and infect domestic animals such as camels coming to drink and rest.

Table. Results of testing of camels for virus antibodies, by location, age group, and sex of camels examined, North Africa, 2009*

Camel characteristic	No. samples	No. positive for antibody			
		RVFV	WNV	AHSV	RVFV and WNV
Origin					
Tata	2	0	0	0	0
Smara-Laayoune	58	13	20	0	11
Dakhla	40	2	9	0	1
Age group, y					
<1	18	1	1	0	1
1–2	25	0	1	0	0
3–5	7	1	3	0	1
6–10	27	6	12	0	10
>10	23	7	12	0	0
Sex					
M	71	10	15	0	7
F	29	5	14	0	5
Total	100	15	29	0	12

*RVFV, Rift Valley fever virus; WNV, West Nile virus; AHSV, African horse sickness virus.

Acknowledgments

We thank the Ministry of Agriculture of Morocco for technical assistance on sample collection.

This research was supported by Biopharma and INIA-MARM agreement no. CC08-020.

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DOI: http://dx.doi.org/10.3201/eid1712.110587

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Brucellosis, Taiwan, 2011

To the Editor: Human brucellosis is the most common zoonosis worldwide (*1–4*). The disease is transmitted to humans through the consumption of infected meat and raw dairy products from domestic livestock or by direct or indirect contact with infected animals (*1–3*). The disease is multisystemic and shows wide clinical polymorphism (*2–4*).

A 54-year-old woman reported high fever, poor appetite, epigastralgia, mild dysuria, generalized myalgia, and mild left side pain for 6 days before she sought care at and was admitted to National Taiwan University Hospital, Taipei, Taiwan. She had a history of ovarian cancer (clear cell, stage Ic), which had been treated with surgery and chemotherapy 7 years earlier at our hospital. She had traveled to many countries, most recently to Algeria and Morocco 2 months before this admission. During her stay in North Africa, she had close contact with camels, ate cheese and yogurt, and drank milk, even in the desert. Fever occurred 1 month after she returned to Taiwan.

On physical examination, her body temperature was 39.9°C, blood pressure was 97/68 mm Hg, and pulse rate was 89 beats/min. There was mild tenderness on palpation in the epigastric area. Laboratory analysis of serum specimens showed elevated levels of alanine aminotransferase (534 U/L), aspartate aminotransferase (841 U/L), and alkaline phosphatase (337 U/L) but a total bilirubin level (0.48 mg/dL) within reference limits. Renal function was within reference ranges (blood urea nitrogen 9.5 mg/dL, creatinine 0.6 mg/dL). C-reactive protein was elevated (4.59 mg/dL), but procalcitonin level was within reference range (0.13 ng/mL). The leukocyte count was 4,710 cells/mm^3, and hemoglobin was 11.4 g/dL. Serologic tests for viral hepatitis were negative for hepatitis B virus, hepatitis A virus, cytomegalovirus, and Epstein-Barr virus infections. Abdominal ultrasound indicated mild splenomegaly and no evidence of vegetation. Abdominal and pelvic computed tomography showed focal splenic infarction with splenomegaly.

Empirical ceftriaxone (1 g every 12 h) and doxycycline (100 mg every 12 h) were administered, and fever subsided 5 days later. Two aerobic culture bottles (BacT/ALERT, bioMérieux Inc., La Balme les Grottes, France) from different sets of blood cultures on the day before admission yielded unidentified gram-negative tiny bacilli after 2 days of incubation. The organism was identified as *Brucella melitensis* by the Vitek 2 GN identification system (bioMérieux Inc.) (probability of identity 99%) and was confirmed by analysis of partial 16S rRNA gene sequencing. Two primers were used: 8FPL (5′-AGAGT TTGATCCTGGCTCAG-3′) and 1492 RPL (5′-GGTTACCTTGTTACGAC TT-3′). We compared the partial sequences with published sequences in the GenBank database by using the BLASTN algorithm (www.ncbi.nlm.nih.gov/blast). The closest match was *B. melitensis* (GenBank accession no. CP001852.1; maximal identity 100%). MICs were determined by the Etest (AB Biodisk, Solna, Sweden) on Mueller-Hinton agar (BBL, Becton Dickinson, Sparks, MD, USA) supplemented with 5% sheep blood and were interpreted 2 days after incubation. The isolate was susceptible to doxycycline (MIC 0.25 μg/mL; susceptible MICs ≤2 μg/mL) but not susceptible to trimethoprim/sulfamethoxazole (MIC 1/19 μg/mL; susceptible MICs ≤0.5 μg/mL) (*5*,*6*). MIC values of tigecycline and gentamicin were 0.125 μg/mL and 2.0 μg/mL, respectively. A serum sample for examination of *Brucella* antibody by Rose Bengal test using *B. abortus* antigen (VLA Scientific,

Winchester, UK) collected 12 days after fever onset was positive.

Antimicrobial drug treatment was changed to doxycycline and gentamicin. However, low-grade fever and low back pain developed 2 days after administration of gentamicin. The back pain was attributed to muscle pain and was almost completely relieved by 2-day treatment with a nonsteroidal antiinflammatory drug. Whole-body gallium scan and spine magnetic resonance imaging suggested osteomyelitis and epidural abscess over the third and fourth lumbar spines. The patient was treated with doxycycline for 6 weeks. A liver function test 2 weeks after admission showed values within reference limits.

Cases of human brucellosis and animal sources of *Brucella* spp. have been reported from Algeria and Morocco (*7*–*10*). The most common laboratory findings in patients with brucellosis are high C-reactive protein levels and anemia (*3*,*4*). The patient had high C-reactive protein levels but procalcitonin values within reference limits at admission. Hepatic involvement of brucellosis has been reported to range from 2% to 25% (*3*). The patient also had acute anicteric hepatitis, and serologic test results were negative for all hepatotropic viruses. The isolate from this patient exhibited high MICs for trimethoprim/sulfamethoxazole, a finding rarely reported (*5*,*6*). The low MIC value of tigecycline suggests the potential role of this agent for the treatment of brucellosis.

This report confirms brucellosis in Taiwan. Brucellosis could become an emerging problem in this country, particularly given the frequency of travel between Taiwan and areas where brucellosis is endemic.

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DOI: http://dx.doi.org/10.3201/eid1712.110739

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Hemoptysis Associated with Leptospirosis Acquired in Hawaii, USA

To the Editor: Severe pulmonary hemorrhagic syndrome (SPHS) is a serious complication of *Leptospira* infection, a globally widespread bacterial zoonosis that is increasing in incidence in tropical and subtropical regions. Despite decreasing endemicity of leptospirosis in industrialized regions, the disease is reemerging in travelers and recreationalists. Leptospirosis is an appreciable attributable cause of travel-related infections (typically associated with waterborne activities), and the incidence of travel-related leptospirosis is proportionally higher than that for endemic leptospirosis. Disease risk epidemiology has shifted concomitantly from occupational to recreational in industrialized countries (*1*–*3*). Risk factors include urbanization, climatic changes, and agricultural practices (*1*–*3*).

Clinical features of leptospirosis range from asymptomatic infections and undifferentiated febrile syndromes to multiorgan dysfunction and death. Weil syndrome (i.e., severe leptospirosis) is characterized by renal and hepatic dysfunction, hyperbilirubinemia (disproportionate to transaminase elevation), and hemorrhage (pulmonary, gastrointestinal, or intracranial). Pulmonary

involvement predicts poor clinical outcome: the case-fatality rate for persons with SPHS is >50% (*4*–*6*). Most US leptospirosis cases are reported from Hawaii, where the annual incidence is 1.63 cases/100,000 person-years (*1*). Leptospirosis is endemic to Hawaii; however, SPHS is uncommonly reported (*7*).

We treated a 21-year-old active-duty Navy sailor for SPHS after he had a 5-day port visit in Hawaii, during which he went cliff-diving in Maunawili Falls. Afterwards, he returned to California and 2 days later sought medical attention in an outpatient clinic. Pharyngitis was diagnosed and azithromycin prescribed.

Two days later, he was hospitalized with fever, chills, pharyngitis, dyspnea, nonproductive cough, headache, myalgias, hemoptysis, epistaxis, diarrhea, nausea, emesis, meningismus, and a lower-extremity rash. Vital signs included temperature 38.3°C, pulse 132 beats/min, blood pressure 128/72 mm Hg, and oxygen saturation 98% on room air. Physical examination noted conjunctival suffusion, epistaxis, posterior cervical and inguinal lymphadenopathy, bilaterally diminished breath sounds, rhonchi and crackles, bloody cough, tachycardia, hepatosplenomegaly, and a macular rash over the lower extremities. Laboratory studies were noteworthy for reference range leukocyte count, hemoglobin (11.8 g/dL), platelets (102 × 10^3/mm^3), creatine phosphokinase (1,719 IU/L), sodium (128 mmol/L), bicarbonate (23 mmol/L), blood-urea-nitrogen (29 mg/dL), creatinine (2.2 mg/dL), aspartate aminotransferase (171 U/L), alanine aminotransferase (147 U/L), bilirubin (1.9 mg/dL), and urinalysis (7 erythrocytes and 9 leukocytes/high-power field). Chest radiography showed multilobar bilateral opacities, and cerebrospinal fluid (CSF) showed mild pleocytosis. The patient received intravenous acyclovir, ceftriaxone, and vancomycin and continued azithromycin.

At hospital admission, the patient experienced respiratory decompensation requiring endotracheal intubation and mechanical ventilation. Results of blood, urine, and CSF cultures and CFS PCR (herpes simplex virus and enterovirus) remained negative at 48 hours, prompting discontinuation of vancomycin and acyclovir. Serologic test results for HIV, dengue fever virus, mycoplasma, and *Chlamydophila* and *Rickettsia* species were negative. Nasopharyngeal influenza PCR, *Streptococcus pneumoniae* and *Legionella* spp. urinary antigen test results, and hepatitis panel results were negative. *Leptospira* spp. test results by culture, PCR, and serologic testing (ELISA and microscopic agglutination testing) were negative.

Given an elevated suspicion for leptospirosis, ceftriaxone and azithromycin were continued through hospital day 7. The patient rapidly improved, was extubated after 48 hours, and was discharged on hospital day 7 with a 7-day course of oral doxycycline. A convalescent-phase serum sample had a titer of 1,600 against *L. interrogans* serovar Copenhageni, as determined by microscopic agglutination testing.

SPHS is associated with infection with *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae (*8*), and the syndrome has been identified in diverse settings, including the Andaman Islands. Recent outbreaks have occurred in Nicaragua and Brazil (*4*,*5*). SPHS pathogenesis remains poorly understood. In animal models and human autopsy studies, immunoglobulin and complement are deposited along alveolar septa without a clear cause-and-effect relationship (*9*). Bacterial virulence factors are postulated but unproven. Leptospires induce endothelial activation and pulmonary endothelial and epithelial injury (possibly by immune-complex deposition and/or autoimmune mechanisms) (*9*). Pulmonary histopathology demonstrates a paucity of leptospires, and antigen levels do not correlate with injury severity (*9*). Steroids, intravenous immunoglobulin, and plasma exchange are of unproven benefit but have been reported to be useful (*9*). Genetically determined responses include associations with human leukocyte antigen–DQ6 and hyperactive Toll-like receptor 4–dependent immunity.

Diagnosis of leptospirosis may have been delayed for this patient because of early empiric azithromycin administration. Azithromycin is increasingly recognized as a potentially effective treatment that is comparable or superior to doxycycline (*10*) and thus warrants testing in human trials. Given the paucity of SPHS in leptospirosis case reports from Hawaii, potential sentinel cases may be harbingers of more virulent disease expression. A potential parallel is the emergence of SPHS in Salvador, Brazil, in 2003. No cases were identified before 2003, but 47 cases and a 75% case-fatality rate were identified during 2003–2005 (*4*,*5*). The entrenched active surveillance and physician awareness of SPHS in neighboring Brazilian cities suggests it is unlikely that this observation stemmed from prior underrecognition of disease; instead, it suggests de novo emergence.

Clinicians should consider leptospirosis (SPHS) in patients with acute fever accompanied by hemoptysis after travel to Hawaii, and leptospirosis should be suspected in any traveler with undifferentiated febrile illness, especially those reporting water exposures (*2*). Vigilant national surveillance is needed to determine further emergence of SPHS in Hawaii.

This study was supported in part by US Public Health Service grants D43TW007120 and K24AI068903 (to J.M.V.).

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DOI: http://dx.doi.org/10.3201/eid1712.110700

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Salmonella enterica in Pinnipeds, Chile

To the Editor: Several wildlife-associated zoonotic agents have played a major role in the emergence of diseases in humans (*1*). However, diseases can also emerge in wildlife as a result of human activities, such as contamination of the marine environment and its fauna by the disposal of nontreated human sewage. *Salmonella enterica* is among the agents identified as causing infection in various marine birds and mammals, including pinnipeds, from different geographic regions (*2*–*4*).

The objective of our study was to determine whether *S. enterica* infection occurs in pinnipeds from the Chilean coast. During August–December 2010, we obtained samples from 13 South American sea lions (*Otaria flavescens*) that the sanitary authority found malnourished and stranded at the northern Chilean beaches of Antofagasta (23°40′S, 70°24′W) and Los Vilos (31°54′S, 71°30′W) (Table). The pinnipeds showed no clinical signs or symptoms of disease; however, rectal swab samples were obtained during their stay for rehabilitation at the Buin Marino facilities (Santiago, Chile). After the animals recovered, they were released to their original habitat.

The swab samples were placed in Cary-Blair transport medium (COPAN, Murrieta, CA, USA) for shipment to the laboratory (Laboratory of Infectious Diseases, University of Chile, Santiago). To isolate bacteria, we placed the swab samples into 5 mL of buffered peptone water (Difco APT broth; Becton Dickinson, Franklin Lakes, NJ, USA), incubated them for 24 h at 42°C with agitation, and then aliquots of the suspension were transferred into the following media: modified semisolid Rappaport-Vassiliadis basal medium (Oxoid, São Paulo, Brazil) with novobiocin (20 μg/mL), selenite cysteine broth base (Oxoid), and xylose lysine desoxycholate agar (Difco XLD; Becton Dickinson). After the aliquots were incubated at 37°C for 24–48 h, we identified suspected colonies by using biochemical tests and *invA* gene detection by PCR (*5*). Results showed that 2 of the 13 animals were infected with *S. enterica* strains, which were serotyped as *S. enterica* serotype Newport and *S. enterica* serotype Havana (Table), according to the Kauffmann-White scheme (*6*). Testing showed that the strains were susceptible to the following antimicrobial drugs: ampicillin, chloramphenicol, tetracycline, amoxicillin/clavulanic acid, trimethoprim/sulfamethoxazole, cefotaxime, nalidixic acid, nitrofurantoin, ciprofloxacin, ceftazidime, and cefoxitin (*7*).

