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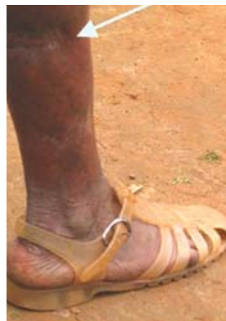

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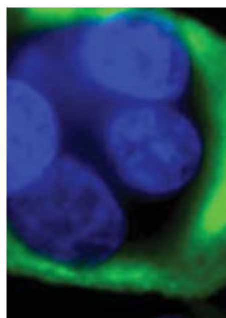

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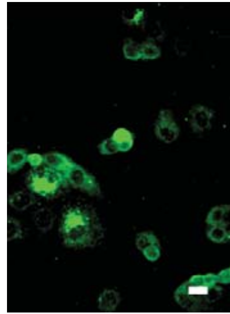

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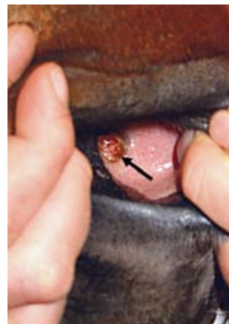

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Threat of Hantavirus Pulmonary Syndrome to Field Biologists Working with Small Mammals

Douglas A. Kelt,* Dirk H. Van Vuren,* Mark S. Hafner,† Brent J. Danielson,‡ and Marcella J. Kelly§

Low risk for hantavirus pulmonary syndrome (HPS) has been reported among biologists engaged in fieldwork with rodents. The overall probability of acquiring HPS when working with rodents appears to be 1 in 1,412 (0.00071). Nonetheless, a causal link between HPS and lack of personal protective equipment (PPE) use is suggested by some investigators. However, supporting data are incomplete and consequently misleading. A recent HPS case was assumed to be acquired during rodent-handling activities, although substantial peridomestic exposure was evident. Regulatory groups interpret inadequate data as evidence of the need for excessive and inappropriate PPE, which can hamper field research and instructional efforts. PPE recommendations should be reviewed and revised to match the risk associated with different types of fieldwork with small mammals.

Hantavirus pulmonary syndrome (HPS) is an uncommon disease associated primarily with exposure to deer mice (*Peromyscus maniculatus*), widespread rodents that serve as the reservoir host for Sin Nombre virus (SNV) (*1*). The virus is shed in the saliva, urine, and feces of the host, and transmission to humans is thought to result primarily from inhaling infectious, aerosolized saliva or excreta, especially when entering or cleaning rodent-infested structures (*2*,*3*). The virus presumably may also be transmitted by a bite from an infected deer mouse, but this type of transmission is considered rare (*4*). The disease is difficult to treat, especially in advanced stages, and mortality rates are ≈30%–35%; thus, prevention is important (*3*). Development of effective preventive measures requires a logical match between mechanisms of transmission and the various protective devices and precautionary measures that have been advocated as well as accurate assessment of occupational risks for exposure.

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Workers who frequently handle wild rodents are presumed to be at greater risk for exposure to SNV (*2*). Thus, in 1994 staff from the Centers for Disease Control and Prevention (CDC) visited several national conferences and took blood samples from field mammalogists whose jobs entailed various levels of direct exposure to small mammals by live and kill trapping. Data for 757 of these donors were recently published in Emerging Infectious Diseases (*5*) and documented that only 4 (0.528%) of 757 active field mammalogists with "a history of exposure to rodents in North America and…of occupational exposure to deer mice …" had positive test results for SNV exposure. The authors concluded (abstract) "that the risk of infection with hantaviruses … is low in persons whose occupations entail close physical contact with … rodents [including deer mice] … in North America." They also cited 3 other studies of workers in occupations with high risk for exposure to rodents in which no SNV-positive cases were documented from 583 (*6*,*7*) and 72 persons (*8*). Summation of results from these 4 studies indicated that only 4 (0.283%) of 1,412 persons in high-risk occupations had antibodies to SNV.

Although their data indicate that fieldwork with mammals has minimal risk for contracting HPS, Fulhorst et al. (*5*) implied a causal link between infection in the 4 HPS-positive mammalogists and their failure to use personal protective equipment (PPE) while handling rodents in the field ("None of the 4 persons in the study who were antibody-positive against SNV had worn gloves, masks, or protective eyewear when handling rodents …"). Such an implication is unwarranted, given that ≈70% of all persons tested by Fulhorst et al. never (or infrequently) wore any protective equipment while handling rodents in the field. Because most of the testing was done before widespread

public awareness of HPS, it is likely that none of the persons tested in 1994 wore protective equipment designed to prevent exposure to hantaviruses in the field.

Only 1 of the 4 SNV-positive persons in the study by Fulhorst et al. reported having been "hospitalized for an illness characterized by fever, headache, and severe shortness of breath (symptoms suggestive of HPS)." The distinction between SNV, the causative agent, and HPS, the manifestation of illness, is important but often overlooked. Whereas 0.528% of samples in the study by Fulhorst et al. had antibodies to SNV, only 0.132% of the persons tested in that study actually exhibited symptoms of HPS. This number decreases to 0.071% (0.00071) if all 1,412 serologic samples (see above) are considered. Thus, in the absence of any data on the proportion of exposed persons (SNV reactive) who become ill with HPS, one could argue that the risk for illness among mammalogists working in the field may be 25% of that reported by Fulhorst et al. Moreover, the single known field mammalogist who contracted HPS (one of the authors, B.J.D.) was living in a mouse-infested building near his field site at the time he was infected.

Fulhorst et al. (*5*) noted that "2 recent HPS cases … underscore the need to use … personal protective equipment and follow recommended safety procedures …" One of these cases was in a field technician who was employed by 2 of us (D.A.K. and D.H.VV.) in a study in the Sierra Nevada of California. Unfortunately, data for this patient were incomplete. Fulhorst et al. (*5*) noted that our employee "was trapping rodents as part of a forest health study in California," but they did not report documented evidence of extended residential exposure to SNV. The implication of Fulhorst et al. was that HPS in this case was acquired through direct contact with rodents in the field. However, our field crew had been living for 2 months in a seasonal cabin that was inhabited by hantavirus-positive deer mice. Testing by the California Department of Health Services Vector-Borne Disease Section (CA-VBDS) documented serum antibodies to SNV in 2 of 4 deer mice trapped in this cabin (*9*). Field sampling by the CA-VBDS resulted in the capture of 50 deer mice, 16 of which (32%) tested positive for antibodies to SNV. These positive samples were found at only 2 of our 18 field sites (in 1 of 5 and 7 of 14 deer mice, respectively) but at all 3 areas sampled at our field camp (3 of 5, 3 of 17, and 2 of 4 deer mice). Field sites ranged from several kilometers to >30 km from our field camp.

Thus, the evidence in this instance points to 2 potential sources of infection: direct handling of rodents in the field or residential exposure to aerosolized hantavirus particles. All data published regarding SNV indicate that the primary route of exposure is by inhalation of aerosolized viral particles in a peridomestic setting (e.g., [*4*]). We acknowledge that our employee may have acquired HPS by occupational exposure in the field, but the available evidence demonstrates that acquisition by peridomestic exposure was at least equally possible. If one considers that 70% of HPS cases are associated with peridomestic exposure (*10*) and that our employee was sleeping, eating, and even shaking out dusty rugs in a cabin inhabited by SNV-positive rodents, a residential source of infection seems most probable. Unfortunately, studies such as that by Fulhorst et al. (*5*) tend to focus the attention of the public and safety administrators on the potential dangers of field mammalogy (e.g., trapping and handling of rodents in the field) and away from the more likely (peridomestic) source of exposure to hantaviruses. Because regulatory bodies, such as institutional animal care and use committees, often "play it safe" by turning CDC safety recommendations into safety requirements for their constituents, many field mammalogists today are required to wear PPE while handling rodents in the field (with a documented 0.071% probability of acquiring HPS [*5*]) but are allowed to sleep and eat unprotected in field cabins potentially infested with viremic rodents.

We are not calling for relaxation or abolition of PPE. Rather, we are trying to emphasize that PPE should be suitable and appropriate for the occupational risk. Additionally, PPE recommendations should be reconsidered when new data suggest that either additional or reduced levels of PPE are warranted. Field work on mammals that involves virologic or blood sampling or other direct contact with body fluids and organs almost certainly involves greater risk for exposure to SNV than mark-recapture live-trapping studies in which the greatest contact with bodily fluids is with rodent urine or during application of an eartag. Although nitrile or latex gloves are reasonable PPE for protecting skin from urine, use of surgical gowns, shoe covers, and high-efficiency particulate air filter–fitted respirators (all recommended by Mills et al. [*2*]) are likely inappropriate and excessive relative to the risk associated with handling, marking, and releasing small mammals in open-air conditions. Current CDC recommendations do not distinguish between invasive and noninvasive studies. As such, these recommendations for PPE against the entire array of potential risk factors lead to cumbersome and likely ineffective PPE for students learning to live-trap small mammals and field workers wishing merely to apply an eartag and release the animal. For such activities, we believe that available data strongly argue against the PPE recommendations currently provided by CDC.

SNV has been present in North America for a long time (*3*,*11*), and all evidence indicates that it will continue to show cyclic increases and decreases in rodent populations coincident with increases and decreases in rodent density (*11*). This virus is unstable in the presence of sunlight, detergents, bleach, and other agents (*12*); combined data of Vitek et al. (*6*), Zeitz et al. (*7*), Fritz et al. (*8*), and Fulhorst et al. (*5*) document that HPS is an uncommon disease that

is difficult to acquire by handling rodents in the field. Although the mortality rate for persons hospitalized with HPS remains high, awareness of HPS symptoms and treatments of the disease are improving, as is prognosis for recovery if the disease is diagnosed promptly.

Fulhorst et al. (*5*) document that field research in ecology and biology of small mammals, including deer mice, poses an extremely low risk for field workers. However, efficacy of protective equipment in reducing exposure to SNV in the field remains unknown. We call for increased objectivity in future studies of HPS risk, especially with regard to possible sources of infection. Open communication between field biologists involved in HPS cases, CDC, and healthcare professionals investigating these cases would be in the best interest of all parties.

Acknowledgment

We thank 2 anonymous reviewers for helping us clarify and hone our message.

Dr Kelt is professor of wildlife ecology at the University of California, Davis. His research interests include population and geographic ecology and conservation of mammals.

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Frequent Travelers and Rate of Spread of Epidemics

T. Déirdre Hollingsworth,* Neil M. Ferguson,* and Roy M. Anderson*

A small proportion of air travelers make disproportionately more journeys than the rest of travelers. They also tend to interact predominantly with other frequent travelers in hotels and airport lounges. This group has the potential to accelerate global spread of infectious respiratory diseases. Using an epidemiologic model, we simulated exportation of cases from severe acute respiratory syndrome–like and influenza-like epidemics in a population for which a small proportion travel more frequently than the rest. Our simulations show that frequent travelers accelerate international spread of epidemics only if they are infected early in an outbreak and the outbreak does not expand rapidly. If the epidemic growth rate is high, as is likely for pandemic influenza, heterogeneities in travel are frequently overwhelmed by the large number of infected persons in the majority population and the resulting high probability that some of these persons will take an international flight.

In today's world of increasing air travel for both business and pleasure, a small proportion of persons make disproportionately more journeys than the rest of the population (*1*,*2*). These frequent fliers tend to travel for business purposes and mix predominantly with other business travelers, stay in particular hotels, and use specific airport lounges. This form of assortative (like with like) mixing means a respiratory infection could potentially spread quickly within this group and thus be disseminated rapidly between countries. This rapid spread was illustrated early in the severe acute respiratory syndrome (SARS) outbreak of 2003. The index SARS case in Hong Kong Special Administrative Region, People's Republic of China, stayed in a hotel and infected 16 persons there. Of these patients with secondary cases, 6 took international flights to Australia, Canada, Singapore, the Philippines, and Vietnam (*3*). The arrival of these infected persons subsequently led to SARS outbreaks in Hanoi, Singapore, and Toronto within a few days of the first case in Hong Kong.

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Recent studies of the role of international air travel on the spread of infectious diseases have highlighted the role of heterogeneities in the connectedness of different airports (*4*–*6*), the length of the latent period of the disease in relation to the duration of the flight (*7*), the possible role of travel restrictions (*8*–*11*) and the role of cooperative strategies to control international spread of pandemic influenza (*10*,*12*). To date, none of these studies has taken into account the effects of heterogeneity in the frequency of travel between persons and the potential role of such heterogeneity on the global spread of a directly transmitted infectious agent. Also of interest is whether targeting interventions specifically at frequent travelers would slow the international spread of a defined pathogen.

Methods

To investigate the role of frequent travelers in the exportation of asymptomatic cases during the early stages of an epidemic, we simulated outbreaks of both a SARS-like and an influenza-like airborne respiratory infection in a population in which a small proportion of the population make many more trips than the rest of the population. In the early stages of an epidemic, chance events are important because the number of infected persons is small. We simulated these early stages by using a stochastic model for which every simulation is different. We present both the mean behavior of the simulations and the range of possible outcomes across a large number of simulations. In a stochastic model, introduction of 1 infected person has a finite probability of resulting in the rapid extinction of an infectious disease. To increase the probability of initiating an outbreak, we introduced 3 asymptomatic persons into the

population. We simulated the outbreak in a large extended metropolitan area with a population of 10^7 persons.

The structure of the model is illustrated schematically in Figure 1A. The population is divided into 2 subpopulations with different frequencies of taking international flights. A small proportion of the population, *r*, are high-frequency fliers. Most of the population, 1 – *r*, are low-frequency fliers. Frequent fliers have contact with other frequent fliers and with the general population. Similarly, the general population has contact with persons in the general population and with frequent fliers. Contacts are more likely to be between persons within each group (frequent fliers or general population), but the level of this assortativeness may vary (parameterized by ϕ). Contacts may be made completely randomly, with the likelihood of meeting a person from the frequent-flying group or the general population being proportional to the number of persons in each population ($\phi = 1$). At the other extreme, persons may only have contact with other persons in the same group ($\phi = 0$). The true mixing pattern is likely to lie between these 2 extremes.

The extent to which the high-frequency and low-frequency fliers mix will determine how quickly a disease will spread from the general population to the frequent fliers and vice versa. We simulated the model for a selection of mixing parameters, ranging from wholly random ($\phi = 1$) to moderate and high levels of assortativeness ($\phi = 0.5$, 0.25, respectively). For comparison, we also simulated a homogeneous model in which the entire population travels equally frequently.

The outbreak is modeled by dividing the population into those who are still susceptible to the disease, those who have contracted the disease and are in the latent stage, those who are infectious and symptomatic, and those who have recovered from the disease (Figure 1). This division is similar to the basic structure used in several recent papers on the role of international travel in the spread of infectious diseases (*8*,*9*,*12*). This model structure can be adapted to many airborne infections because it allows for an asymptomatic period, which may or may not be infectious, followed by a potentially symptomatic period during which transmission can also occur.

In our stochastic model, events (such as infection or a person leaving the source area) occur by chance. For example, the time after symptom onset at which a person recovers from infection with SARS is not a fixed quantity; rather, it is a randomly chosen time from an exponential probability distribution with a mean of 10 days. Table 1 shows the average latent and infectious periods used. The probability of leaving the country is constant for all persons (Table 1). The probability of a susceptible person becoming infected increases as a larger proportion of the population becomes infected and is chosen so that the average number of new

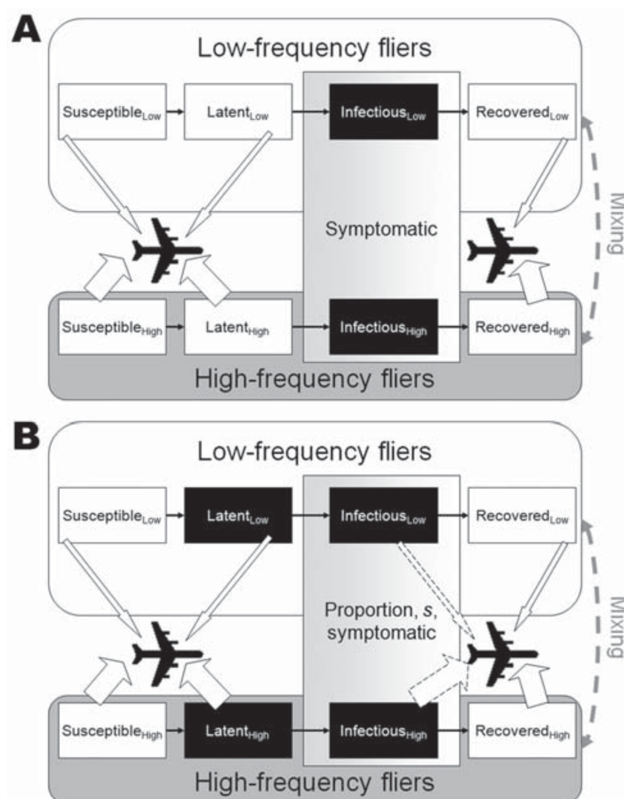

Figure 1. Schematic representation of the model structure. Black boxes represent infectious stages and arrows indicate that persons in these stages are allowed to fly. A) Severe acute respiratory syndrome. Persons with latent infections are not infectious, and all infectious persons are symptomatic and prevented from traveling. B) Possible scenario for pandemic influenza. Persons with latent infections are infectious, and a proportion (1 – *s*) of infectious persons are asymptomatic and allowed to travel (indicated by the dotted arrows). The size of the arrows indicates that the persons in the high-frequency flier group have a higher probability of flying per day.

infections caused by each infected person in the early stages of the epidemic is equal to the basic reproductive number R_0 (2.5 for SARS, 1.8 for influenza; Table 1). The epidemic is simulated by evaluating the probability that any person is infected, becomes symptomatic, or recovers in any short time interval (we divide time into sequential short intervals of one fiftieth of a day), and then testing whether that event occurs. The simulation can be thought of as generating a random number between 0 and 1 for each person in each time step. If this random number is less than the probability of a particular event occurring to that person, then the event occurs. Otherwise, the person is left in his or her current state. The model does not store the details of every person separately but keeps track of the number of persons who are susceptible (*S*), latently infected (*E*), infectious (*I*), and recovered (*R*) at any point in time. As events occur, these variables change. For example, when a person becomes infected, *S* decreases by 1 and *E* increases by 1. Because the