Our results confirm *S. enterica* infection in pinnipeds from Chile and,

Table. Characteristics of South American sea lions (*Otaria flavescens*) tested for infection with *Salmonella* spp., Chilean coast, August–December 2010*

Identification no.	Sex	Age†	Source, city in Chile	*S. enterica* serotype isolated
p070810	F	Juvenile	Antofagasta	ND
p240810	F	Juvenile	Los Vilos	ND
p260810	F	Juvenile	Los Vilos	ND
p090910–1	M	Pup	Antofagasta	Havana
p090910–2	F	Pup	Antofagasta	ND
p140910	M	Pup	Antofagasta	Newport
p011210–1	F	Pup	Antofagasta	ND
p011210–2	F	Pup	Antofagasta	ND
p011210–3	F	Pup	Antofagasta	ND
p011210–4	F	Pup	Antofagasta	ND
p011210–5	F	Pup	Antofagasta	ND
p011210–6	F	Pup	Antofagasta	ND
p011210–7	F	Pup	Antofagasta	ND

*ND, no detection.
†Juvenile, animal 1–5 years of age; pup, animal <1 year of age.

more broadly, the South American coast and contrast with previous unsuccessful attempts to detect *Salmonella* spp. in pinnipeds from Valdivia, 2,200 km to the south (*8*). This finding suggests geographic variability in the epidemiology of infection; however, this possibility must be confirmed in additional studies with more samples and additional regions.

S. enterica is an endemic bacterium in Chile that causes infection in humans and domestic animals. The Chilean sanitary authority includes *S. enterica* infection among the list of notifiable diseases, but surveillance is not conducted for *S. enterica* in wildlife. However, consideration should be given to changing this situation, given a report suggesting *S. enterica* as a priority for active surveillance (*9*). In addition, *S. enterica* serotypes Newport and Havana have been detected in Chile's human population (*10*), strengthening the necessity for official support for initiatives addressing the need to elucidate the epidemiology of *Salmonella* in aquatic animals.

This work was supported by a grant from the International Society for Infectious Diseases.

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DOI: http://dx.doi.org/10.3201/eid1712.111103

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Changing Perception of Avian Influenza Risk, Hong Kong, 2006–2010

To the Editor: Since 1997, routine surveillance has demonstrated periodic reemergence of influenza A (H5N1) viruses (avian influenza) in retail markets in Hong Kong, People's Republic of China (*1*,*2*), leading to stepped implementation (progressively implementing more measures over time) of measures to reduce human exposure to influenza subtype H5N1. From 2006 through November 2008, progressive importation and farm restrictions and curtailed retail capacity cut Hong's live poultry supply in half, from 40,000 to <20,000 chickens daily (*3*,*4*).

To determine whether the decline in the Hong Kong live poultry supply was paralleled by declines in avian influenza risk perceptions and protective hygiene behavior, we conducted a telephone survey. During December 2005–March 2006, we recruited 1,760 adults ≥17 years of age. We randomly called households and then interviewed 1 adult (randomly selected by Kish grid) within each household (*5*,*6*). Ordering by age and starting from the oldest eligible member in the household, 1 selected member was then invited to participate in the survey. Of 1,613 (92%) respondents consenting to follow-up survey, 680 (42%) were resurveyed during July–August 2010. The same items were used in both surveys to measure avian influenza risk perceptions, personal live poultry exposures, and hygiene practices.

Overall, 461 (68%) respondents completed the initial (2006) and follow-up (2010) surveys. Compared with respondents lost to follow-up, these 461 respondents were more likely to be female, slightly older, and married; they were comparable with the general population (*7*), except more respondents were older (data not shown).

Respondents perceived that their likelihood of contracting avian influenza was the same in 2010 as in 2006, but they reported worrying less about contracting avian influenza and risks from buying live poultry in 2010 than in 2006 (Table). When categorized into "unchanged," "increasing," and "declining" in 2010 relative to 2006, these groups were comparable demographically, except younger respondents more often perceived declining likelihood of contracting avian influenza (odds ratio [OR] 2.30, 95% confidence interval [CI] 1.25–4.24 for those 18–34 years of age); declining worry about contracting avian influenza (OR 2.01, 95% CI 1.10–3.66 for those 18–34 years of age; OR 1.87, 95% CI 1.13–3.09 for those 35–54 years of age), and declining risk from buying live poultry (OR 2.31, 95% CI 1.33–4.01 for those 35–54 years of age); respondents who had completed secondary education were more likely to report declining worry about contracting avian influenza (OR 1.90, 95% CI 1.09–3.31).

The percentage of respondents who reported household buying of live poultry declined from 73% in 2006 to 41% in 2010 (Table); 22% of nonbuying households in 2006 were again buying in 2010, and 52% of those buying in 2006 had stopped buying in 2010. After adjustment for demographics, perceived increased risk from buying was associated

Table. Changes in perception of risk for avian influenza, live poultry exposure, and hygiene practices, Hong Kong, 2006–2010*

Perception or practice	2006 survey	2010 survey	Differences, p value
Risk perception			
Perceived likelihood (likely/very likely/certain)	18	14	0.201†
Worry (worry a bit/a lot/all the time)	26	21	<0.001†
Perceived risk from buying live poultry (somewhat agree/agree/strongly agree)	42	31	<0.001†
Live poultry exposure			
Buying live poultry (yes)	73	41	0.009‡
Frequency of purchase (at least monthly or more frequently)	50	15	<0.001†
Purchase rate among buyers (chickens/household/y)	14.4	11.4	
Frequency of touch among buyers (sometimes/usually/always)	8	6	0.326§
Averaged touch rate among buyers	0.05	0.05	
Averaged exposure among buyers (exposure/household/y)	0.72	0.57	0.011†
Personal hygiene practices			
Frequency of washing hands (at least hourly)	49	44	0.023†
Covering mouth when sneezing or coughing (usually/always)	91	85	0.003†
Washing hands after sneezing, coughing, or touching nose (usually/always)	72	75	0.027†
Using liquid soap when washing hands (usually/always)	63	74	<0.001†
Using serving utensils when dining with others (usually/always)	40	62	<0.001†

*Total population surveyed = 461. Values are % unless otherwise stated.
†Wilcoxon signed rank test.
‡McNemar test.
§χ^2 test.

with not buying live poultry in 2010 (OR 0.34, 95% CI 0.19–0.60).

In contrast, rates of touching poultry during buying (5%) were unchanged (Table). Using a standardized estimate (*5*), we determined that purchasing households bought on average 11.4 live chickens/household/year in 2010 versus 14.4 in 2006 (Table). Purchase rate × touch rate gave an estimated average of 0.57 exposures/household/year in 2010, a 21% decline from 0.72 exposures/household/year in 2006 (p = 0.011) (Table).

Substantial improvement was noted for most personal hygiene practices, except frequencies for daily handwashing and covering the mouth when sneezing or coughing were each lower in 2010 than in 2006 (Table). Changed hygiene practices were independent of demographic factors except that male respondents more often reported less covering of the mouth when sneezing or coughing (OR 1.60, 95% CI 1.00–2.56) and less use of liquid soap for handwashing (OR 1.64, 95% CI 1.04–2.60); immigrants were more likely to have reduced daily handwashing frequency (OR 1.58, 95% CI 1.04–2.41). Only perceived declining worry about contracting avian influenza was significantly associated with declining frequency of handwashing after sneezing, coughing, or touching the nose (OR 1.61, 95% CI 1.04–2.47).

The 21% decline in exposure from less buying, but not touching, of live poultry suggests that limiting poultry availability, but not health education efforts, was responsible. Perceptions of avian influenza risk and worry also mostly declined, as did frequency of some personal hygiene practices, including handwashing, particularly among younger male and immigrant respondents. Although our previous studies suggest that public health education might have contributed to an ≈43% reduction in rate of touching when buying live poultry in Hong Kong from 2004 to 2006 (*5*,*6*), the prolonged warning that a future pandemic is likely to be sparked by influenza A (H5N1) viruses is likely to cause pandemic fatigue in the public and therefore would not change their perception of avian influenza risk and associated protective behavior. As exposure risk has declined (as a result of government policy), so has perceived infection risk also declined, paradoxically increasing population vulnerability to other influenza viruses through reductions in preventive hygiene behavior.

Acknowledgments

We thank Ella Ho for coordinating the data collection of the 2006 survey and HKU Public Opinion Programme for assistance in administering the 2010 follow-up survey.

The 2006 and 2010 surveys were supported by the Research Fund for the Control of Infectious Disease, Food and Health Bureau, Government of Hong Kong Special Administrative Region (grant nos. 02040172 and 09080732, respectively). This work was also supported by the Area of Excellence Scheme of the Hong Kong University Grants Committee (grant no. AoE/M-12/06).

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DOI: http://dx.doi.org/10.3201/eid1712.110298

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Bat Rabies and Human Postexposure Prophylaxis, New York, USA

To the Editor: The New York State Department of Health (NYSDOH) assessed the effect of terrestrial rabies on human postexposure prophylaxis (PEP) during the first 10-year period of computerized reporting (1993–2002) (*1*). We assessed the effect of bat rabies during the same period, when guidelines for PEP were changing (*2*). NYSDOH developed local health department and public education programs to reduce bat encounters, increase testing of bats involved in

encounters, and improve reporting of bat encounters (*3*).

Use of PEP for all New York counties was included in the study; PEP in New York, New York, and from other states was excluded. Analyses of reasonable probability exposures, age, and sex were conducted for 1998–2002. Population data from 2000 (www.factfinder.census.gov) were used to calculate rates. Epi Info (Centers for Disease Control and Prevention, Atlanta, GA, USA) and SAS (SAS Institute, Cary, NC, USA) were used for χ^2 statistical analyses. We considered p values ≤ 0.05 significant.

During 1993–2002, a total of 6,320 bat-associated rabies exposure incidents and 11,365 PEPs were reported (Table). Incidents increased 7-fold, and use of PEP increased 9-fold. More than three quarters of all incidents were reported in June, July, and August. The number of persons who received PEP per incident ranged from 1 to 40, with an increase in mean from 1.3 to 1.8.

Nonbite exposures (scratch, direct and indirect contact with saliva, reasonable probability of exposure, and other unspecified exposures) accounted for 88% of PEP, with a significant increasing trend. During 1998–2002, "reasonable probability" and "bat in the bedroom" accounted for 79% and 53% of bat-associated PEP, respectively.

Table. Bat-associated rabies exposure incidents, PEP, and bats received for testing, New York, USA, 1993–2002*

	No. (%)										
Incidence data	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	Total
Total incidents†	137 (100.0)	116 (100.0)	290 (100.0)	527 (100.0)	672 (100.0)	764 (100.0)	964 (100.0)	924 (100.0)	973 (100.0)	953 (100.0)	6,320 (100.0)
Bats tested†	42 (30.7)	43 (37.1)	57 (19.7)	111 (21.1)	111 (16.5)	116 (15.2)	112 (11.6)	110 (11.9)	113 (11.6)	124 (13.0)	939 (14.9)
Bats not tested	95 (69.3)	73 (62.9)	233 (80.3)	416 (78.9)	561 (83.5)	648 (84.8)	852 (88.4)	814 (88.1)	860 (88.4)	829 (87.0)	5,381 (85.1)
Total PEP	184	131	440	968	1,326	1,512	1,755	1,641	1,735	1,673	11,365
Average/incident	1.3	1.1	1.5	1.8	2.0	2.0	1.8	1.8	1.8	1.8	1.8
Bat rabies status											
Positive†	49 (26.6)	17 (13.0)	34 (7.7)	74 (7.6)	88 (6.6)	111 (7.3)	110 (6.3)	110 (6.7)	99 (5.7)	98 (5.9)	790 (7.0)
Negative	6 (3.3)	24 (18.3)	17 (3.9)	37 (3.8)	36 (2.7)	33 (2.2)	22 (1.3)	32 (2.0)	21 (1.2)	23 (1.4)	251 (2.2)
Untestable	18 (9.8)	10 (7.6)	34 (7.7)	110 (11.4)	89 (6.7)	69 (4.6)	74 (4.2)	76 (4.6)	114 (6.6)	115 (6.9)	709 (6.2)
Not tested	111 (60.3)	80 (61.1)	355 (80.7)	747 (77.2)	1,113 (83.9)	1,299 (85.9)	1,549 (88.3)	1,423 (86.7)	1,501 (86.5)	1,437 (85.9)	9,615 (84.6)
Bat exposure type											
Bite†	43 (23.4)	71 (54.2)	124 (28.2)	160 (16.5)	188 (14.2)	134 (8.9)	186 (10.6)	163 (9.9)	150 (8.6)	145 (8.7)	1,364 (12.0)
Scratch or saliva contact	73 (39.7)	50 (38.2)	102 (23.2)	259 (26.8)	429 (32.4)	168 (11.1)	147 (8.4)	131 (8.0)	152 (8.8)	126 (7.5)	1,637 (14.4)
Reasonable probability	NA	NA	NA	NA	NA	1,145 (75.7)	1,365 (77.8)	1,299 (79.2)	1,382 (79.7)	1,367 (81.7)	6,558 (57.7)
Other	68 (37.0)	10 (7.6)	214 (48.6)	549 (56.7)	709 (53.5)	65 (4.3)	57 (3.2)	48 (2.9)	51 (2.9)	35 (2.1)	1,806 (15.9)
Bats received for rabies testing											
Total	420 (100.0)	419 (100.0)	386 (100.0)	764 (100.0)	741 (100.0)	868 (100.0)	923 (100.0)	1,220 (100.0)	1,421 (100.0)	1,487 (100.0)	8,649 (100.0)
By bat rabies status											
Positive‡	20 (4.8)	17 (4.1)	19 (4.9)	23 (3.0)	28 (3.8)	38 (4.4)	34 (3.7)	36 (3.0)	45 (3.2)	34 (2.3)	294 (3.4)
Negative	342 (81.4)	375 (89.5)	315 (81.6)	653 (85.5)	667 (90.0)	769 (88.6)	833 (90.2)	1,112 (91.1)	1,300 (91.5)	1,363 (91.7)	7,729 (89.4)
Untestable	58 (13.8)	27 (6.4)	52 (13.5)	88 (11.5)	46 (6.2)	61 (7.0)	56 (6.1)	72 (5.9)	76 (5.3)	90 (6.1)	626 (7.2)
By exposure type											
Bite†	77 (18.3)	106 (25.3)	103 (26.7)	118 (15.4)	98 (13.2)	139 (16.0)	141 (15.3)	131 (10.7)	131 (9.2)	148 (10.0)	1,192 (13.8)
Nonbite§	343 (81.7)	313 (74.7)	283 (73.3)	646 (84.6)	643 (86.8)	729 (84.0)	782 (84.7)	1,089 (89.3)	1,290 (90.8)	1,339 (90.0)	7,457 (86.2)

*PEP, human rabies postexposure prophylaxis; NA, data not collected for this time period.
†Test for trend, p<0.0001.
‡Test for trend, p<0.005.
§Includes scratch, saliva, and reasonable probability.