Table 1. Parameter descriptions and values for epidemiologic model that simulates exportation of cases from SARS-like and influenza-like epidemics*

Description	Parameter	Value (reference) SARS	Influenza
Infection			
Basic reproductive number	R_0	2.5 (*13*)	1.8 (*14*)
Latent period, d	T_L	4 (*13*)	1.5 (*14*)
Infectious period, d	T_I	10 (*13*)	1.1 (*14*)
Generation time, d	$T_g = T_L + T_I$	14	2.6
Epidemic doubling time, d	$t_d = T_g / (R_0 - 1) \ln 2$	6.5	2.3
International travel			
Proportion of population who are high-frequency fliers	r	0–0.5	
Mixing between groups: $\phi = 1$, random mixing; $\phi = 0$, assortative mixing	ϕ	0–1	
Relative probability of flying of high-frequency fliers	f	20	
Mean probability of flying per day	ε	0.005 (*9*)	
Probability of flying per day of high-frequency fliers	$\varepsilon_H = f / 1 + (f-1) r \varepsilon$	0.084	
Probability of flying per day of low-frequency fliers	$\varepsilon_L = 1 / 1 + (f-1) r \varepsilon$	0.042	
Probability of a case being exported			
Homogeneous flying patterns	$L = T_L \varepsilon$	0.02	0.008
High-frequency fliers	$l_H = T_L \varepsilon_H$	0.34	0.13
Low-frequency fliers	$l_L = T_L \varepsilon_L$	0.017	0.006

*SARS, severe acute respiratory syndrome.

events occur by chance, the total number of persons who are in each state, including the number of infected persons taking flights, varies stochastically.

In our model, we assume that those who are in the latent stage of the disease are not infectious for SARS and influenza. This is generally accepted to be a good model for SARS because isolation of symptomatic persons prevented onward transmission of SARS, which indicated that the latent period has limited or no infectivity (*15*). We also assume that all infectious persons are symptomatic. This is a conservative assumption, but serosurveillance studies for SARS have shown low prevalence of seropositivity in persons who did not show symptoms of disease (*16–21*). Lastly, we assume that all symptomatic persons are prevented from traveling because of symptom severity or effective screening. The model equations are shown in the online Technical Appendix (available from www.cdc.gov/EID/content/13/9/1288-Techapp.pdf).

The disease course of a possible future influenza pandemic is not known. However, studies of previous pandemics and seasonal epidemics suggest a possible scenario in which the latent period of influenza may be infectious and not all infected persons will show symptoms (*14*,*22–24*). This means that a larger proportion of cases could be allowed to travel on international flights, even with 100% effective screening, because they are asymptomatically infected (Figure 1, panel B). We have modeled a conservative scenario, in which influenza has a disease life history similar to that of SARS, but with shorter latent and infectious periods (Table 1). The inclusion of partially effective screening or, equivalently, the inclusion of asymptomatic cases would lead to more cases being exported than is shown here.

Little data are available across a population for the relative frequency of flying. The mean probability of flying for the whole population can be approximated by the number of airline passengers divided by the population of a country or city. This calculation gives estimates of 0.005 for Hong Kong, 0.0005 for Beijing, and 0.0002 for Thailand (*9*). We modeled a population of 10 million persons with a 0.005 probability of flying per day as an example of an outbreak in a well-connected city. A study on domestic flying in Norway suggested that ≈2% of a survey population who take domestic flights in Norway make >20 journeys a year (*1*). This survey did not include persons who do not take flights. Therefore, the proportion of the total population who make this many journeys is likely to be lower. On the basis of this data, we present results for a population in which 1% of the population travel 20× more frequently than the rest of the population and discuss results for different values of these parameters.

We investigated the effect of setting where the outbreak is initiated by using 2 scenarios. In the first scenario, the outbreak begins among the general, infrequently flying population. Cases subsequently occur among high-frequency fliers as a result of contact between the 2 subpopulations. The mean time until the first high-frequency flier becomes infected is a function of the incidence rate in the main population and level of mixing between the 2 groups. In the second scenario, the outbreak begins among the high-frequency fliers. The disease again spreads to the main population because of contacts between the groups, with the mean time until this occurs being a function of the incidence rate in the main population and the level of mixing between the 2 groups.

The mean cumulative number of cases exported (across 50,000 simulations) is presented for both SARS-like and influenza-like parameters (Table 1), for initiation of the epidemic among the low-frequency and high-frequency fliers, and for a range of mixing between the high-frequency and low-frequency travelers. We also illustrate variability in simulated outcomes by presenting snapshots of the distributions of the cumulative number of exported cases.

Results

As an epidemic progresses, the cumulative number of cases increases, and therefore the number of asymptomatic cases exported from a source area increases for all travel patterns (Figure 2). If a SARS-like epidemic is seeded in the group of frequent fliers, then the initial rate of international spread is accelerated relative to the rate for the homogeneous case (Figure 2, panel A, open symbols). If the frequent travelers contract the infection early, more exclusivity of mixing (smaller ϕ) serves to speed international spread, and this effect may last well into the epidemic (Figure 2, panel A, open triangles). If the epidemic is initiated in the low-frequency fliers, the mean number of exported cases is similar to results of the homogeneous model (Figure 2, panel A, closed symbols). Heterogeneities in travel patterns increase the variability between simulated epidemics; higher variability results from more assortative mixing (Table 2; online Appendix Figure 1, available from www.cdc.gov/EID/content/13/9/1288-appG1.htm).

In an outbreak in which the infection spreads rapidly, such as could potentially occur with pandemic influenza A (Figure 2, panel B), heterogeneities in travel patterns have less effect on the rate of exportation of cases early in the epidemic than they would for SARS (Figure 2, panel A), particularly after the first weeks of the epidemic. The overall pattern of the exportation of cases is similar for SARS and influenza, but the time scale for influenza is much shorter because of the short doubling time (Table 1). For example, the number of exported cases is in the thousands for influenza by day 50 (Figure 2, panel B), when it is <20 for SARS (Figure 2, panel A).

Later in an epidemic, the mean number of exported cases is similar, regardless of where the epidemic is seeded or the mixing patterns of the high-frequency fliers and low-frequency fliers (Figure 2, panel B, inset for influenza, not shown for SARS). The variability between simulated epidemics becomes large, with some simulations resulting in hundreds of exported cases and many resulting in only a few exported cases (Table 2; online Appendix Figure 1, panel D).

Heterogeneities in travel patterns increase the number of exported cases to a greater extent and for a longer period if the relative frequency of flying of the high-frequency fliers, f, is higher or if the proportion of the population who are high-frequency fliers, r, is smaller (online Appendix Figure 2, available from www.cdc.gov/EID/content/13/9/1288-appG2.htm) because the probability that any frequent flier will fly per day is higher (Table 1, ε_H). However, if r becomes small, the epidemic among this group peaks and then decreases quickly because of the limited number in the group. In this case, the period in which there are enough infected persons in this group who can contribute to an increased rate of spread of exportation of cases is short (online Appendix Figure 2, panel B).

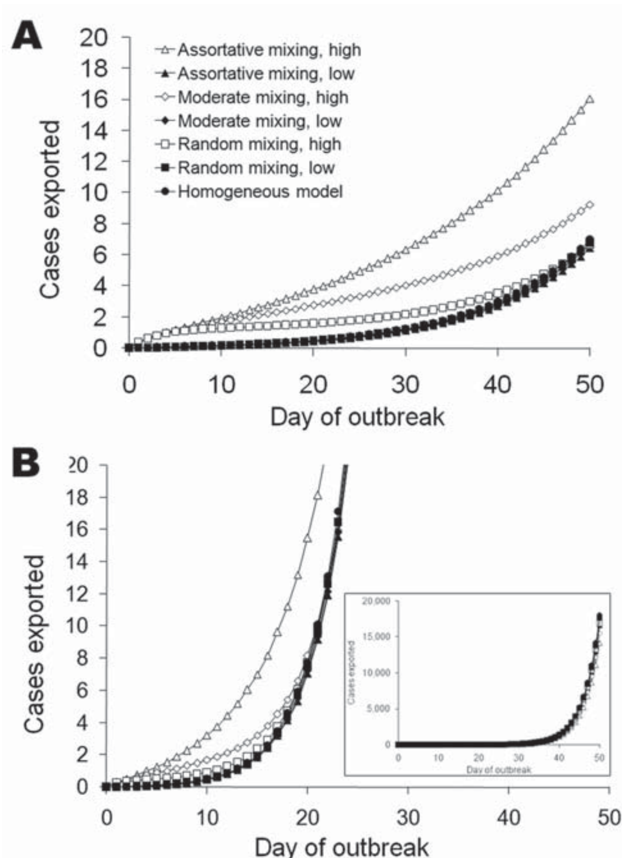

Figure 2. Mean number of cases exported from a single simulated source epidemic for severe acute respiratory syndrome–like parameters (A) and influenza-like parameters (B) (50,000 runs; parameters are listed in Table 1). Results are shown for a population in which everyone travels equally frequently (homogeneous model, circles), for a population in which 1% travel 20 times more frequently than the rest of the population, and for the 2 populations mixing randomly (ϕ = 1, squares), for moderate levels of mixing between the groups (ϕ = 0.5, diamonds), and for low levels of mixing in which most contacts are assortative (ϕ = 0.25, triangles). The first cases are either in the majority population of low-frequency fliers (solid symbols) or high-frequency fliers (open symbols). Inset in B shows a greater range on the y-axis. Variability about these means is shown in Table 2 and online Appendix Figure 1.

Table 2. Variability between runs in an epidemiologic model that simulates exportation of cases from SARS-like and influenza-like epidemics*

Mixing pattern	First case	No. cases exported, mean, median (5th–95th percentile) Day 10	Day 20	Day 30	Day 40	Day 50
SARS						
Homogeneous flying patterns		0, 0 (0–0)	0, 0 (0–1)	1, 0 (0–3)	3, 1 (0–7)	7, 5 (1–16)
Random mixing	High	1, 0 (0–2)	2, 0 (0–2)	2, 1 (0–3)	4, 2 (1–7)	7, 5 (2–14)
	Low	0, 0 (0–0)	0, 0 (0–1)	1, 0 (0–3)	3, 1 (0–6)	7, 5 (1–15)
Moderately assortative	High	2, 1 (0–3)	3, 2 (1–4)	4 (3, 1–7)	6 (4, 2–12)	9, 7 (2–20)
	Low	0, 0 (0–0)	0, 0 (0–1)	1, 0 (0–2)	3, 1 (0–6)	7, 5 (0–15)
Highly assortative	High	2, 1 (0–3)	4, 2 (1–7)	5, 5 (2–13)	10, 8 (3–22)	16, 12 (4–38)
	Low	0, 0 (0–0)	0, 0 (0–1)	1, 0 (0–2)	3, 1 (0–6)	6, 4 (1–15)
Influenza						
Homogeneous flying patterns		1, 0 (0–1)	8, 5 (0–20)	107, 85 (1–251)	1,268, 1,069 (7–3,118)	15,729, 13,541 (73–35,132)
Random mixing	High	1, 0 (0–2)	7, 5 (0–18)	89, 74 (1–233)	1,341, 940 (1–3,049)	14,592, 11,990 (1–35,632)
	Low	0, 0 (0–1)	7, 5 (0–18)	95, 78 (1– 246)	1,264, 1,057 (7–3,256)	15,668, 13,651 (74– 35,231)
Moderately assortative	High	2, 0 (0–3)	8, 6 (0–32)	93, 72 (1–231)	1,288, 1,138 (1–3,387)	15,505, 14,362 (1–32,134)
	Low	1, 0 (0–1)	7, 5 (0–20)	104, 83 (0–264)	1,411, 1,213 (0–3,526)	17,081, 15,850 (0–35,403)
Highly assortative	High	3, 2 (0–7)	15, 10 (2–41)	106, 81 (2–291)	1,166, 840 (2–2,923)	14,145, 10,770 (2–34,351)
	Low	0, 0 (0–2)	12, 0 (0–33)	164, 139 (0–246)	1,312, 967 (1–3,231)	16,592, 12,607 (28–36,643)

*Means are shown in Figure 2 and distributions in online Appendix Figure 1. SARS, severe acute respiratory syndrome.

Discussion

The probability that an infected person will make an international flight while still incubating infection and nonsymptomatic is higher for a high-frequency flier than for a low-frequency flier (Table 1). In the early stages of an epidemic in which most cases occur in high-frequency fliers, the expected number of cases exported will therefore be higher than if the early cases occur in predominantly low-frequency fliers (Figure 2). Heterogeneity in flying patterns also increases the variability between simulated outbreaks (Table 2; online Appendix Figure 1).

Wherever the epidemic is initially concentrated, the disease will spread to all parts of the population because of contacts between persons in both groups. The speed with which this occurs will be a function of the level of mixing between the groups. If high-frequency fliers mix almost exclusively among themselves, they are unlikely to acquire cases early in an epidemic in which the first cases emerge in the general population. If, however, they contract the infection early, this exclusivity serves to speed international spread and this effect may last well into the epidemic (Figure 2, open triangles). If mixing is less assortative, then the epidemic will spread to the general population more rapidly. Because most of the population are low-frequency fliers, the number of infected persons in the main population will quickly exceed those in the small group of high-frequency fliers.

When the number of cases becomes large, the expected number of exported cases (which may be approximated as the probability of flying while asymptomatic multiplied by cumulative incidence [*9*]) will be large, even if the probability that any person travels is small. Once the epidemic takes hold in the general population, the number of cases being exported from the majority low-frequency flier population exceeds those being exported from the much smaller group of high-frequency fliers. Regardless of where most initial cases occur, the contribution of high-frequency fliers to international spread is eventually overwhelmed by the large epidemic in the general population, despite their lower probability of flying per day. Thus, the average behavior of epidemics is eventually similar, whether they start in high-frequency fliers, or in groups with no heterogeneities in travel (Figure 2, panel B, inset), but the variability between simulations is large (Table 2; online Appendix Figure 1).

The latent period for influenza is likely to be shorter than that for SARS, which reduces the probability that any infected person will travel before exhibiting symptoms (Table 1). However, the doubling time for an influenza pandemic is less than half that for SARS because of the much shorter generation time for influenza (Table 1). Therefore, the number of cases exported from a local influenza epidemic will increase far more rapidly than those from a SARS epidemic (Figure 2, panel B). This rapid growth means that any increased rate of export caused by early concentration

of infection among the high-frequency fliers will be quickly overcome by the number of cases being exported from the general population (Figure 2, panel B), which indicates that heterogeneities in travel have little effect.

We have simulated an outbreak in a single population by using a relatively simple model. Similar models have been used for the dynamics of single epidemics in a network of countries or areas connected by a complex airline network (*6*,*8*,*12*), and more complex, person-based, within-country models have been used to simulate epidemics within smaller groups of countries (*10*,*14*). Our results show that in the event of an influenza pandemic, interventions such as travel restrictions will have to be implemented rapidly and effectively to have a substantial effect (*8*–*10*,*12*). We have shown that high-frequency fliers have the potential to spread infection even more rapidly than previously indicated by models that assume homogenous travel behavior.

Our study and the relatively simple structure of the model were limited by the lack of available data on the travel patterns of persons. Travel patterns may vary with age, sex, occupation, and district or country of origin. To increase our knowledge of these patterns, existing surveys of airline passengers at airports could be extended to ask additional questions on number of journeys per year. However, these surveys would necessarily omit those persons who do not take international flights, who are believed to make up a large proportion of many populations. Any additional information could be valuable for assessing the risk for international spread of diseases from affected areas.

The SARS epidemic in Hong Kong satisfied the criteria we have identified for frequent travelers to accelerate the international spread of an outbreak. The first case-patient with SARS in Hong Kong had contact with other frequent travelers in a hotel and seeded the epidemic in high-frequency travelers. However, SARS has long incubation and infectious periods and only moderate transmissibility. For influenza A, which has much shorter incubation and infectious periods, such heterogeneities have a limited effect on the rate of exportation of cases. Because frequent travelers play a role mainly in the early stages of an epidemic, targeting interventions to these persons is unlikely to be an effective control strategy because such a plan would have to be in place almost immediately.

Finally, estimates of the rate of international spread of respiratory infections that do not consider heterogeneities in behavior may be misleading. If an outbreak begins in a rural area, where persons have a low probability of traveling abroad and mixing with frequent fliers, the time until cases are exported is longer than in outbreaks in which frequent travelers contract infection early in the course of the outbreak. When combined with the vagaries of chance early in the evolution of a new epidemic and the complexities of the international airline network, this variability makes early prediction of the pattern and speed of global spread difficult. This difficulty in predicting whether a particular country is likely to import cases from a currently unknown source area highlights the need for developing a strategy for controlling an outbreak caused by imported cases.

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Dr Hollingsworth is a mathematical modeler at Imperial College London. Her research interests include developing models for the design of effective interventions to control epidemic outbreaks of directly transmitted pathogens.

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Midnight Cave, Texas: The Experiment

— For Michel Siffre

Al Zolynas

A man descends into a cave
long abandoned by bats. For six months
the electrodes and wires of science
bristle from his head.
in the dark chest of the earth,
a hundred feet beneath the seasons
and with no clock but
the wound timepiece of himself,
he seeks his own rhythms.
Above him colleagues monitor
his vital functions
and turn the lights on
and off at his request.
His dreams, of course, are his own,
part of the self's short-circuit,
not to be monitored by the surface crew.
After the 130th cycle
there are no days), after waking
in panic in absolute darkness,
he writes, "When you find yourself
alone, isolated
in a world totally without time,
face-to-face with yourself, all
the masks that you hide behind——
those that preserve your own illusions,
those that protect them before others——
finally fall, sometimes brutally."
The man sits on a rock
in the circle of light
around his pale-blue tent
for a succession of eternities
swaying mindlessly. He daydreams
of the dense jungles of Guatemala,
the sunlight filtering
through wet leaves. His boyhood
fantasy of finding Mayan relics
somehow sustains him:
"I will go to Central America
and I will regain control of my soul."
On the floor of the cave
the dust of ancient bat guano
filters, particle by fine particle,
through itself.