Rabies-positive bats accounted for 7% of PEP, with a significant decreasing trend. Untested bats accounted for 89% of the increase in PEP. Three quarters of PEP was administered for nonbite exposures to untested bats.

Of 8,244 PEPs since 1998, a total of 4,384 (53.2%) were for female patients, for whom the age-adjusted rate was 15.6 PEPs per 100,000 persons per year, compared with 14.3 for male patients ($p = 0.0003$). Persons ≤14 years of age received PEP twice as often as did persons ≥15 years of age. More persons ≤14 years of age (86%) received PEP for reasonable probability of exposure than did persons ≥15 years of age (76%) ($p = 0.001$).

During the study period, a total of 8,649 bats were received for rabies testing with concerns reported at the time of submission about the possibility of human contact, although further epidemiologic review would not classify them all as exposure incidents (Table). The number of bats submitted increased almost 4-fold. Similar to the seasonal pattern of exposure incidents, three quarters of bats were received for testing during June through August, with most (40%) received during August. Three percent of submitted bats were rabies positive, 89% were rabies negative, and 7% were unsatisfactory for testing. There was a significant decreasing trend in the proportion of tested bats that were rabid.

Bats for which nonbite contacts were reported accounted for 86% of those received for testing and 93% of the increase in bats received. There was a significant increasing trend in the proportion of bats reported with nonbite contacts.

For bats not tested, encounters resulted in an average of 1.8 PEP per incident, at an estimated cost for biologics of $10.9 million based on an average of $1,136 per PEP (*4*). Capturing and testing the 7,729 rabies-negative bats precluded the need for ≈14,000 PEP at an estimated savings for biologics of $15.8 million.

Encounters with bats are fairly common in New York State. Eidson et al. reported that one-third of survey respondents reported a bat in their house, including 10% who had seen a bat in their bedroom (*3*). Less than 20% knew a bat found indoors should not be released until rabies exposure is ruled out.

Similar rabies patterns have been reported from other states and Canada. In Massachusetts the number of bats submitted for rabies testing increased substantially during 1985–2009 (*5*). South Carolina reported an increase in administration of bat-associated PEP during the same period as this study (*6*). The seasonal pattern of bat encounters in New York was similar to those reported in Colorado (*7*), Minnesota (*8*), and Quebec, Canada (*9*), reflecting the pattern of bat hibernation and reproduction (*10*). As in New York, "bat in bedroom" was the most common exposure in Minnesota and 1 of the more frequent exposures in Colorado and Quebec.

In conclusion, during PEP guideline revision, which expanded the recommendation for PEP beyond persons with known bite exposures, numbers of bats submitted for testing, reported exposure incidents, and instances of PEP administration increased significantly in New York. Although the cause of the increases cannot be definitively determined, the increases were consistent with changes in guidelines and public education. With 89% of bats confirmed as rabies negative that were submitted because of possible human contact, improving bat capture and testing should be considered as a strategy for excluding rabies exposures and thus reducing the number of PEPs administered.

Acknowledgments

We thank local health department and NYSDOH staff for reporting data, including the former Zoonoses Program and the Wadsworth Center's Rabies Laboratory under the former direction of Charles Trimarchi.

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DOI: http://dx.doi.org/10.3201/eid1712.102024

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Chrysosporium sp. Infection in Eastern Massasauga Rattlesnakes

To the Editor: During 2008, the ninth year of a long-term biologic monitoring program, 3 eastern massasauga rattlesnakes (*Sistrurus catenatus catenatus*) with severe facial swelling and disfiguration died within 3 weeks after discovery near Carlyle, Illinois, USA. In spring 2010, a similar syndrome was diagnosed in a fourth massasauga; this snake continues to be treated with thermal and nutritional support and antifungal therapy. A keratinophilic fungal infection caused by *Chrysosporium* sp. was diagnosed after physical examination, histopathologic analysis, and PCR in all 4 snakes. The prevalence of clinical signs consistent with *Chrysosporium* sp. infection during 2000–2007 was 0.0%, and prevalence of *Chrysosporium* sp.–associated disease was 4.4% (95% confidence interval [CI] 1.1%–13.2%) for 2008 and 1.8% (95% CI 0.0%–11.1%) for 2010.

Clinical and gross necropsy abnormalities were limited to the heads of affected animals. In each case, a unilateral subcutaneous swelling completely obstructed the nasolabial pits (Figure, panel A). In the most severely affected snake, swelling extended to the cranial aspect of the orbit and maxillary fang (Figure, panel B). Notable histologic lesions were restricted to skin, gingiva, and deeper tissues of the head and cervical region and consisted of cutaneous ulcers with granulomas in deeper tissues (Figure, panel C). Ulcers had thick adherent serocellular crusts and were delineated by small dermal accumulations of heterophils and fewer macrophages. Crusts contained numerous 4–6-μm diameter right-angle branching fungal hyphae with terminal structures consistent with spores. In 1 snake, infection was associated with retained devitalized layers of epidermis consistent with dysecdysis. In the same snake, the eye and ventral periocular tissues were effaced by inflammation, but the spectacle and a small fragment of cornea remained; the corneal remnant contained few fungal hyphae.

In all snakes, in addition to deep cutaneous ulceration, the dermis, hypodermis and skeletal muscle of the maxillary and or mandibular region contained multiple granulomas, centered on variable numbers of fungal hyphae (Figure, panel D). In 1 snake, similar granulomas were also observed in maxillary gingival submucosa and subjacent maxillary bone.

Five frozen skin biopsy samples from 4 snakes were thawed and plated on Sabaroud agar; however, no fungal growth was recovered. Genomic DNA was extracted from tissue, and PCR was performed by using 2 sets of fungus-directed rRNA gene primers. The DNA was sequenced, and the 4 amplicons showed >99% homology with *C. ophiodiicola* (GenBank accession no. EU715819.1).

Fungal pathogens have been increasingly associated with free-ranging epidemics in wildlife, including the well-known effects of

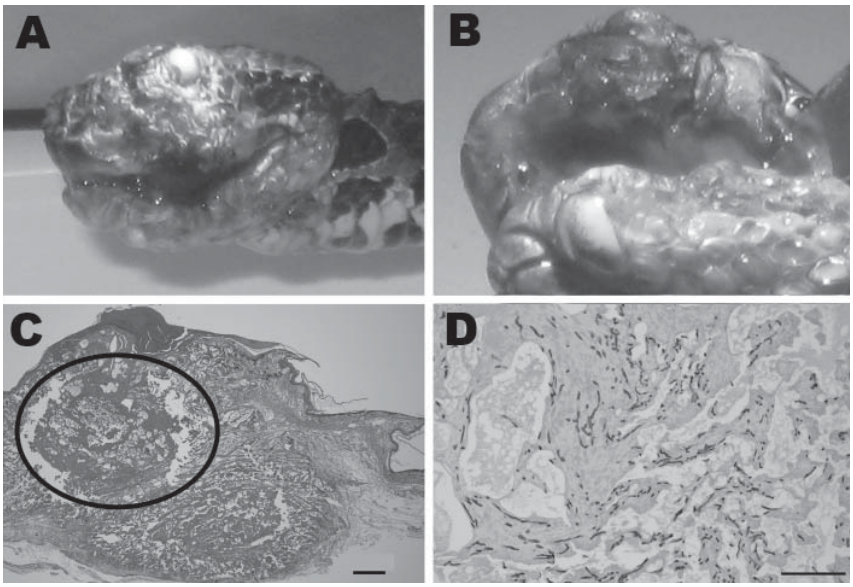

Figure. *Chrysosporium* sp. fungal infection in eastern massasagauga rattlesnake (*Sistrurus catenatus catenatus*). A) Facial dermatitis and cellulitis caused by *Chrysosporium* sp. infection in rattlesnake from Carlyle, Illinois, USA; B) close-up showing maxillary fang destruction. C) Maxillary dermal and subcutaneous fungal granuloma (circled area). Hematoxylin and eosin stain, original magnification ×2, scale bar = 500 μm. D) Granuloma center with large numbers of fungal hyphae. Grocott methenamine silver stain, original magnification ×10, scale bar = 100 μm. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/17/12/11-0240-F1.htm).

Batrachochytrium dendrobatidis on frog populations globally (*1*) and white-nose syndrome in bats (*2*). Both of these diseases cause widespread and ongoing deaths in these populations that seriously threaten biodiversity across the United States (*1,2*). Furthermore, the emergence of keratinophilic fungi, *Chrysosporium* anamorph *Nannizziopsis vriesii*, caused fatal disease in captive bearded dragons within the past decade (*3,4*). Keratinophilic fungi have received considerable interest recently because of pulmonary or dermatologic disease caused in immunocompromised humans and prevalence in hospitals (*5,6*).

The *Chrysosporium* sp. fungi recently identified in the snakes from the Carlyle Lake area is molecularly related to a *Chrysosporium* sp. from diseased skin in a captive snake (*7*). Fungal diseases in reptiles are commonly secondary or opportunistic pathogens. However, *Chrysosporium* anamorph *Nannizziopsis vriesii* (*3,4,8*) and the *Chrysosporium* sp. reported here in massasaugas are occurring in animals as primary pathogens.

We describe evidence of *Chrysosporium* sp. causing death in free-ranging snakes. To our knowledge, this is the first reported occurrence of any similar disease syndrome in this population. Before 2008, these clinical signs had not been witnessed during radiotelemetry and mark-recapture studies or in health monitoring studies (*9,10*). More intensive health monitoring programs are warranted at this site, as well as across this species' range. Whether this disease represents isolated emerging incidents in Illinois or indicates more widespread concern for this species, as has been documented in bats with white-nose syndrome (*2*), is unclear.

Origin, transmission, and treatment of *Chrysosporium* sp. are unknown. The eastern massasaugas in this investigation carrying the fungal infection were from 2 discontiguous sites; therefore, direct transmission is not necessary. The occurrence across different locations and in different years suggests the organism is present in the environment, and histopathologic results indicative of primary skin involvement were consistent with environmental acquisition of infection. Potential causes for the development of lesions specifically to the head include primary trauma, high local environmental load, or disruption of the normal skin defense mechanisms.

This fungal pathogen has serious long-term implications for this population of endangered snakes. There is no indication that hikers in this environment are at risk, but continued monitoring of human and wildlife health is essential to assess environmental and zoonotic disease risks. Furthermore, if human behavior can alter disease transmission (e.g., through hiking behaviors), disease prevention at Carlyle Lake, which hosts >1 million visitors annually, will likely be unsuccessful.

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DOI: http://dx.doi.org/10.3201/eid1712.110240

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Cutaneous Myiasis in Traveler Returning from Ethiopia

To the Editor: Myiasis is an infestation of human tissue by the larval stage of flies of the order Diptera. There are 3 clinical manifestations of myiasis: localized furuncular myiasis typically caused by *Dermatobia hominis*, *Cordylobia anthropophaga*, *Wohlfahrtia vigil*, and *Cuterebra* spp.; creeping dermal myiasis caused by *Gasterophilus* spp. and *Hypoderma* spp.; and wound and body cavity myiasis caused by *Cochliomyia hominivorax*, *Chrysomya bezziana*, and *Wohlfahrtia magnifica* (*1*). The Tumbu fly (*C. anthropophaga*) and the human botfly (*D. hominis*) are the most common vectors for myiasis in Africa and the tropical Western Hemisphere, respectively (*2*). The genus *Cordylobia* also contains 2 less common species (*C. ruandae* and *C. rodhaini*) (*3*). Infection with *C. rodhaini* (Lund's fly) is less common.

A review of the literature showed only 7 reports of *C. rodhani* myiasis in travelers from countries such as Australia (*3*), Italy (*4*), Canada (*5*), France (*6*), and Israel (*7*). All travelers were infested after travel to eastern and western regions of central Africa. In humans, the skin lesion starts as a painful red papule that gradually enlarges and develops into a furuncle. Typically, the center of the lesion has an opening, through which the larva breaths and discharges its waste products. Cutaneous myiasis is usually an uncomplicated and self-limiting disease. The flies have adapted to tropical environments, and spread to areas in which this disease is not endemic is unlikely.

In the emergency department, cellulitis or furuncular lesions are common with a broad differential diagnosis. With the introduction of bedside ultrasonography in the emergency department, ultrasonographic evaluation of soft tissue infections is more accurate than clinical examination in detecting abscesses (*8*,*9*). Ultrasonographic examination of soft tissue infections enables more accurate localization of an associated abscess and the potential to more specifically identify etiology such as a foreign body (*10*). We report a rare case of cutaneous myiasis caused by *C. rodhaini* larvae in a traveler returning from tropical Africa.