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Detection of Group 1 Coronaviruses in Bats in North America

Samuel R. Dominguez,*[1] Thomas J. O'Shea,†[1] Lauren M. Oko,* and Kathryn V. Holmes*

The epidemic of severe acute respiratory syndrome (SARS) was caused by a newly emerged coronavirus (SARS-CoV). Bats of several species in southern People's Republic of China harbor SARS-like CoVs and may be reservoir hosts for them. To determine whether bats in North America also harbor coronaviruses, we used reverse transcription–PCR to detect coronavirus RNA in bats. We found coronavirus RNA in 6 of 28 fecal specimens from bats of 2 of 7 species tested. The prevalence of viral RNA shedding was high: 17% in *Eptesicus fuscus* and 50% in *Myotis occultus*. Sequence analysis of a 440-bp amplicon in gene *1b* showed that these Rocky Mountain bat coronaviruses formed 3 clusters in phylogenetic group 1 that were distinct from group 1 coronaviruses of Asian bats. Because of the potential for bat coronaviruses to cause disease in humans and animals, further surveillance and characterization of bat coronaviruses in North America are needed.

Emerging diseases are frequently zoonoses caused by RNA viruses (*1*,*2*). Defense against emerging infectious diseases, identification of reservoirs for such viruses, surveillance for host-jumping events, and elucidation of viral and host factors that may facilitate such events are warranted. The epidemic of severe acute respiratory syndrome (SARS) in 2002–2003 was caused by a newly emerged zoonotic coronavirus (SARS-CoV) (order Nidovirales, family *Coronaviridae*, genus *Coronavirus*). Other coronaviruses have also jumped to new host species and caused emerging diseases. For example, porcine epidemic diarrhea virus emerged in European pigs from an unknown host species during the late 1970s and caused severe enteric disease (*3*). Human coronavirus OC43 is believed to have been derived from bovine coronavirus (*4*). In addition, the genomes of canine and feline coronaviruses can recombine in vivo and have developed into different biotypes that are serially transmissible in their new host species (*5*).

SARS-CoV entered the human population as a result of a zoonotic transmission in southern People's Republic of China in 2002. Epidemiologic studies demonstrated that the first human cases of SARS were caused by coronaviruses closely related to viruses found in masked palm civets (*Paguma larvata*) and raccoon dogs (*Nyctereutes procyonoides*) in live animal markets (*6*). Subsequently, surveys of coronaviruses in domestic animals, livestock, poultry, and wildlife were conducted in Southeast Asia to identify the reservoir(s) of SARS-CoV. On the basis of low prevalence of SARS-like CoVs in wild and farmed masked palm civets, these animals are now believed to be an intermediate host rather than a primary reservoir for SARS-CoV (*7*). During these surveys, a wide variety of coronaviruses were detected in many bat species in Asia (*8*–*11*).

Horseshoe-nosed bats of several species (suborder Microchiroptera, family Rhinolophidae, genus *Rhinolophus*) from different locations in southern People's Republic of China and the Hong Kong Special Administrative Region were found to be infected with SARS-like CoVs, and some of the bats had antibodies to these newly recognized coronaviruses (*10*,*12*). Phylogenetic analysis of the complete genome sequences of the bat SARS-like CoVs showed that they form a large and diverse clade within phylogenetic group 2b (also called group 4), which includes SARS-CoVs from palm civets and humans obtained during the 2002–2003 outbreak (*10*,*12*,*13*). Thus, the virus responsible for the SARS pandemic may have originated from bats, perhaps with the palm civet as an intermediate host. In addition to SARS-like CoVs, RNAs of many other viruses

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belonging to coronavirus groups 1 and 2a, and proposed new group 5, were detected in several species of Asian bats (*8*,*9*,*14*,*15*). To date, no infectious bat coronavirus has been isolated in cell culture.

We investigated whether bats in North America also harbor coronaviruses. To our knowledge, we provide the first evidence of coronaviruses in bats in the Western Hemisphere. We studied oral, anal, and fecal specimens from 57 bats in the Rocky Mountain region and detected coronavirus RNA in 6 of 28 fecal specimens from 2 of 7 bat species tested. Limited sequence analysis showed that these viruses are in phylogenetic group 1 and that they differ from group 1 coronaviruses of Asian bats.

Materials and Methods

Sample Collection

Bats were sampled at 4 sites in the Rocky Mountain region in August 2006. At sites 1 and 2, bats of 2 species were sampled in colonies inhabiting 2 buildings 480 km apart on opposite sides of the continental divide of the Rocky Mountains. Eight occult myotis (*Myotis occultus*) and 1 Brazilian free-tailed bat (*Tadarida brasiliensis*) were captured in mist nets as they emerged from a roost in a building in Mancos in Montezuma County in southwestern Colorado (site 1) at dusk on August 19. *M. occultus* was previously thought to be conspecific with the little brown bat (*M. lucifugus*) that is common throughout North America (*16*). Big brown bats (*Eptesicus fuscus*) were sampled at a roost in a building in Fort Collins in Larimer County in north-central Colorado (site 2) on August 7. Other bats (n = 27) were sampled at sites 3 and 4 incidental to ongoing, unrelated bat faunal surveys. One western small-footed myotis (*M. ciliolabrum*) and 1 long-eared myotis (*M. evotis*) were captured in mist nets over water on August 8 at Soapstone Prairie Natural Area in Larimer County (site 3). Four big brown bats, 3 long-eared myotis, 8 occult myotis, 1 Brazilian free-tailed bat, 7 long-legged myotis (*M. volans*), and 2 silver-haired bats (*Lasionycteris noctivagans*) were trapped in mist nets during the nights of August 14–20 as they drank or foraged near open water at 2 sewage treatment lagoons (9 km apart) (site 4) in Montezuma County, Colorado. Bats were captured under authority of a Colorado Division of Wildlife Scientific Collection License following procedures approved by the Institutional Animal Care and Use Committee of the US Geological Survey, Fort Collins Science Center. Typically, each bat was sampled within 5–10 minutes of capture and then released.

Whenever possible, 3 sample types were taken from each bat (Table). Sterile calcium alginate swabs were used for oral or anal area samples that were immediately placed into 2 mL of RNAlater (Ambion, Austin, TX, USA). Fecal samples were collected if the bat produced a fresh bolus during handling. Disposable latex gloves were changed between samples, and multiple forceps used to collect fecal boluses were rinsed, wiped in ethanol, and air-dried between samples. Samples were numbered, kept in a cooler in the field, stored at 4°C, and delivered to the laboratory on August 28.

RNA Extraction and Reverse Transcription (RT)

RNA from 140 µL of each of the 79 samples was extracted by using the QIamp viral RNA mini kit (QIAGEN, Valencia, CA, USA) following the manufacturer's instructions. Extracted RNA was eluted in 50 µL of RNase-free water and stored at –80°C. We used Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random hexamers in a 20-µL reaction to generate cDNAs by using 10 µL of RNA as a template according to the manufacturer's instructions. All samples were extracted and analyzed in triplicate. RT products were stored at –20°C.

PCR and Sequencing

All samples were screened by PCR and nested PCR. On the basis of previous reports, PCR with a pair of consen-

Table. Reverse transcription–PCR analysis of coronaviruses in Rocky Mountain bats*

Location	Bat species	No. bats tested	Anal (positive/total)	Oral (positive/total)	Fecal (positive/total)	Positive samples
Site 1	*Tadarida brasiliensis*	1	ND	0/1	ND	
	Myotis occultus	8	0/3	0/6	1/2	Bat 27
Site 2	*Eptesicus fuscus*	21	0/21	ND	1/3	Bat 65
Site 3	*M. ciliolabrum*	1	ND	ND	0/1	
	M. evotis	1	ND	ND	0/1	
Site 4	*E. fuscus*	4	0/1	0/2	0/3	
	M. evotis	3	0/2	0/1	0/1	
	Lasionycteris noctivagans	2	ND	0/2	0/2	
	M. volans	7	0/2	0/4	0/6	
	T. brasiliensis	1	ND	0/1	0/1	
	M. occultus	8	ND	0/5	4/8	Bats 3, 6, 11, 48
	Total	57	0/29	0/22	6/28	

*ND, not determined (no samples available for analysis).

sus primers that target a highly conserved region of coronavirus gene *1b* was used to screen the cDNA samples (*8*). Three microliters of cDNA was amplified in a 50-μL reaction containing 1.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleoside triphosophates, 2.5 U of HotStarTaq (QIAGEN), and 0.2 μmol/L of primers 1 and 2: 5′-GGTTGGGACTATCCTAAGTGTGA-3′ (primer 1) and 5′-CCATCATCAGATAGAATCATCATA-3′ (primer 2) by using the following PCR program: 15 min at 95°C; 45 cycles for 1 min at 95°C, 1 min at 48°C, and 1 min at 72°C; and 10 min at 72°C.

For nested PCR, 5 μL from each PCR was amplified in a 50-μL reaction with primer 2 and primer 3 (5′-GTTGTACTGCTAGTGACAGG-3′), an internal primer based on nucleotide sequences of the PCR amplicons by using 40 cycles of the same PCR program. All RT-PCRs were conducted in an enclosed nucleic acid workstation equipped with a UV light (Clone Zone; USA Scientific, Ocala, FL, USA) in a room separate from the main laboratory. Water controls in all RT-PCRs did not show false-positive results. To overcome possible PCR inhibitors in fecal samples, PCR was performed both on the cDNA and on a 1:10 dilution of the cDNA. Amplicons were analyzed by agarose gel electrophoresis. For each positive specimen, amplicons from 2 independent RT-PCRs were sequenced on an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) at the University of Colorado Health Science Center Cancer Center DNA Sequencing and Analysis Core. Numbered specimens were then correlated with lists of bat samples.

Data Analysis

Viral sequences were analyzed and aligned by using ClustalW (http://workbench.sdsc.edu). Phylogenetic trees were constructed by using the neighbor-joining method in the program PAUP* version 4.0 (Sinauer Associates, Inc., Sunderland, MA, USA) rooted with porcine respiratory and reproductive syndrome virus (GenBank accession no. NC_001961). Sequences used for alignment were AF304460 (HCoV-229E), AY567487 (HCoV-NL63), DQ648858 (BtCoV 512), AY594268 (BtCoV HKU2), DQ249224 (BtCoV HKU6), DQ249226 (BtCoV HKU7), and DQ249228 (BtCoV HKU8). The deduced sequences from this study were deposited in GenBank under accession nos. EF544563–EF544568.

Results

Identification of Rocky Mountain Bat Coronaviruses (RM-Bt-CoVs)

A total of 79 samples (28 fecal samples, 29 anal swab specimens, and 22 oral swab specimens) were collected from 57 bats of 7 species in 4 locations in the Rocky Mountain region during a 2-week period in August 2006 (Table). PCR amplicons that target a conserved region in gene *1b* containing the RNA-dependent RNA polymerase common to all coronaviruses were detected in reversed-transcribed RNA from 6 of the 79 samples. All samples positive for coronavirus RNA were from the 28 fecal samples tested (Table). None of the anal region or oral swab specimens were positive for coronavirus RNA.

Despite the small number of bats sampled, there was a high prevalence of coronavirus RNA shedding in fecal samples of 2 species of bats. Five (50%) of 10 fecal samples from occult myotis and 1 (17%) of 6 fecal samples from big brown bats were positive for coronavirus in screening tests. The 1 coronavirus-positive sample from big brown bat (bat sample 65) was from feces of 1 (33%) of 3 big brown bats sampled at site 2 in north-central Colorado, whereas the positive samples from the occult myotis (bat samples 3, 6, 11, 27, and 48) were from sites 1 and 4 in southwestern Colorado, ≈480 km from site 2 (Table). Most of the fecal samples were only positive in the PCRs with cDNA diluted 1:10, which suggested that PCR inhibitors were present in feces. In addition, most of the samples were positive only in the nested PCRs, which indicated that either the RNA was present in small amounts or that the primers used were not an optimal match for these viruses.

Phylogenetic Analysis of RM-Bt-CoVs

A 440-nt sequence in the RNA-dependent RNA polymerase region of gene *1b* was amplified by RT-PCR from the 6 positive samples. Analysis of nucleotide sequences of these amplicons showed that all 6 RM-Bt-CoVs are members of coronavirus group 1 (Figure 1). Although these sequences were similar to those published for Asian bat group 1 coronaviruses, there was enough dissimilarity in this highly conserved region to suggest that the Rocky Mountain specimens represent unique coronaviruses (*8*,*9*). Phylogenetic analysis of this region of gene 1b suggests that the RM-Bt-CoVs cluster in 3 subgroups within group 1. Three of the 5 specimens from the occult myotis (samples 6, 11, and 48) were in 1 cluster and the other 2 (samples 3 and 27) formed a second cluster within group 1 coronaviruses. The 1 specimen from the big brown bat (sample 65) was a more distantly related group 1 coronavirus (Figure 2).

Discussion

To our knowledge, this is the first report of coronaviruses in bats in the Western Hemisphere. With >1,100 species, bats are among the most divergent and widely distributed nonhuman mammals (*17*). Bats are reservoirs for rabies virus and other lyssaviruses and were recently shown to be reservoirs for other important emerging viruses. Old World fruit bats (family Pteropodidae) are reservoirs for Hendra virus, which caused small outbreaks of severe re-

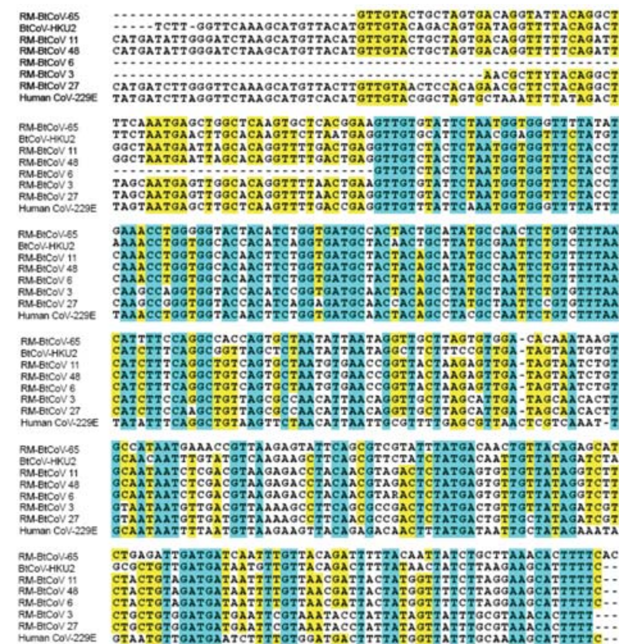

Figure 1. Nucleotide sequence alignment of amplicons from a 440-nt region of gene *1b* of Rocky Mountain bat coronaviruses (RM-Bt-CoVs) compared with group 1 coronaviruses of Asian bats (BtCoVs) and human coronavirus 229E. Identical residues are shaded in blue and similar residues are shaded in yellow. Hyphens indicate positions where sequences are not available.

spiratory illnesses in horses and humans in Australia (*18–24*) and Nipah virus, which caused large outbreaks of lethal encephalitis and respiratory illnesses in humans and pigs in Malaysia and Singapore (*25–28*). Old World fruit bats may also be the long-sought reservoir hosts for Ebola and Marburg viruses (*29*,*30*). More than 60 different RNA viruses have been isolated from and detected in bats, which play important roles in maintaining and transmitting zoonotic viruses (*31–33*).

The need for understanding the ecology and evolution of coronaviruses in wildlife was highlighted by the observation that SARS-CoVs that caused 4 sporadic human cases of SARS in 2003–2004 were more closely related to viruses from palm civets found in 2004 than to the human epidemic strain of SARS-CoV (*34*). The gene encoding the viral spike glycoprotein that binds the virus receptor human angiotensin-converting enzyme 2 was one of the fastest-adapting genes of SARS-CoV during the 2002–2003 epidemic. Nonsynonymous amino acid substitutions in the spike protein that were selected during the epidemic optimized binding of the spike to its human receptor and enhanced human-to-human transmission (*34*,*35*). Sequencing of SARS-CoV genomes during and after the epidemic suggests that multiple independent species-jumping events of SARS-CoV from animals to humans have occurred.

Although all samples we tested were from apparently healthy wild bats, a high prevalence of coronavirus RNA was detected in 2 of the 7 species of bats tested. Five (50%) of 10 occult myotis and 1 (17%) of 6 big brown bats tested contained low levels of coronavirus RNA in feces. No coronavirus RNA was detected in the oral or anal region swabs tested. Similarly in Asian bats, coronavirus RNA was found in a higher percentage of fecal samples than saliva samples (*8*,*9*,*14*). Thus, bats may be persistently infected carriers that shed low levels of coronaviruses in feces. Persistent fecal shedding of coronaviruses has also been detected in pigs, cats, dogs, and cattle (*36*). The mechanisms for persistent fecal shedding of viruses in bats without apparent disease have not yet been determined (*32*,*33*).