The patient was a 26-year-old woman who came to the emergency department at Toronto East General Hospital with a 10-day history of a painful red lesion on her left upper arm. She had first assumed it to be an insect bite, but during the preceding few days the swelling had greatly increased. She had no constitutional symptoms other than a persisting mild cough for which she had taken a 5-day course of amoxicillin ≈2 weeks before coming to the hospital. Her medical history was noncontributory. She reported that 7 days earlier she had returned from a 1-month trip to Ethiopia. During her stay in Ethiopia, she had been primarily in rural areas but did not report contact with sick persons. Her vital signs were normal.

Physical examination showed a 2.5-cm^2 erythematous area on the lateral aspect of the upper arm (Figure, panel A). There was a 1-mm central punctum and local tenderness. Discharge, streaking, or proximal adenopathy were not present. Other results of the examination were noncontributory. Results of a complete blood count and liver function tests were within reference ranges. Results of a chest radiographic were normal.

Bedside ultrasonography was performed to assess possible abscess. During ultrasonography, the patient reported a biting sensation and increased pain in the area of the lesion. Ultrasonographic images of the lesion showed an area of spontaneous movement just below the skin, suggestive of cutaneous myiasis (online Video, wwwnc.cdc.gov/EID/article/17/12/11-1062-V1.htm).

Treatment for myiasis can be conservative or surgical. Surgical treatment consists of mechanical removal of the larva. After consultation with infectious disease specialists, we covered the lesion with standard lubricating jelly and Op Site Flexfix transparent adhesive (Smith and Nephew, St. Laurent, Quebec, Canada) to obtain a seal.

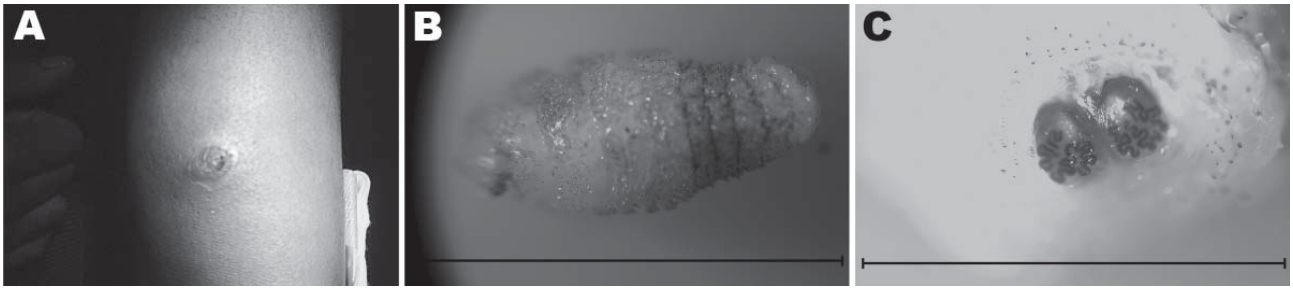

Figure. A) Lateral aspect of the upper arm of a 26-year-old woman showing cutaneous myiasis and an erythematous lesion 2.5 cm in diameter, Canada. B) *Cordylobia rodhaini* larva (length ≈1 cm) isolated from the erythematous lesion. Scale bar = 10 mm. C) Characteristic posterior spiracles of a *C. rodhaini* larva. Scale bar = 3 mm. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/17/12/11-1062-F1.htm).

Approximately 45 minutes later, a 1-cm, white–yellow larvae emerged from the area and was removed intact with the dressing (Figure, panel B). *C. rodhaini* was identified by its characteristic posterior spiracles and the pattern of the larvae (Figure, panel C). Another occlusive dressing was applied before patient discharge. At follow-up 4 days later, the lesion was no longer symptomatic and the patient refused further treatment.

Physicians should consider myiasis in patients who have a furuncular lesion after returning from tropical countries. Bedside ultrasonography rapidly confirmed the diagnosis of myiasis, enabling immediate and appropriate treatment. Travelers should be aware of this potential infestation with the less common Lund's fly and not only avoid direct contact with clothes left outside but also avoid direct contact with infested material (*5*).

Acknowledgments

We thank clinical laboratory staff and public health departments for expert assistance.

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DOI: http://dx.doi.org/10.3201/eid1712.111062

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Brucella suis Infection in Dogs, Georgia, USA

To the Editor: Brucellosis is a serious, globally distributed zoonotic disease. Humans are susceptible to infection by *Brucella suis*, *B. abortus*, *B. melitensis*, and *B. canis* and can have lifelong symptoms of undulating fever, enlarged lymph nodes, malaise, and arthritis (*1*). In 2009, the United States was officially classified free of *B. abortus*. All states except Texas are classified as stage III (free) for swine brucellosis caused by *B. suis* (*2*).

Consumption of unpasteurized dairy products confers the highest risk for brucellosis in disease-endemic areas (*3*). However, hunters and owners of hunting dogs are at high risk for transmission of brucellosis from wildlife. Sporadic transmission of *B. canis* from pet dogs to their owners has been reported (*4*–*6*). We describe a recent increase in *B. suis* detection in dogs in southern Georgia, USA, and caution the public about the potential for transmission to humans in contact with infected dogs and wild hogs.

Smooth *Brucella* spp. express the immunodominant O side chain on the lipopolysaccharide of their surface. Therefore, this side chain forms the antigenic basis of diagnostic tests, such as the card test. The *B. abortus* plate antigen (BAPA) test can detect smooth species. Because *B. canis* does not express the O side chain on its surface, serologic tests for *B. canis* differ from tests for *B. abortus*, *B. suis*, or *B. melitensis* (*7*). Therefore, *B. suis*–infected dogs are unlikely to have positive results for *B. canis* tests and vice versa.

During June 2010–July 2011, a total of 674 canine serum samples submitted by veterinarians servicing 207 kennels or pet owners in Georgia were tested by using the BAPA and card agglutination tests. Positive dogs were not detected until late

March 2011. However, 9 dogs from 4 counties (Laurens, Worth, Tift, and Dougherty) were seropositive by BAPA and card agglutination tests. The same dogs were seronegative by *B. canis*–specific tube agglutination and agar gel immunodiffusion tests. Results indicated exposure to *B. abortus*, *B. suis*, or *B. melitensis* (*7*). Examination of case histories showed that all seropositive dogs had been recently exposed to feral swine during hunting expeditions, which led to a presumptive diagnosis of *B. suis* infection in the exposed dogs. All dogs were subsequently euthanized.

Testicles from 2 of the dogs were subjected to *Brucella* spp. culture. These dogs were a hunting dog and a pet dog owned by a hog hunter. Culture of testicles from both animals showed a *Brucella* sp., which was identified as *B. suis* by using conventional biochemical testing and sequencing of the 16S rRNA gene. Both isolates were destroyed ≤7 days after confirmation according to select agent guidelines. Histopathologic examination of testicular tissue from affected dogs showed severe necrotizing, suppurative to pyogranulomatous epididymitis and orchitis.

Although transmission of *B. suis* from dogs to humans has not been reported, *B. suis* is second only to *B. melitensis* in its pathogenicity to humans (*1*). Therefore, dogs exposed to feral hogs should be tested for *Brucella* sp. and monitored for clinical signs, while keeping in mind that sensitivity and specificity of *B. abortus*–specific tests is unknown. If a pet is infected with *B. canis*, a long course of antimicrobial drugs and spaying or neutering to prevent breeding is advisable but might not be completely effective.

Because *B. suis* is more pathogenic to humans than *B. canis*, and its mechanisms of pathogenesis in dogs can vary, the same recommendations might not be true for *B. suis*–infected dogs. Given the serious zoonotic implications of *B. suis* infections, euthanasia of the affected pet may be advocated by regulatory agencies and physicians treating exposed humans.

Little information, including that for pathogenesis or duration of bacteremia, is available for *B. suis* infections in dogs. Therefore, blood cultures might not reliably detect *B. suis*–infected dogs. For *B. canis* infections, only animals certified free of *Brucella* spp. by 2 consecutive serologic or blood culture tests conducted 4–6 weeks apart can be used as breeding stock in kennels. If an outbreak occurs in a kennel, all infected animals should be isolated and euthanized after showing positive test results. The premises should also be thoroughly disinfected before restocking (*8*).

Classification of the United States as free of swine brucellosis is based on surveillance of domestic swine populations (*2*). However, many states classify swine into 3 categories: domestic swine that have no contact with feral swine, transitional swine that might have contact with feral swine, and feral swine. If a domestic swine herd is infected with *B. suis*, many states then reclassify that herd as transitional. Therefore, classification of a state as free of swine brucellosis does not mean that transitional herds or infected feral swine do not exist. Our results indicate possible underestimation of the role of feral swine in the sylvatic transmission of *B. suis* (*9*,*10*). Future surveillance of feral swine populations in southern Georgia is warranted to determine the prevalence of *B. suis*.

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DOI: http://dx.doi.org/10.3201/eid1712.111127

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Iridovirus Infection in Chinese Giant Salamanders, China, 2010

To the Editor: The Chinese giant salamander (*Andreas davidianus*) is one of the world's largest amphibian species and is often referred to as a living fossil. They primarily inhabit drainage basins of the Yangtze River, the Yellow River, and the Pearl River in the People's Republic of China (*1*). Because of habitat loss, pollution, and overharvesting, the population of wild Chinese giant salamanders has dropped sharply (*2*,*3*). As a result, the Chinese giant salamander is artificially farmed in mesocosms for research and conservation. The mesocosms (ambient temperature <20°C) are maintained primarily in mountainous caves and ditches. During June–October 2010, a high mortality rate was reported in salamanders in ditch mesocosms in Shaanxi, Sichuan, and Henan, reaching an epidemic peak in July. Mortality rate reached 95% in the affected areas. Although bacteria, including *Aeromonas hydrophila* (*4*), were isolated from sick salamanders, antimicrobial drug treatment did not successfully improve the situation. Further pathologic analysis and viral testing were subsequently performed.

Pathologic changes were similar among the affected salamander populations. Gross anatomical changes included palpebral hyperemia or swelling; mouth pouch erythema; ecchymoses in the oral cavity; petechiae, ulceration; and erythema on the dorsal and ventral body surface; toe necrosis (online Technical Appendix Figure 1, panels A, B, wwwnc.cdc.gov/EID/pdfs/10-1758-Techapp1.pdf); emaciation; friable and gray-black liver; and mottled, friable lesions of the kidney and spleen (online Technical Appendix Figure 1, panel C). Histologic examination showed hyperplastic lymphoid nodules in the spleen (Figure, panel A). Additionally, nuclear debris, macrophages (Figure, panel A), and intracytoplasmic inclusion bodies (Figure, panel B) were observed in the lymphoid nodules. Liver sinusoids were enlarged and contained large numbers of macrophages. Degenerating hepatocytes were noted (online Technical Appendix Figure 1, panel D). Degenerate renal epithelial cells were shed from the basement membrane and were found in the lumen of the renal tubules (online Technical Appendix Figure 1, panel E). A large number of viral particles were observed in renal epithelial cells (online Technical Appendix Figure 1, panel G). Virus was isolated from the liver, kidney, and spleen. Electron microscopy was performed on random tissue samples from organs positive for an unidentified virus. Icosahedral viral particles ≈150 nm in diameter were observed in the cytoplasm of some cells (Figure, panel B; online Technical Appendix Figure 1, panels F, G).

On the basis of the gross lesions and the appearance of the virus, we suspected that it was a member of the iridovirus family. To test this hypothesis, genomic DNA (gDNA) was extracted from the isolated virus by using a commercial kit (Genray, Shanhai, China). PCR was performed by using 3 sets of primers targeting 681 bp, 568 bp, and 616 bp iridoviral fragments respectively, from the major capsid protein gene (GenBank accession no. U36913; 5′-CCCTCCCATTCTTCTTCTCC-3′, 5′-GGCGTTGGTCAGTCTACCGTA

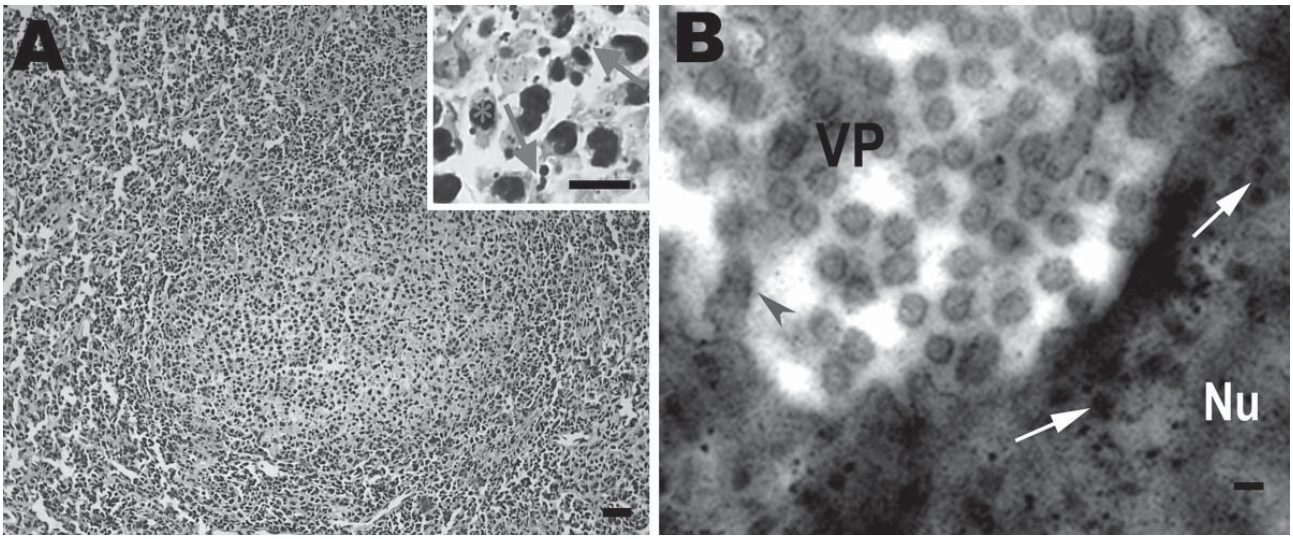

Figure. Histologic changes in the spleen of sick Chinese giant salamanders (*Andrias davidianus*), People's Republic of China, 2010. Electron microscopy shows virus particles in splenocytes. A) Hyperplasia of lymphoid nodules in the splenic white pulp. Inset: Some splenocytes contain nuclear debris (arrows) and macrophages (asterisk). Hematoxylin and eosin stain; scale bars = 80 μm. B) Electron microscopy image of viral particles in splenocytes. Many viral particles are cytoplasmic and appear hexagonal or round. Scale bar = 200 nm. VP, viral particles in cytoplasm; Nu, nucleus; arrowhead, provirus in nuclear membrane; arrows, provirus in nucleus.

AT-3′), the ATPase gene (GenBank accession no. M80551; 5′-CCAAGAG GCACATCATACCG-3′,5′-GCT GGACATCTCCTACGACCC-3′), and the thymidine kinase gene (GenBank accession no. AY837779; 5′-GGGCTAATGTATTGAAGA CGC-3′,5′-TTGTAAACTTGGAGTG GAGGG-3′). Resulting PCR products from 10 salamanders were sequenced and compared with the corresponding sequences of the 5 known iridovirus strains by using a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (frog virus 3, GenBank accession no. AY548484; soft-shelled turtle iridovirus, GenBank accession no. EU627010; tiger frog virus, GenBank accession no. AF389451; epizootic hematopoietic necrosis virus GenBank accession no. FJ433873; and Ambystoma tigrinum stebbensi virus, GenBank accession no. AY150217). The sequences of the 3 PCR products from the virus-infected Chinese giant salamanders (GenBank accession nos. HQ829176, HQ829177, and HQ829178) showed >96% homology with the corresponding sequences of the 5 iridovirus strains. Additionally, neighbor-joining tree analysis showed that the virus was clustered in 1 lineage with frog virus 3, soft-shelled turle iridovirus, and tiger frog virus (online Technical Appendix Figure 2). These results suggest that the high mortality rates in Chinese giant salamanders were caused by a virus in the iridovirus family.

The iridoviruses are carried in the bodies of vertebrates such as gopher tortoises (*Gopherus polyphemus*) (*5*), Chinese forest frogs (*Rana dybowskii*) (*6*), and fish (*7*,*8*). Iridoviruses are thought to be transmitted horizontally in lower vertebrates, such as bullfrogs (*7*,*9*,*10*). In addition, some iridovirus infections may be chronic or conditional (*7*). In this study, the virus was isolated from the liver and spleen of 30 sick (n = 7) or dead (n = 23) salamanders that were farmed in ditch mesocosms, where ambient temperatures were unusually high (>25°C) at the time of the epidemic. Although the virus also was isolated from animals living in cooler cave mesocosms (ambient temperature <18°C), these animals showed no apparent signs of illness. Studies have reported that, when infection is detected early in the course of the disease and when exogenous stress is minimized, mildly affected bullfrogs are able to clear the virus (*9*,*10*). The high water temperatures in the ditch mesocosms (i.e., >25°C) and the associated stress on the animals may have increased disease in ditch-dwelling Chinese giant salamanders. This seems particularly likely, given the absence of clinical signs of disease in infected salamanders that lived in the cooler cave mesocosms (i.e., <18°C). In addition, absence of exposure of Chinese giant salamanders to other animal carriers of the virus may prevent horizontal transmission of iridovirus.

Acknowledgments

We are indebted to Regina Turner for a critical reading and editing of the manuscript. We also thank Zhang Chi for collecting the samples and Guoyun Zhang and Hongchao Zhou for conducting histopathologic analysis.

This work was supported by the Finances Special-purpose Fund of Northwest A & F University to W.D. (no. Z109021001) and the startup fund of Northwest A & F University to W.Z. (no. Z111020902).

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DOI: http://dx.doi.org/10.3201/eid1712.101758

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Vertical Transmission of Avian Bornavirus in Psittacines

To the Editor: Proventricular dilatation disease (PDD) is a fatal disease in psittacines that jeopardizes critical species conservation projects, such as that involving the Spix's macaw (*Cyanopsitta spixii*), the world's most endangered bird species (*1*). The disease is characterized by lymphoplasmacytic infiltrations in the enteric and central nervous systems (*2*). Consequently, gastrointestinal and neurologic disorders are the major clinical manifestations. Only recently has the cause of the disease been identified by characterization of a newly discovered member of the family *Bornaviridae*, the avian bornavirus (ABV), which has been detected in affected psittacines (*3*,*4*). The relationship of an infection with ABV and the occurrence of PDD has been described in natural cases (*5*,*6*) and in experimental trials (*7*,*8*). However, birds that are infected with ABV but that are clinically healthy have also been recognized (*6*). Infected birds can shed viral RNA intermittently (*9*), and not all infected birds seroconvert (*5*). For psittacine flock management, control of an ABV infection is critical, e.g., by repeated testing of breeding stock and removal of ABV-positive birds (*2*,*5*). However, in breeding projects of rare species, every individual is genetically important and cannot be lost. Therefore, pairing infected, but clinically healthy, birds separately from birds that test negative for the virus might represent an option. For this possibility to be viable, whether vertical transmission of ABV can take place must be further clarified. A study investigating the distribution of ABV in tissues of PDD-positive birds has demonstrated ABV antigen in follicular cells, which may point toward vertical transmission (*9*).

To investigate vertical transmission of ABV, we examined 30 dead-in-shell embryos of various psittacine species that originated from ABV-infected flocks with a history of PDD. First, the eggshell was disinfected and opened at the blunt end by using sterile equipment. The brain and proventriculus of each embryo were analyzed for the presence of ABV RNA by using 2 different real-time reverse transcription PCRs, as described by Honkavuori (*4*), with the primer pair 1034–1322 and, in case of a negative result, the additional primer set 1367. Sampling, RNA extraction, and PCR were repeated by using the same methods to exclude possible cross-contamination of samples. Afterwards, the complete embryo was placed in 10% buffered formalin, and histopathologic examination and immunohistochemical (IHC) testing were carried out (*10*) with antibodies directed against the viral phosphoprotein and X protein. If ABV RNA or ABV antigen was demonstrated, crop and cloacal swab specimens and serum of the parents of the positive embryo were immediately taken and used either for ABV RNA detection with the above described PCR or for the detection of specific ABV antibodies by indirect immunofluorescence assay (*10*). This procedure was chosen because earlier sampling of the parents might have caused breeding interruption, and which eggs of which parents would be available for investigation was not clear.

In 2 of the 30 embryos investigated, ABV RNA was detected by using the 1034 PCR. The repeated procedure provided the same results in the same embryos. One embryo was a Major Mitchell cockatoo (*Cacatua leadbeateri*) (cycle threshold 31.41) and the other a red-crowned Amazon (*Amazona viridigenalis*) (cycle threshold 35.1). None of the investigated embryos demonstrated histopathologic lesions typical of an ABV infection. IHC testing did not show any positive results. However, in the ABV-positive Amazon embryo, an equivocal result was obtained.

The swab specimens of both parents of the Major Mitchell cockatoo tested positive for ABV RNA, but serum did not demonstrate specific ABV antibodies. The crop swab specimen of the female red crowned Amazon was positive for ABV RNA, but serum was negative for ABV antibodies; the male bird tested negative by PCR but demonstrated an ABV-specific antibody titer of 80.

These results highlight the potential risk for vertical transmission of ABV and the conclusion that ABV-infected parents can most likely produce infected offspring. However, test results were positive for only 2 of 30 eggs. Because potentially dead-in-shell embryos are usually further incubated by the breeder to ensure embryonic death, the possibility cannot be excluded that ABV RNA was already degraded in some cases, thus causing false-negative results. On the other hand, these eggs might have originated from ABV-negative parents. The quality of the samples might also have caused the questionable IHC results. In PDD-affected, ABV-positive flocks >30% of the birds could be infected (*5*,*6*) and the virus is shed intermittently (*9*). Therefore, pairing ABV-positive birds, incubating their eggs artificially, and raising the chicks separately until they show negative test results, might be an option for breeding projects. However, when vertical transmission occurs (and, if so, its incidence) is unknown.

Whether ABV infection of the embryos was the cause of death remains unclear. Even if typical lesions were not detected, the poor quality of the material might have hidden such lesions. However, embryonic infection that does not result in embryonic death is the basic requirement for successful vertical transmission. These preliminary results warrant further

studies investigating the possibility of vertical transmission by ABV-infected pairs, especially to minimize the risk for such transmission to endangered species with restricted breeding opportunities.

Acknowledgment

We thank the Loro Parque Fundación, Tenerife, Spain, for financial support (LPF-Project no.: PP-65-2010-1).

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DOI: http://dx.doi.org/10.3201/eid1712.111317

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Novel Astroviruses in Children, Egypt

To the Editor: Human astroviruses (HAstVs) are a common cause of gastroenteritis in children, the elderly, and immunocompromised persons (*1*). Up to 10% of acute viral gastroenteritis in children and 0.5%–15% of diarrheal outbreaks are attributed to astroviruses (*2*). Until 2008, eight classical astrovirus serotypes were known to cause human disease; in Egypt, HAstV-1 is the most frequent astrovirus serotype detected (*3*). Recently, 5 novel astroviruses have been discovered in human fecal samples from patients with diarrhea or acute flaccid paralysis (*4*–*7*). Because the prevalence of these virsues in the Middle East is unknown, we screened fecal samples from children with diarrhea residing in Egypt to ascertain the prevalence and diversity of these novel astroviruses.

Fecal samples were collected from a cohort of 364 symptomatic children <5 years of age who had diarrhea and were seeking medical care at Abu Homos Hospital in the Nile Delta of Egypt from September 2006 through September 2007. RNA was extracted from 10% fecal suspensions and reverse transcription PCR for astrovirus was performed as previously described (*4*–*6*). Astrovirus consensus primer pair SF0073/SF0076 amplified an ≈409-bp region of open reading frame (ORF) 1b, encoding the RNA polymerase gene. PCR-positive samples were then tested by using primer sets Mon269–Mon270 (*8*) and SF0053–SF0061, amplifying either a 449-bp or a 402-bp product of the ORF2 capsid gene from classical HAstVs (serotypes I–VIII), or astrovirus MLB1, respectively. DNA sequences of PCR products were determined by using Big Dye Terminator Cycle technology (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were compared with sequences obtained from GenBank. Phylogenies

were constructed with the MEGA4 software (www.megasoftware.net) by using the neighbor-joining method and a p-distance algorithm. Bootstrap resampling was performed by using 2,000 replicates to demonstrate robustness of grouping. The nucleotide sequences determined in this study were assigned GenBank accession nos. HQ674630–HQ674650.

Consensus astrovirus reverse transcription PCR results were positive in 23 (6.3%) of 364 fecal samples. Five common serotypes of classical HAstV were identified constituting 16 (70%) of 23 positive samples; HAstV type I was most prevalent (n = 9). Alignment of the partial amino acid sequences of the ORF2 capsid region indicates that Egyptian HAstV type I strains share 99%–100% identity (Figure, panel A).

Five of the 7 remaining positive samples were most closely related to MLB1 on the basis of the partial ORF2 sequence analysis. Egypt MLB1 samples shared ≈99%-nt identity with each other, and all grouped in 1 phylogenetic cluster (Figure, panel B) along with a recently identified MLB1 strain (GenBank accession no. HM450380 [*9*]) isolated from a human fecal sample in Hong Kong. The Egypt MLB1 strains shared ≈97%-nt identity with the prototype MLB1 Australia strain but were more divergent from an isolate recently described in the United States (92% nt identity, GenBank accession no. FJ222451). However, all of the observed nucleotide differences represented silent mutations between Egypt MLB1 and Australia and US isolates; comparison of partial capsid protein sequences indicated no amino acid changes.

The 2 remaining HAstV-positive samples were phylogenetically most similar to astrovirus VA2 (VA2) (Figure, panel B). On the basis of the sequence of the amplicon from the ORF1b region, the Egyptian VA2 isolates shared 96.1%–100% aa identity to previously described VA2 and astrovirus human/mink/ovine genomes in GenBank.

Our study describes the detection of the recently identified viruses MLB1 and VA2 in a cohort of symptomatic children with diarrhea residing in Egypt. This study expands the geographic range of these viruses to include northern Africa. The consensus primers used in our study were able to detect a higher percentage of positive HAstV serotypes I–VIII than the Mon340/Astman-2 primers (*10*) (data not shown), a finding that encourages use of these primers to screen humans with gastroenteritis for astroviruses. Increased understanding of the genetic diversity within viral families infecting humans will assist in future studies of their pathogenicity and the design of specific diagnostic assays. Further epidemiologic studies, including clinical cases and demographically matched healthy con-

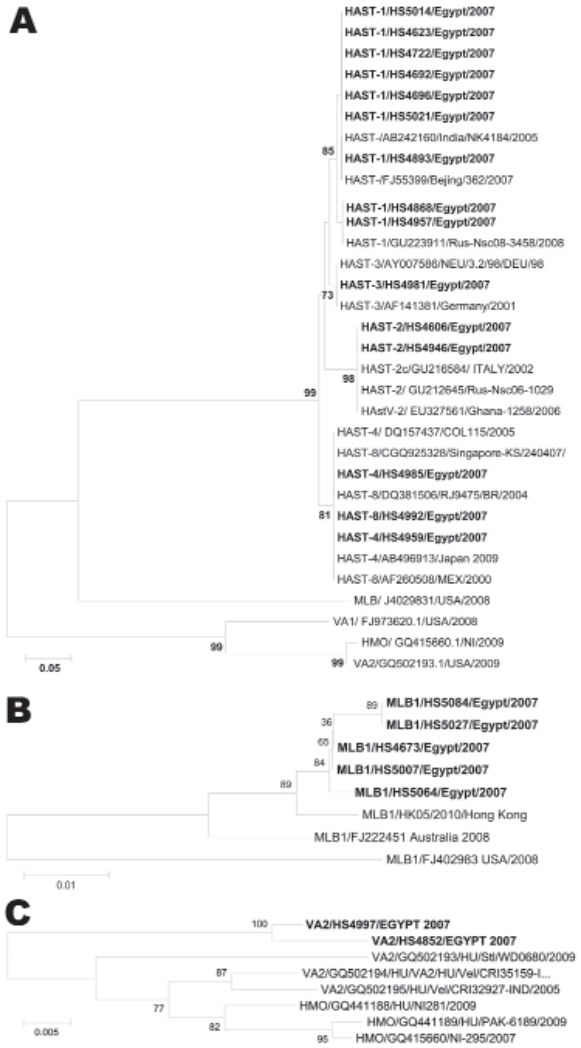

Figure. A) Phylogenetic analysis of the partial amino acid sequences of the open reading frame (ORF) 2 capsid region of human astrovirus (HAstV) types I–VIII (using Mon primer) with other sequences from GenBank. B, C) Phylogenetic trees based on partial nucleotide sequences of MLB1 ORF2 (B) and ORF1b (VA2) (C). Egyptian isolates are shown in **boldface**. Sequence alignment was performed by using ClustalW in the BioEdit software package (www.clustal.org). Dendrograms were constructed by using the neighbor-joining method as in MEGA4 (www.megasoftware.net). Values on the branches represent the bootstrap values from 2,000 replicates. Scale bar in A indicates amino acid substitutions per site; scale bars in B and C indicate nucleotide substitutions per site.

trols, are required to better define their pathogenic potential.