No bat of any species occurs in both the Eastern and Western Hemispheres (*37*). Therefore, it is of great interest that group 1 coronaviruses have now been found in bats in North America as well as in Asia. Comparison of the nucleotide sequences of related coronaviruses from different species of bats on different continents is likely to provide information about coronavirus evolution. Figure 2 shows the phylogeny of RM-Bt-CoVs in relation to group 1 coronaviruses from Asia on the basis of the 440-nt amplicon in gene *1b*. Bats of the genera *Myotis* and *Eptesicus* are in the family Vespertilionidae, which has diversified into many different species in the Eastern and Western Hemispheres (*17*). Amplicons of 3 of the 5 coronaviruses (samples 6, 11, and 48) from occult myotis in Colorado have the highest nucleotide sequence identity with the HKU6 bat coronavirus found in an Asian bat of the same genus but a different species, Rickett's big-footed myotis (*M. ricketti,* subfamily Myotinae) (*11*,*17*). The coronavirus RNA in the big brown bat (sample 65) from Colorado (subfamily Vespertilioninae) was most similar to HKU2 bat coronavirus found in Asian bats in the family Rhinolophidae (*11*) (Figure 2). Rhinolophid bats are not found in the Western Hemisphere

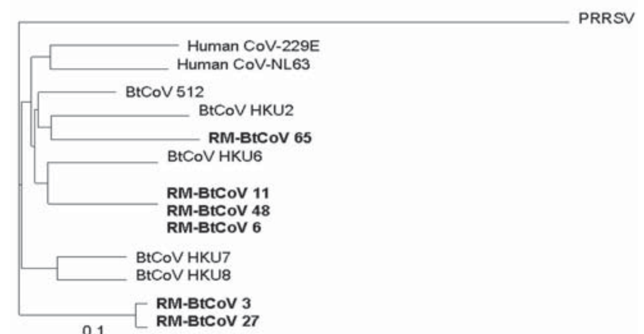

Figure 2. Phylogenetic relationships based on a 440-nt sequence in a conserved region of gene *1b* of Rocky Mountain bat coronaviruses (RM-Bt-CoVs) (shown in **boldface**), group 1 coronaviruses of Asian bats (BtCoVs), and human coronaviruses 229E and NL63. Porcine respiratory and reproductive syndrome virus (PRRSV) was used as the outgroup to root the tree. Scale bar at the lower left indicates 0.1 nucleotide substitutions per site.

and are phylogenetically far removed from the big brown bat (*37*,*38*).

In our small, initial study of coronaviruses in North American bats, samples were restricted in size, location, and variety of bat species, and we found only group 1 coronaviruses. When larger numbers of bats and additional bat species in North America are studied, additional bat coronaviruses with complex phylogenetic attributes, biogeographic patterns, and perhaps epizootiologic attributes may be discovered. For example, determining if North American bat coronaviruses are species-specific will provide useful information. In Asia, different species of bats roosting in the same cave host different coronaviruses (*9*). However, bats of 1 species can also harbor different types of coronaviruses at different geographic locations (*9*).

A recent analysis of genome sequences of coronaviruses of bats, other animals, humans, and birds suggested that bats may be the original hosts from which all coronavirus lineages were derived (*15*). We find this hypothesis intriguing, in light of the high prevalence and diversity of coronaviruses in bats in North America found in our initial small survey. The North American species of bats found to harbor group 1 coronaviruses commonly roost in buildings inhabited by humans (*39*), which provides ecologic overlap between these bats and humans. Before the SARS epidemic of 2002–2003, only 2 coronaviruses, HCoV-229E and HCoV-OC43, were known to cause human disease, primarily mild upper respiratory tract infections. In contrast, SARS-CoV caused severe lower respiratory tract disease with a death rate of 10%. Recently, 2 additional human coronaviruses, HCoV-NL63 and HCoV-HKU1, were discovered and found to cause both upper and lower respiratory tract infections worldwide (*40*).

It is possible that another epidemic caused by an emerging coronavirus could occur in the future. As in the SARS epidemic, bats could play a role in future emergence of coronaviruses in humans or other species. Isolation of infectious bat coronaviruses and elucidation of their host ranges, receptor specificities, and genetic diversity will greatly aid in our understanding of their potential for emergence.

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Landscape Elements and Hantaan Virus–related Hemorrhagic Fever with Renal Syndrome, People's Republic of China

Lei Yan,*[1] Li-Qun Fang,†[1] Hua-Guo Huang,* Long-Qi Zhang,* Dan Feng,‡ Wen-Juan Zhao,‡ Wen-Yi Zhang,‡ Xiao-Wen Li,* and Wu-Chun Cao‡[1]

Hemorrhagic fever with renal syndrome (HFRS) is an important public health problem in the People's Republic of China, accounting for 90% of human cases reported globally. In this study, a landscape epidemiologic approach, combined with geographic information system and remote sensing techniques, was applied to increase our understanding of HFRS due to Hantaan virus and its relationship with landscape elements in China. The landscape elements considered were elevation, normalized difference vegetation index (NDVI), precipitation, annual cumulative air temperature, land surface temperature, soil type, and land use. Multivariate logistic regression analysis showed that HFRS incidence was remarkably associated with elevation, NDVI, precipitation, annual cumulative air temperature, semihydromorphic soils, timber forests, and orchards. These findings have important applications for targeting HFRS interventions in mainland China.

Hemorrhagic fever with renal syndrome (HFRS) is a zoonosis caused by different species of hantavirus (HV). It was first recognized in northeastern China in 1931 and has been prevalent in many other parts of China since 1955. At present, HFRS is endemic in 28 of 31 provinces of the People's Republic of China, autonomous regions, and metropolitan areas and accounts for 90% of the HFRS cases reported globally (*1*). The disease has taken a heavy toll on the health of the Chinese people, having been responsible for 1.2 million symptomatic infections and 44,300 deaths from 1950 to 1997.

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In China, HFRS is mainly caused by 2 HVs, i.e., Hantaan virus (HTNV) and Seoul virus (SEOV), each with a distinct rodent host. HTNV, which causes more severe disease, is carried by *Apodemus agrarius*. SEOV, which causes a less severe form of HFRS, is carried by *Rattus norvegicus*. A novel HV named Amur virus (AMRV) was identified recently in *A. peninsulae* from far eastern Russia and subsequently identified in a few patients from China (*2*,*3*). Another HV, designated as Soochong virus, was recently isolated from *A. peninsulae* in Korea and was described as an antigenically and genetically distinct HV species, which was monophyletic with AMRV but not with *A. agrarius*–associated HTNV (*4*). HVs are primarily transmitted from rodent host to human by aerosols generated by contaminated urine and feces and possibly by contaminated food or rodent bites (*5*,*6*).

Previous studies indicated that HFRS incidence seemed to be associated with environmental factors, including topography, hydrologic features, and rainfall. HFRS cases were mainly reported from areas <500 m above sea level and in the regions with very moist soil. HFRS cases were rarely reported in areas that were very dry or very wet (*7–10*).

Recently, we analyzed the distribution of HFRS cases in China based on geographic information system (GIS) spatial analysis (*11*) and found areas where the population had a high risk of acquiring the disease. That study demonstrated a new approach to integrating such tools into the epidemiologic study and risk assessment of HFRS.

Our objective for the current study was to identify the relationship between the incidence of HFRS due to HTNV and landscape elements by using the concepts of landscape epidemiology as well as GIS and remote sensing techniques. The major landscape elements considered in this

[1]These authors contributed equally to this article.

study were elevation, normalized difference vegetation index (NDVI), precipitation, annual cumulative air temperature, land surface temperature (LST), soil type, and land use. The study focused on HFRS cases caused by HTNV only and restricted study sites to rural areas of the country and the areas with population density <1,000/km^2.

Methods

Data Collection and Management

All the cases reported in mainland China from 1994 through 1998 were obtained from the National Notifiable Disease Surveillance System (NNDSS). NNDSS is supported by a special monitoring network and produces these data annually according to county, a political subdivision of a province, which usually contains several townships and has a population of ≈500,000 persons.

Because the number of cases was small and varied yearly in each county, we used the mean number of HFRS cases from each county from 1994 to 1998. All HFRS cases were coded according to geographic area (geo-coded) and matched to the corresponding polygon and its label point on a digital map of China by using the software ArcGIS 9.1 (ESRI Inc., Redlands, CA, USA). The NNDSS HFRS data do not differentiate HTNV from SEOV infections. The study was limited to the rural areas of the country and areas with population density <1,000/km^2, to capture most, if not all, of the patients infected with HNTV.

Demographic data at the county level were obtained from the 1995 and 2000 censuses. To overcome difficulties due to changes in administrative boundaries, the vector map of the demographic data was converted to a raster map of the population with a 1-km grid (*12*). Based on the 1995 and 2000 maps for the population and the map of the administrative units, the average population of each county was calculated.

Digital topographic map information was used to generate a digital elevation model (DEM) with a 1:100,000 scale. The elevation data obtained from DEM was transferred into a raster map with a 1-km grid (*12*). Based on DEM and the map of the administrative units, the average elevation of each county was calculated. Counties were then classified into 8 levels (meters above sea level): ≤100, 101–200, 201–500, 501–1,000, 1,001–1,500, 1,501–2,000, 2,001–3,000, and >3,000.

The NDVI was derived by the National Satellite Meteorological Center in China by using advanced, high-resolution radiometer (AVHRR) images. Monthly and annual NDVI in 1998 were calculated by using ERDAS Imagine 8.7 (Leica Geosystems Geospatial Imaging, LLC., Norcross, GA, USA) (*12*). Counties were classified according to 4 NDVI levels: <0.1, 0.101–0.2, 0.201–0.3, and >0.3.

The annual precipitation data were based on the average of the cumulative annual precipitation in China from 1994 to 1998, obtained from 700 weather stations (*12*). The inverse distance weighting (IDW) method was applied to interpolate and generate its raster map for annual precipitation with a 1-km grid (*13*,*14*). Annual precipitation values were divided into 4 levels: ≤400, 401–800, 801–1,200, and >1,200 mm. These levels corresponded to arid, semiarid, semihumid, and humid areas, respectively.