This work was supported by the Global Emerging Infections and Surveillance System, a Division of the Armed Forces Health Surveillance Center and in part by the National Institutes of Health under KL2 RR024994. The study protocol DOD NAMRU3.2000.0002 (IRB Protocol No. 096) was hospital-based surveillance for enteric pathogens associated with severe diarrhea in Egyptian children.

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DOI: http://dx.doi.org/10.3201/eid1712.110909

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Humans as Source of *Mycobacterium tuberculosis* Infection in Cattle, Spain

To the Editor: *Mycobacterium tuberculosis* is the main causative agent of tuberculosis in humans. However, little attention has been paid to its transmission from humans to animals. We report *M. tuberculosis* infections in 3 cattle farms in Spain. The epidemiologic investigation traced humans as the source of infection, with 1 of the strains showing multidrug resistance.

Recent studies have reported isolation of *M. tuberculosis* in cattle with prevalences of 4.7%–30.8% in African and Asian countries (*1*–*3*). In cattle, this infection occurs in countries with the highest incidence of human tuberculosis in the world. In Europe, only 14 cases of *M. tuberculosis* infection have been described in 3 eastern countries since implementation of eradication programs (*4*,*5*). The only reported cases of *M. tuberculosis* in cattle in western Europe were described in Great Britain and date back to the 1950s (*6*).

During 2007–2009, three cases of tuberculosis caused by *M. tuberculosis* were detected in 3 unrelated cattle farms, 2 of them free of tuberculosis (farms 1 and 2). As part of the surveillance system of bovine tuberculosis, a pool of tissue samples from each cow (respiratory lymph nodes and lung) were homogenized with sterile distilled water, and culture was carried out by the BACTEC mycobacteria growth indicator tube 960 system (Beckton Dickinson, Madrid, Spain). Members of the *M. tuberculosis* complex were identified and genotyped by direct variable repeat spacer oligonucleotide typing and mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) typing (*7*).

The 3 *M. tuberculosis*–infected animals were <9 months of age (Table). As described (*6*), the possibility of infection in young animals could be more probable than infection in older cows.

M. tuberculosis–infected animals from farms 1 and 3 were detected by the intradermal tuberculin test (Table). The animal without immunologic response (farm 2) was detected because an *M. bovis* infection was confirmed in the herd, and all animals were slaughtered. Confirmation of infection by culture without immunologic response is rare, although the high sensitivity of the mycobacteria growth indicator tube system could detect a low bacterial load in the initial stages

of infection. Recent implementation of liquid systems in animal health laboratories has enabled detection of *M. tuberculosis* when it is compared with results using only conventional methods. Moreover, no tuberculosis-compatible lesions were observed in the 3 animals, similar to previous studies (*6*). On the basis of these facts, *M. tuberculosis* transmission was not detected among cattle in the following intradermal tuberculin tests.

Co-infection with other mycobacteria (*M. avium* subsp. *hominissuis*) was found in the same animal from farm 1 (Table). This co-infection suggested the immunocompromised status of the animal and hence a high susceptibility to *M. tuberculosis* infection. Moreover, *M. bovis* was isolated from 52% (16/31) of all animals from farm 2 that showed a positive reaction to the intradermal tuberculin skin test, making remarkable the absence of co-infection with *M. bovis* in the *M. tuberculosis*–infected animal. Therefore, the lack of *M. tuberculosis* transmission within this herd contrasts with the *M. bovis* dissemination.

The veterinary services reported these findings to the National Public Health System, and an epidemiologic investigation was conducted on the cattle farms to determine the source of infection. In all cases, staff of the farms had active tuberculosis (Table). Three different strains were characterized: SIT2537 (octal code 777617777720771), 2535332334332362522114233423 (farm 1); SIT1564, 3′5233423245545725121 3423 (farm 2); and SIT58, 254343 243233232526221342 3 (farm 3) (Table). The MIRU-VNTR pattern and spoligotype are shared by Spanish human and cattle isolates from farm 1; SIT2537 is an uncommon profile that has been detected in Brazil and Spain (according to the SITVIT2 database). The human strain showed multidrug resistance to isoniazid, rifampin, and ethionamide. In cattle and human isolates, genes associated with isoniazid and rifampin resistance were studied (*8*) and *rpoB* analysis confirmed rifampin resistance (Ser531Leu). In farm 2, the origin of the farm worker was eastern Europe and the cattle isolate showed an SIT1564 profile, which is found only in 6 human isolates in the SpolDB4 database, all from Poland, Bulgaria, and Russia. On farm 3, human and cattle isolates from Spain shared identical spoligotype and MIRU-VNTR patterns. The profile SIT58 is frequent in Spain (*9*) and other countries with historical links to Spain, mainly the south American countries (79/114 according to SpolDB4).

A well-designed program for eradicating bovine tuberculosis helps to detect *M. tuberculosis* infection by immune response or by bacteriologic culture. The use of liquid systems and results of epidemiologic studies (Spanish Database of Animal Mycobacteriosis, mycoDB.es) (S. Rodríguez, unpub. data) are recommended for prompt confirmation of the *M. tuberculosis* complex infection and for enhancing the sensitivity of culture. In addition, the Spanish Ministry of Environment, Rural and Marine Affairs has reinforced the need to improve cooperation between human and animal health systems to minimize the risk for *M. tuberculosis* complex transmission from animals to humans or vice versa and to control infection in all susceptible animal species (*10*).

Table. Relevant information about *Mycobacterium tuberculosis* infection in 3 cattle farms in Spain*

Variable	Farm 1	Farm 2	Farm 3
Cattle herd			
No. animals	6	54	>200
Production	Beef	Beef	Dairy
Previous status	TB free	TB-free	Non–TB free
No. reactors	1	31	12
M. tuberculosis infection in cattle			
Year of isolation	2007	2008	2009
Age, mo	9	4	3
IDTB/interferon-γ	Pos/not determined	Neg/neg	Pos/not determined
TB-compatible lesion	No	No	No
Spoligotyping profile	SIT2537	SIT1564	SIT58
MIRU-VNTR profile†	2535332334332362522114233423	3′5233423245545725121 3423	254343243232325262213423
Co-infection with other mycobacteria	Yes (*M. avium* subsp. *hominissuis*)‡	Yes (*M. bovis*)	No
M. tuberculosis infection in human			
Active tuberculosis	Yes	Yes	Yes
Spoligotyping profile	SIT2537	Not available	SIT58
MIRU-VNTR profile†	2535332334332362522114233423	3′5233423245545725121 3423	254343243232325262213423
Origin	Spain	Eastern Europe	Spain

*TB, tuberculosis; IDTB, intradermal tuberculin tested according to the European Council Directive 64/432/EEC; pos, positive; neg, negative; MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number tandem repeat.
†MIRU-VNTR profile on the basis of the 24 MIRU-VNTR loci (*7*).
‡Co-infection in the same animal.

Acknowledgments

We thank L. Carbajo, J. Carpintero, E. Fernández, and L.M. Álvarez for their continuous encouragement; F. Lozano, N. Moya, A. Gutiérrez, J. Gimeno, A. Penedo, A. Menéndez, C. Martínez, and J.A. Anguiano for technical help; R. Daza and M.S. Jiménez for providing the human strains and related data; Nalin Rastogi for comparison of spoligotyping results with the SITVIT2 proprietary database; and M. Gilmour for careful revision of the manuscript.

This research was funded by project TB-STEP FP7-KBBE-2007-212414 of the European Union and by the Spanish Ministry of the Environment, and Rural and Marine Affairs and partially by Fondo de Investigaciones Sanitarias (FIS S09/02205). B.R. received a research contract funded by Comunidad de Madrid (IV regional framework program of research and technological innovation 2005-2008).

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DOI: http://dx.doi.org/10.3201/eid1712.101476

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Porcine and Human Community Reservoirs of *Enterococcus faecalis*, Denmark

To the Editor: *Enterococcus faecalis*, which exists commensally in the gut in warm-blooded animals and humans, is an opportunistic pathogen that causes a variety of community-acquired and health care–associated infections, such as urinary tract and intraabdominal infections, bacteremia, and endocarditis (*1*). Only a few studies have assessed the relationships between clinical *E. faecalis* strains; strains endemic to the health care setting; and community strains residing in humans, animals, or animal-origin food (*2*).

Recently we showed that the emergence of high-level gentamicin-resistant (HLGR) *E. faecalis* among patients with infective endocarditis (IE) coincided with an increase in HLGR *E. faecalis* in the pig population in Denmark (*3*). The majority of isolates belonged to the same clonal group (sequence type [ST] 16), suggesting that pigs constitute a community reservoir of HLGR *E. faecalis*.

We investigated human and porcine community reservoirs of other *E. faecalis* clonal types associated with IE in humans in Denmark.

A total of 20 consecutive gentamicin-susceptible *E. faecalis* isolates were obtained from IE patients in North Denmark Region during 1996–2002 (online Appendix Table, wwwnc.cdc.gov/EID/article/17/12/10-1584-TA1.htm). Cases of IE were classified as definite (n = 12) or possible (n = 8) according to the modified Duke criteria (*4*). A case of community-acquired *E. faecalis* infection (n = 6) was defined in accordance with strict criteria applied for methicillin-resistant *Staphylococcus aureus* (*5*); otherwise, cases were deemed to be health care associated (n = 14) (online Appendix Table). HLGR ST16 isolates recovered from 2 IE patients during the study period have been characterized (*3*) and were excluded from the present study.

Using multilocus sequence typing (*6*), we identified 14 STs among the 20 IE isolates (online Appendix Table), then compared them with STs from 2 collections of *E. faecalis* isolates collected as part of the Danish Integrated Antimicrobial Resistance Monitoring and Research Program (www.danmap.org): 1) all 14 isolates recovered from community-dwelling humans in North Denmark Region during 2002–2006 with approval from the local ethics committee ([KF] 01-006/02), which were classified into 10 STs in this study (online Appendix Table); and 2) 19 pig isolates from 2001 that were shown in a previous study to belong to 12 STs (*7*).

Among the 14 STs identified in IE isolates, 4 (ST19, ST21, ST72, and ST306) and 2 (ST40 and ST97) were also found among isolates from community-dwelling humans and pigs, respectively (online Appendix Table). Isolates belonging to these 6 STs were further characterized by pulsed-field gel electrophoresis (PFGE) by using *Sma*I and grouped into PFGE pulsotypes as described (*3*). STs and PFGE pulsotypes (A–F) were largely concordant (ST97:A, ST72:B, ST19:C, ST40:D, ST21:E, and ST306:F), except for 2 isolates belonging to ST72 and ST40, for which PFGE banding patterns (U1 and U2, respectively) were unrelated to the major PFGE pulsotypes (A–F), and 1 ST306 isolate exhibiting the ST21-like PFGE banding pattern E (online Appendix Table).

These findings confirm the genetic relatedness of IE isolates with those from community-dwelling humans (ST72:B, ST19:C, ST21:E, and ST306:F) and pigs (ST97:A and ST40:D). Seven (64%) of 11 IE isolates belonging to these 6 clonal types originated from IE patients with health care–associated risk factors (online Appendix Table), which suggests that health care users are predisposed to colonization and infection with *E. faecalis* strains residing in human and porcine community reservoirs.

Previous reports have shown that epidemiologically distinct *E. faecalis* populations differ in terms of biofilm formation, virulence gene content, and antimicrobial drug susceptibility profiles (*2*,*8*). Therefore, we characterized all isolates with respect to these traits. Isolates were categorized into strong, medium, weak, and nonbiofilm formers by using the method of Mohamed et al. (*8*). The presence of 12 virulence-associated and pathogenicity island genes (*ebpA*, *gelE*, ef1824, *hylA*, ef1896, ef2347, ef2505, *hylB*, *ace*, *cbh*, *esp*, and ef0571) was investigated by using colony lysates and probes that have been described elsewhere (*9*). The antimicrobial drug susceptibility profiles (ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, kanamycin, linezolid, penicillin, streptomycin, teicoplanin, tetracycline, and vancomycin) were determined by the Sensititre system (Trek Diagnostic Systems, East Grinstead, UK) in accordance with Clinical and Laboratory Standards Institute guidelines (*10*). The isolates were generally homogenous within each clonal type in terms of biofilm formation, presence of virulence-associated and pathogenicity island genes, and resistance profiles (online Appendix Table), further supporting that IE isolates are genetically related to those from community-dwelling humans and pigs, respectively. Notably, most IE isolates were susceptible to ampicillin (100%), penicillin (100%), vancomycin (100%), high-level gentamicin (100%), and high-level streptomycin (80%), which are the drugs of choice in therapeutic regiments for *E. faecalis* endocarditis.

In conclusion, our results suggest that the normal intestinal microflora of humans and pigs are community reservoirs of clinical *E. faecalis* and link 2 porcine-origin clonal types of gentamicin-susceptible *E. faecalis*, ST97:A, and ST40:D to IE in humans in Denmark. This finding strengthens existing evidence that pigs can be a source of serious infections in humans.

Acknowledgments

We thank Karin S. Pedersen for help with antimicrobial drug susceptibility testing and genotyping and Lena Mortensen for providing clinical isolates.

This work was supported by grant 271-06-0241 from the Danish Medical Research Council, the Danish Ministry of Family and Consumer Affairs, and the Danish Ministry of the Interior and Health as part of the Danish Integrated Antimicrobial Resistance and Research Program, and the European Union Sixth Framework Program "Approaches to Control Multiresistant Enterococci: Studies on molecular ecology, horizontal gene transfer, fitness and prevention" under contract LSHE-CT-2007-037410.

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DOI: http://dx.doi.org/10.3201/eid1712.101584

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West Nile Fever Outbreak in Horses and Humans, Spain, 2010

To the Editor: *West Nile virus* (WNV) is a member of the genus *Flavivirus* within the Japanese encephalitis antigenic complex. The enzootic virus cycle involves transmission between avian hosts and ornithophilic mosquitoes, whereas humans and horses are considered dead-end hosts. Given the recent increase of WNV infection in humans and horses in Europe, concern has been raised regarding public and animal health.

In Spain, WNV seropositivity has been reported for humans (2001), horses (2005–2008), and wild birds (2007–2008) (*1*–*3*). Clinical disease has been described for humans (2004) and raptors (2001–2005) (*4*,*5*) but not for horses. We report the main epidemiologic and clinical findings of a WNV outbreak in horses and humans in Spain in 2010.

After the first clinical case of West Nile fever was detected in a horse in September 2010 in Andalusia (southern Spain), a control program for WNV was initiated that included symptomatic treatment of animals, protection of horses in shelters during hours of higher vector activity, vaccination (not mandatory), vector control using pyrethroid-based insecticides, and elimination of mosquito breeding habitats. Horses with neurologic signs were confirmed as WNV positive by detection of serum IgM against WNV by using a competitive ELISA (IDEXX IgM WNV Ab; IDEXX Laboratories, Westbrook, ME, USA). To assess level of WNV infection within affected herds, samples from sick and clinically healthy unvaccinated horses were collected 2 months after the last case. Serum was tested for IgG against WNV by using a blocking ELISA (Ingezim West Nile compac R.10. WNV.K3; Ingenasa, Madrid, Spain). Positivity was confirmed by a serum microneutralization test (SNT) against WNV (strain Eg101) according to World Organisation for Animal Health guidelines. Blood and cerebrospinal fluid samples from clinically affected horses were analyzed by real-time reverse transcription PCR (*6*).

IgM against WNV was detected in 51 (50%) of 102 clinically ill horses; 15 died and 3 were euthanized. The most common clinical signs were

ataxia, disorientation, and weakness, followed by fever, muscular tremor, cranial nerves deficit, and photophobia. Of the 36 infected herds, 30 were located in the province of Cádiz, 5 in Seville, and 1 in Málaga (Figure). The first WNV case was reported on September 10, 2010; the number of cases peaked at 17 in mid-September, then decreased until the last case reported on December 15, 2010. In the second survey, IgG seroprevalence within the 36 infected herds was 51.7% (46/89). All IgM-positive horses and 23 (34%) of 68 clinically healthy horses had antibodies against WNV by blocking ELISA and SNT, indicating intense local transmission in 2010, which contrasts with previous observations (*2*).

On September 20, 2010, the first case of WNV infection in a 60-year-old man was confirmed by detection of IgM by competitive ELISA and SNT. On October 6, 2010, a 77-year-old case-patient was reported. Both patients, detected in the same area and period of the WNV outbreak (Figure), showed signs of encephalitis but were discharged after several days' hospitalization. After the human cases were confirmed, control measures, such as inclusion of West Nile fever in the differential diagnosis of neurologic diseases and the control of blood samples from suspected cases and donors, were implemented.

WNV lineage 1 RNA was detected by real-time reverse transcription PCR in blood and cerebrospinal fluid of 1 of the 51 horses analyzed. Despite evidence that WNV persisted during the winter and reemerged during spring in the western Mediterranean, the Spanish strain (JF719069-Spain/10/H-1b) and the strains isolated in Spain in 2007 and 2008 belong to clade 1a and clade 2, respectively (*7*). The closest relatives of the Spain/2010 strain are the 2008 and 2009 Italian strains, with which it seems to share a common ancestor (*7*). Therefore, WNV may have circulated silently in the western Mediterranean region, establishing an endemic cycle after a single introduction. Alternatively, because Andalusia is located within the migratory flyways for wild birds between Europe and Africa, the Spain/2010 strain might have been introduced putatively from Africa from the same source as the Italian strains. Further studies are needed to elucidate the origin of the Spain/2010 strain.

Previous serologic surveys in migratory and resident wild birds from the affected area indicated WNV circulation during 2007 and 2008 (*3*). Although high numbers of dead birds were reported in resident wild birds concurrently with the equine WNF outbreak, it appeared to be caused by another flavivirus, Bagaza virus, not previously found in Europe (*8*). In 2010, Andalusia had the highest rainfall during spring and the hottest summer in the past decade (*9*), which provided optimal conditions for *Culex* spp. mosquitoes. An entomologic survey in the affected area in 2010 showed that the most abundant species was *Cx. pipiens*, with maximum abundance during June and September. The abundance of competent vectors and the high number of wild bird nesting areas in Andalusia provide ideal conditions for the maintenance and circulation of WNV. Therefore, the risk for reemergence of WNV in Spain should be considered high. To improve the early detection of WNV cases and prevent new outbreaks, a surveillance program of passive surveillance in humans, equids, and wild birds; serosurveillance in sentinel horses and wild birds; and entomologic surveillance was initiated after the 2010 outbreak (*10*).

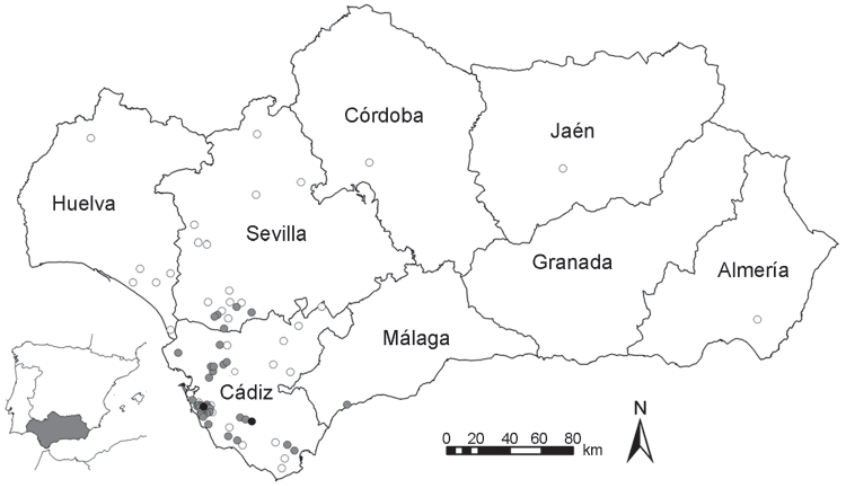

Figure. Spatial distribution of West Nile virus–infected horse herds (gray dots), virus-negative horse herds (white dots), and human cases (black dots) in Andalusia (southern Spain) at the end of 2010.

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DOI: http://dx.doi.org/10.3201/eid1712.110651

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Correction

Vol. 17, No. 11

In the article Lessons Learned during Public Health Response to Cholera Epidemic in Haiti and the Dominican Republic (J.W. Tappero, R.V. Tauxe), data in Figure 2 were published incorrectly. The corrected figure is provided here; the article has also been corrected online (wwwnc.cdc.gov/EID/article/17/11/11-0827_article.htm).

DOI: http://dx.doi.org/10.3201/eid1712.C11712

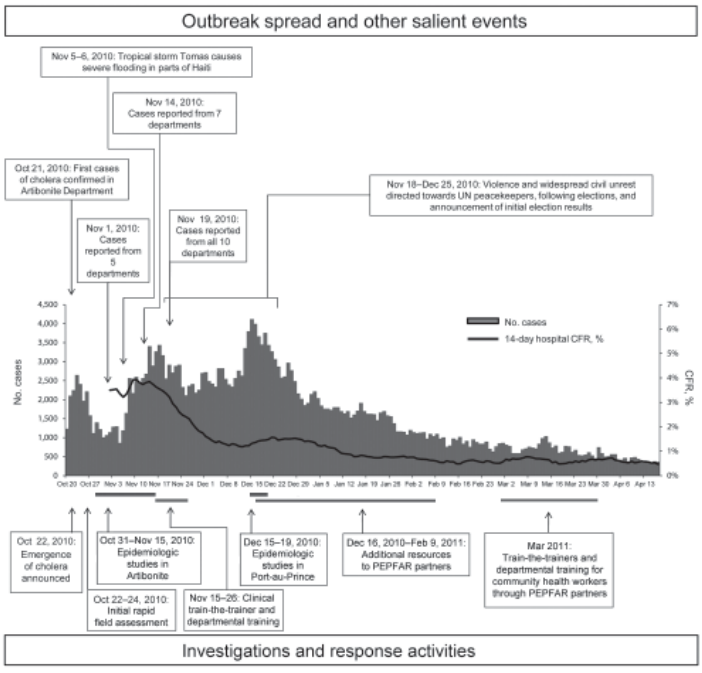

Figure 2. Reported cases of cholera by day, and 14-day smoothed case-fatality rate (CFR) among hospitalized cases, by day, Haiti, October 22, 2010–July 25, 2011. UN, United Nations; CDC, Centers for Disease Control and Prevention; PAHO, Pan American Health Organization; MSPP, Ministère de la Santé Publique et de la Population.

In Memoriam: Washington C. (Wash) Winn Jr. (1941–2011)

David H. Walker, Rocco LaSala, Bobbi Pritt, Elmer Koneman, and J. Michael Miller

Washington (Wash) C. Winn Jr., (Figure) died suddenly and unexpectedly on July 3, 2011. A remarkable Renaissance man; a warm, humane person; and outstanding academic physician and scientist, Dr. Winn is remembered vividly as a contributor to understanding of emerging infectious diseases; a contemporary, efficient diagnostic clinical microbiologist; and a treasured educator. Born in Richmond, Virginia, on April 2, 1941, he was a true scholar, graduating magna cum laude with honors in English from Yale University in 1963. All who have been a co-author with, been edited by, or shared an educational venue with him have experienced a captivating creativity, mastery of expression, and improvement in one's own communication through his skills with the language.

Dr. Winn earned his doctor of medicine degree from the University of Virginia (1967) and deepened his experience as a physician during an internship in medicine at Tufts–New England Medical Center where he received the Medical Intern Award. Subsequently, he served as a resident in pathology at Washington University in St. Louis (1968–1970). Dr. Winn also earned a master's degree in business administration at from the University of Vermont in 1993.

A critical period in his life (1970–1973) occurred when he was a medical officer at the Center for Disease Control in Atlanta. He served in the Arbovirus Reference Unit and the Viral Pathology Branch, Division of Viral and Rickettsial Diseases, where he attributed the mentoring influence of Frederick A. Murphy on the subsequent direction of his professional career. His research on rhabdoviruses and arenaviruses led to his first 7 scholarly publications, including contributions to our knowledge of the pathology of Lassa fever, an archetypal emerging infectious disease. His colleagues of that era remember him as "a warm and wonderful guy and a true Virginia gentleman" (Thomas Monath, pers. comm., 2011), "who lived a life of real grace in everything he did or thought" (Karl Johnson, pers. comm., 2011).

After 4 years as assistant professor of pathology at the University of Virginia (1973–1977), Dr. Winn moved to the University of Vermont where he served as director of the clinical microbiology laboratory for 34 years. An outbreak of Legionnaires' disease in the hospital led to investigations of this disease, which was emerging at the time. As

Figure. Washington C. Winn, Jr.

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DOI: http://dx.doi.org/10.3201/eid1712.IM1712

principal investigator of a National Institutes of Health grant, he contributed to our knowledge of the pathology of *Legionella pneumoniae* and the basic pathogenesis of legionellosis and to methods for the clinical microbiologic diagnosis of the disease, with 29 peer-reviewed publications on these topics.

Dr. Winn then made a conscious positively motivated decision to focus his career on clinical microbiology, with resulting tremendous benefits to the field nationally and internationally and to patients in his institution. His clinical microbiology laboratory at Fletcher Allen Health Care, the clinical program of the University of Vermont, was a model of success. Through the decades, he assembled an exceptional technical staff. He had honed laboratory policies and optimized procedural details to such a degree that problems were essentially nonexistent. He kept the laboratory up to date with the latest technologies. In 2006, his goal was to incorporate real-time PCR into the laboratory—something that was not available in any clinical or anatomic pathology laboratory at the institution. Within just 5 years, he transitioned an essentially nonmolecular test menu to one with a full spectrum of qualitative and quantitative real-time PCRs that encompassed locally developed and Food and Drug Administration–approved assays.

His national contributions to clinical microbiology included membership on the Microbiology Test Committee of the American Board of Pathology and service as chairman, vice chairman, and member and advisor of the Microbiology Resource Committee of the College of American Pathologists. In these roles, he is remembered not only as a knowledgeable scientist and diagnostician but also as a person of character and genuine concern for those with whom he worked. He was always willing to share what he knew in an unselfish way that was endearing to everyone who learned from him.

Another great contribution to the field of clinical microbiology was Dr. Winn's involvement in the influential textbook *Color Atlas and Textbook of Diagnostic Microbiology*, in which he joined Elmer Koneman as co-author of the third edition (1988) (*1*). He wrote chapters on virology, antimicrobial drug susceptibility testing, and new technologies in the diagnosis of infectious diseases in the next 3 editions and added a section on ectoparasites to the parasitology chapter in the fifth edition. He became chief editor of the sixth edition (2006) (*2*), which in the opinion of Dr. Koneman was the crown jewel in the life of the book (E. Koneman, pers. comm.).

Memories of Dr. Winn are indelible and include his extraordinary wealth of knowledge and passion for opera, particularly when the scenes and music were linked to medical practice, such as the final arias sung by Violetta (*La Traviata*) and Mimi (*La Boheme*) before dying of tuberculosis. He will be remembered for his joy in playing the banjo and fiddle, love of good wines and diverse cuisines, learning the German language and Egyptian hieroglyphics, expressing staunch Republican opinions in a sea of liberal colleagues, and cultivating bonsai and ancient varieties of roses. His wisdom, humor, insight, honesty, and kindness will be missed tremendously. Dr. Winn was also a member of the Emerging Infectious Diseases review panel.

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Jan Fyt and others (1611–1661) *Atalanta and Meleager Hunt the Calydonian Boar* (1648) Oil on canvas (243.8 cm × 411.5 cm)
Bequest of John Ringling, 1936, Collection of The John and Mable Ringling Museum of Art http://www.ringling.org/, The State Art Museum of Florida, a division of Florida State University

When snouted wild-boars routing tender corn / Anger our huntsman[1]

Polyxeni Potter

"A monster sent by the gods to ravage the vineyards" is how the Calydonian boar was described in mythologic accounts. King Oeneus did not offer proper thanks to Artemis, goddess of the hunt. In retaliation, she unleashed the monster on the fertile fields of Calydon, "an ornament to Greece," terrorizing adjacent communities and wreaking havoc with local agriculture. "Now it trampled the young shoots of the growing crops, now cut short the ripeness, longed-for by the mournful farmer, and scythed down the corn in ear," wrote Ovid in his Metamorphoses. "Its tusks were the size of an Indian elephant's: lightning came from its mouth: and the leaves were scorched by its breath."

Oeneus' son, Meleager assembled a team of braves to go after the beast. Among these were Jason, leader of the Argonauts; Theseus king of Athens; and swift-footed Atalanta, "the warrior girl of Tegea," a creature of the forest, suckled by a she-bear after being abandoned by her father, who had wished for a son.

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DOI: http://dx.doi.org/10.3201/eid1712.AC1712

The boar, "flame burning in its eyes," charged the hunters "like lightening forced from colliding clouds," killing several before it was finally wounded by Atalanta's arrow and finished off by Meleager who, impressed by the heroine's performance, offered her the pelt as trophy. Meleager's gesture offended the team. In the scuffle that followed, he killed two of his uncles. His mother, distraught by the loss, allowed her son's death to avenge them.

The story of the Calydonian boar hunt was mentioned in The Iliad and was retold many times, in greatest detail by Ovid. "The boar was furiously twisting its body round and round, its jaws slavering with foam and fresh blood …. The hero who had dealt the wound came up close to the animal and roused his foe to fury, before finally burying his shining spear in its shoulder."

Member of the species *Sus scrofa*, family Suidae, and ancestor of the domestic pig, the wild boar roamed much of the world. Native to Europe and the Mediterranean region, the large-bodied, short-legged, coarse and bristled beast, was one of the first to be tamed and live among humans, featured in the mythology of many nations: Egypt, Persia, China, New Guinea, India. In the wild, it embodied, along-

[1]John Keats, "Endymion," http://www.gutenberg.org/files/24280/24280-h/24280-h.htm.

side a penchant for savage behavior, other, much coveted, virtues: power, courage, nobility, cleverness, initiative. In the hierarchy of wild animals, it was right up there, just below the lion, followed by birds of prey.

Legends we read for their timelessness and to understand the world and its complexities, ancient and modern: angry gods, fearless heroes, family strife, the female athlete, men and women, and no less the epic combat between humans and animals, their common destiny, and their close evolutionary and spiritual connection. The Atalanta tale from classical antiquity has had many interpretations. One, most persistent, concerns the relationship between nature and culture—animals and their behavior in the wild, humans and their behavior in societies, and the inevitable link between them.

Artists have long drawn inspiration from legends, and the Calydonian boar hunt was depicted often, as early as 2,500 years ago by the potter Ergotimos and the painter Kleitias, and throughout art history. Peter Paul Rubens, an avid painter of historical and mythologic themes, created his own version. Another famous presentation is Jan Fyt's effort on this month's cover. Fyt, a native of Antwerp, who trained with accomplished still life painter and animalier Frans Snyder, traveled to Italy and France but returned to live and work in his home town. A draftsman and etcher as well as painter, he excelled in the depiction of animals, which he presented with flourish in hunting scenes or within compositions filled with exquisite porcelain, fabrics, flowers, and fruit. At times he collaborated with other artists, among them Willeboirts Bosschaert and Jacob Jordaens.

In his version of the myth, Fyt captured with the brush the fierceness of the battle as effectively as Ovid did in words. "There was a deep valley … and it held in its depths pliant willows, smooth sedges, and marsh grasses." The swift arrow from the girl from Tegea "grazed the top of the boar's back, and fixing itself below one ear, reddened the bristles with a thin stream of blood." The braves circled as the hunting dogs, dwarfed by the massive beast, lay injured and worse.

The symbolic nature of the myth lies in the struggle between the wild animal and the human community, with the beast destabilizing the social fabric and triggering mayhem, much to the detriment of both. Animal and human communities have undergone massive changes since the crisis in Calydon. Although wild boars still occasionally tear up rural villages, they pose scarcely a risk to urban areas, which themselves hardly resemble the mythologic vineyards. Yet the struggle persists and not only on the level of the hunt but also on the microbiologic level, come to the surface far too late for mythologic coverage but filled with intrigue nonetheless.

Instead of wild boars plundering the countryside, we now have in much of the world domesticated swine herds, although in developing countries, pigs are often allowed to roam in the community, where they no longer terrorize the populace with oversized tusks but cause other problems. In the domesticated setting, pigs and humans share commensal organisms in the gut that can cause extraintestinal disease when opportunities arise, as in the health care setting. But often, where there is disease in pigs, there is also disease in humans.

With some zoonotic infections, such as in this issue trichinellosis and swine brucellosis, infected pigs continue to be a threat. *Streptococcus suis*, which causes meningitis and septicemia in piglets and occupational disease in humans, is reported to be a cause of adult streptococcal infection in Vietnam and Thailand and of outbreaks in the People's Republic of China. Humans and pigs can harbor clinical *Enterococcus faecalis.* A link of two intestinal commensal porcine-origin *E. faecalis* strains in Denmark strengthens evidence that pigs can be a source of these infections in humans.

While contact with our wild counterparts will continue to result in emerging human disease, effective measures have limited exposure to many zoonotic agents in regions with strong programs in domestic animal disease control. But until control of livestock infections is universally used to safeguard public health, these infections will continue to pose a threat to humans and their domesticated animal partners around the world. Or as Keats predicted in "Endymion," "Again my trooping hounds their tongues shall loll / Around the breathed boar."

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Emerging Infectious Diseases thanks the following reviewers for their support through thoughtful, thorough, and timely reviews in 2011. We apologize for any inadvertent omissions.

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Article Title

Risk for Rabies Importation from North Africa

CME Questions

1. What is the most common animal vector associated with fatal cases of rabies?

A. Bat
B. Cat
C. Squirrel
D. Dog

2. Which of the following statements regarding efforts to prevent rabies in North Africa is most accurate?

A. Greatest access to rabies postexposure prophylaxis (PEP) is in Sudan
B. Least access to rabies PEP is in Tunisia
C. Mass vaccination campaigns and the elimination of free-roaming dogs have been somewhat effective in Tunisia
D. The elimination of free-roaming dogs has been largely effective in Egypt

3. Which of the following statements regarding the management of rabies cases in France is most accurate?

A. Most patients in ARMC were exposed to animals outside of France
B. The use of rabies PEP for injuries incurred in other countries increased between 1996 and 2009
C. Sudan is the country at highest risk for rabies exposure requiring rabies PEP
D. The use of rabies PEP is similar regardless of injury in North Africa vs. other international locations

4. Which of the following groups of patients should be most strongly considered for rabies preexposure vaccination prior to travel to North Africa?

A. Tourists visiting for a limited time
B. Older adults
C. Visitors to countries with the mouse brain rabies vaccine
D. Women

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Worldwide Occurrence and Impact of Human Trichinellosis, 1986–2009

CME Questions

1. You are seeing a 40-year-old man with a 1-month history of myalgia, diarrhea, and intermittent fever. He has a history of eating raw meat on a hunting trip 6 weeks ago.

You suspect that this patient might have trichinellosis. What should you consider regarding the epidemiology of trichinellosis in the current study?

A. Trichinellosis was particularly common among Muslims
B. Western Europe had higher rates of trichinellosis compared with Eastern Europe
C. The prevalence of trichinellosis in the United States has risen dramatically
D. Argentina has the highest prevalence of trichinellosis in South America

2. Which of the following epidemiologic trends in trichinellosis was most pronounced in the current study?

A. Children had higher rates of trichinellosis compared with adults
B. Adults had higher rates of trichinellosis compared with children
C. Women had higher rates of trichinellosis compared with men
D. Men had higher rates of trichinellosis compared with women

3. What should you consider regarding the diagnosis and treatment of trichinellosis in this patient?

A. Symptoms of myalgia, diarrhea, and fever are common
B. Symptoms usually resolve within a few days of the initiation of therapy
C. No long-term sequelae to infection have been described
D. Trichinellosis is still usually fatal

4. Which of the following animals was the most common source of trichinellosis in the current study?

A. Horse
B. Dog
C. Cow
D. Pig

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Intestinal Toxemia Botulism in 3 Adults, Ontario, Canada, 2006–2008

Haemophilus influenzae Type b Disease, Transmission, and Vaccine Programs

Accelerating Control of Pertussis in England and Wales

Enhancing Capacity for Pandemic Influenza Surge in Public Health Laboratories

Increase in Invasive Meningococcal Capsular Group Y Disease, England and Wales

The 1918 Influenza Pandemic in Boyacá, Colombia

Serious Invasive Saffold Virus Infections in Children, 2009

Assessing Prion Infectivity of Urine in Sporadic Creutzfeldt-Jakob Disease

High Prevalence of Multidrug-Resistant Tuberculosis, Swaziland, 2009–2010

Differential Mortality for New Zealand Maori in 3 Influenza Pandemics

Dengue Fever Epidemic Viewed through Daily Newspaper, Athens, Greece, 1927–1931

Early Detection of Pandemic (H1N1) 2009 Virus and Oseltamivir Treatment, Bangladesh

Human Herpesvirus 8 Seroprevalence, People's Republic of China

Atypical Presentation and Cranial Nerve Demyelination in Adult with Type F Botulism

Disseminated Infections Caused by *Blastoschizomyces capitatus*, Central Europe

Dengue Outbreak in Key West, Florida, 2009

Legionella longbeachae and Endocarditis

MRSA USA300 at Alaska Native Medical Center, Anchorage, Alaska, 2000–2006

Mutation I117V and Neuraminidase Inhibitor Sensitivity of Pandemic (H1N1) 2009 Viruses

Complete list of articles in the January issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

2012

March 5–8, 2012
19th Conference on Retroviruses and Opportunistic Infections (CROI 2012)
Washington State Convention Center
Seattle, WA, USA
http://www.retroconference.org

March 11–14, 2012
ICEID 2012
Atlanta, GA, USA

June 13–16, 2012
15th International Congress on Infectious Diseases (ICID)
Bangkok, Thailand
http://www.isid.org/15th_icid

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

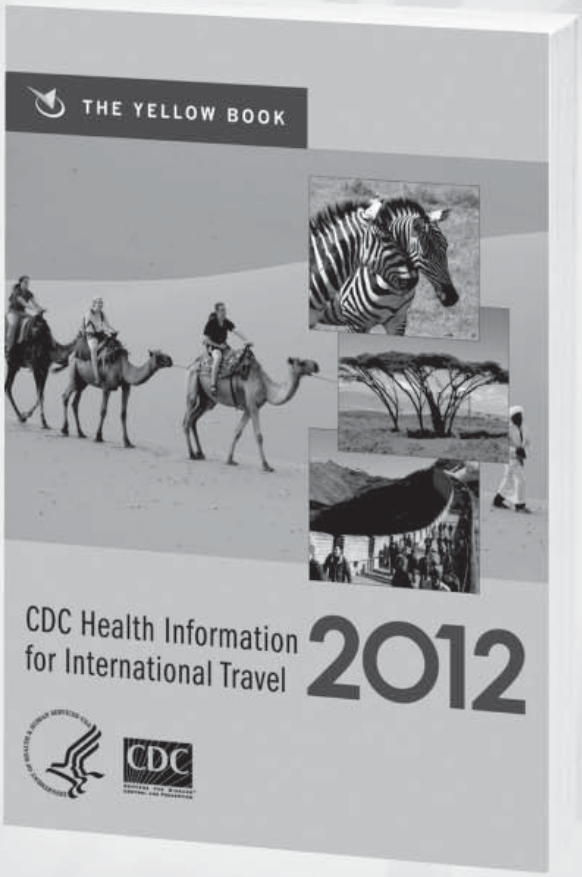
Announcements may be posted on the journal Web page only, depending on the event date.

CDC Health Information for International Travel 2012

CDC

Health risks are dynamic and ever-changing, both at home and while traveling abroad. To stay abreast of the most up-to-date health recommendations, for decades health care professionals and travelers have relied on the Centers for Disease Control and Prevention's user-friendly Health Information for International Travel (commonly referred to as the The Yellow Book) as a trusted reference. Updated biennially by a team of experts, this book is the only publication for all official government recommendations for international travel.

The book's features include clear and easy-to-read disease risk maps, information on where to find health care during travel, specific health information and itineraries for popular tourist destinations, detailed country-specific information for yellow fever and malaria, advice for those traveling with infants and children, and a comprehensive catalog of diseases, their clinical pictures, and their epidemiologies. The Yellow Book addresses the pre-travel consult and provides post-travel clinical guidance on ways to approach common syndromes of returned travelers who are ill.



May 2011 640 pp.
9780199769018 Paperback $45.00

FEATURES

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November 2011

Cholera

Courtesy of Patrick Lemmie

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The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

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Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit figures as separate files, in the native format when possible (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpg or .tif files. Other file formats may be acceptable; contact fue7@cdc.gov for guidance. Figures should not be embedded in the manuscript file. Use color only as needed. Use Arial font for figure lettering. Figures, symbols, lettering, and numbering should be clear and large enough to remain legible when reduced to print size. Large figures may be made available online only. Place figure keys within the figure; figure legends should be provided at the end of the manuscript file.

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Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words 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. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only and should contain 500–1,000 words. They should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.