Air temperature data were obtained from 700 weather stations through the country from 1970 to 2001 (*12*). The IDW method was applied to interpolate and generate its raster map with a 1-km grid. The average daily temperature of each county was added to derive the annual cumulative air temperature, and it was divided into 5 temperature ranges: ≤1,600°C, 1,601°C–3,400°C, 3,401°C–4,500°C, 4,501°C–8,000°C, and >8,000°C. These levels represent frigid-temperate, mid-temperature zone, warm-temperate, semitropical, and tropical zones, respectively.

LST data at county level were also obtained from the monthly AVHRR 1998 data (*12*). LST values were divided into 5 levels: <28°C, 28°C–31°C, 32°C–34°C, 35°–37°C, and >37°C.

The soil types in the map were grouped into 12 categories, i.e., argosols, semiluvisols, caliche soils, arid soils, desert soils, skeletol primitive soils, semihydromorphic soils, hydromorphic soils, saline soils, anthrosols, alpine soils, and ferralisols. These categories are based on the Classification and Codes of Soil in China (*12*).

The types of land use in the map were categorized as rice land, irrigated land/nonirrigated farmland, timber forest land, orchard land, sparse woods, bush, prairie and grassland, hilly/mountainous grassland, desert (desert, Gobi, cold desert), wetland, saline-alkali land, and bare land (*12*). The timber forest land is used to produce timber for building and furniture; orchard land produces fruits and raw materials for industry or for beverages and medicines, for example. The Gobi is a large desert region of southeast Mongolia and northern China, which consists mainly of series of shallow alkaline basins. Bush has been defined as land covered with dense vegetation or undergrowth.

Data Analyses

To process the data for landscape elements at county level, we overlaid the map of administrative units on the raster map of each landscape element. The average elevation, NDVI, air temperature, LST, precipitation, area proportions with different type of soils, and land use were then calculated for each county by using ArcGIS 9. The average annual HFRS incidence of each county was calculated as well. Through the linkage of the 6-digit county geo-code, the incidence of HFRS at county level was displayed on

the base map with administrative boundaries and then converted to a raster map, which was overlaid on the thematic maps of the landscape elements.

HFRS incidence was also calculated for each category of the landscape elements by overlaying maps of HFRS with the different thematic maps. For example, elevation was divided into 8 levels and then displayed on the map of elevation for the whole country. According to the area proportions of each level of elevation, the population and the number of HFRS cases at the county level were displayed as HFRS incidence data at each elevation level were then obtained.

Univariate analysis (χ^2) was used to compare HFRS incidence across the different levels of each landscape element, including elevation, NDVI, precipitation, annual cumulative air temperature, and LST; odds ratios (ORs) were obtained by comparing the HFRS incidence of different categories of the landscape elements. To determine the associations between HFRS and soil type as well as land use, univariate logistic analysis was conducted, and ORs were computed by comparing counties where HFRS was found with non-HFRS–endemic counties. Through GIS, different thematic maps were also generated to facilitate graphic and spatial visualization of HFRS occurrence at the county level in China and geographic distribution of the different landscape elements (*15*).

Multivariate logistic regression analysis was then performed. The dependent variable was whether HFRS occurs; independent variables were landscape elements (elevation, NDVI, precipitation, annual cumulative temperature and LST, type of soil, and land use). Backward stepwise selection was performed with the criterion of p>0.05. The possible interaction between individual elements was considered.

Condition indexes and variance decomposition proportions were used to test colinearity among the independent variables and identify the sources of colinearity. When the condition index was >30, the independent variables had strong colinearity. If a large condition index is associated with variables that have variance decomposition proportions >0.5, these variables may be causing colinearity problems (*16*).

Results

The average HFRS incidence of each county in mainland China is displayed in Figure 2, with an overlaid map of *A. agrarius* capture points (*17*). The top 6 incidence rates were 20.3, 18.9, 8.2, 7.7, 5.0, and 4.6/100,000 population in Heilongjiang, Shandong, Zhejiang, Hunan, Hebei, and Hubei Provinces, respectively. Approximately 70% of HFRS cases were reported from the above provinces. Only Xingjiang, Tibet autonomous regions, and Qinghai Province never reported any HFRS cases.

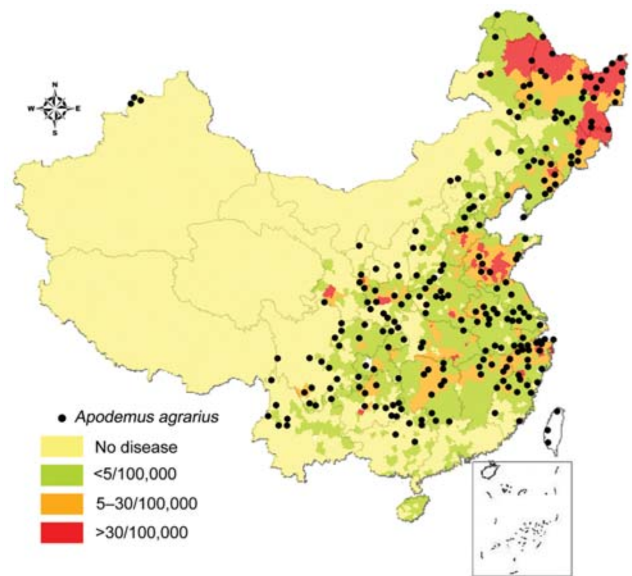

Figure 1. Geographic distribution of incidence of hemorrhagic fever with renal syndrome (HFRS) in the People's Republic of China and relationship with capture points *of Apodemus agrarius.* Black dots show the capture points of *A. agrarius.* Internal borders indicate provinces.

HFRS incidence significantly declined as elevation increased (χ^2 for trend test, p<0.001; Spearman correlation test r = –0.466, p<0.01). The highest incidence (7.3/100,000 population) was observed in areas with elevation of 100–200 m. No cases were reported in areas >3,000 m except in 3 counties of Gansu Province (XiaHe, Diebu, and Zhuoni). Approximately 86.4% HFRS cases occurred in areas with 0–500 m elevation in the eastern part of China and the Sichuan Basin (Figure 2).

HFRS incidence was 3–4× higher in areas with an NDVI 0.1–0.3 than in areas with NDVI <0.1 (Table 1). There were significant differences in HFRS incidence in regard to NDVI (df = 3, p<0.001). However, the peak incidence of 4.6/100,000 population was observed at an NDVI level of 0.2–0.3. These areas are mainly located in the eastern and middle part of China.

The highest HFRS incidence of 6.4/100,000 occurred in the semihumid areas, where precipitation levels are 400–800 mm. The HFRS incidence was ≈50% in areas with precipitation >800 mm. No cases were reported from the arid areas, where the precipitation was <200 mm. The difference in HFRS incidence was statistically significant among different precipitation level (df = 4, p<0.001).

The frigid-temperate zone, with annual cumulative temperature of <1,600°C, had the highest HFRS incidence at 10.2/100,000. This was followed by the warm zone (3,400–4,500°C) and semitropical (4,500°C–8,000°C) zones with HFRS incidences of 8.0 and 2.6 per 100,000,

Table 1. HFRS incidence at different levels of landscape elements, China*

	Incidence (95% CI)†	p value‡	Odds ratio (95% CI)
Elevation, m			
<100§	3.48 (3.41–3.52)	–	1.00
100–200	7.29 (7.16–7.43)	<0.001	2.10(2.05–2.16)
201–500	5.17 (5.09–5.26)	<0.001	1.49(1.46–1.53)
501–1,000	2.97 (2.88–3.06)	<0.001	0.86(0.83–0.89)
1001–1,500	1.76 (1.71–1.88)	<0.001	0.36(0.34–0.38)
1,501–2,000	0.32 (0.27–0.38)	<0.001	0.09(0.08–0.11)
2,001–3,000	0.85 (0.75–0.96)	<0.001	0.24(0.22–0.28)
>3,000	0.71 (0.53–0.92)	<0.001	0.20(0.15–0.27)
NDVI		<0.001‡	
0–0.1§	1.14 (1.06–1.23)		1.00
0.101–0.2	4.21 (4.13–4.29)	<0.001	3.69 (3.42–3.99)
0.201–0.3	4.55 (4.51–4.61)	<0.001	3.99 (3.70–4.30)
>0.3	1.43 (1.36–1.50)	<0.001	1.25 (1.15–1.37)
Precipitation, mm/y		<0.001‡	
0–400§	0.18 (0.14–0.22)	–	1.00
401–800	6.42 (6.34–6.51)	<0.001	36.21 (29.23–45.41)
801–1,200	3.65 (3.58–3.70)	<0.001	20.51 (16.56–25.74)
>1,200	2.64 (2.60–2.69)	<0.001	14.91 (12.03–18.70)
Annual cumulative air temperature, °C		<0.001‡	
0–1,600§	10.18 (9.96–10.39)	–	1.00
1,601–3,400	1.44 (1.38–1.51)	<0.001	0.15 (0.14–0.16)
3,401–4,500	8.01 (7.89–8.12)	<0.001	0.84 (0.82–0.86)
4,501–8,000	2.56 (2.52–2.59)	<0.001	0.27 (0.26–0.27)
>8,000	0.19 (0.10–0.34)	<0.001	0.02 (0.01–0.03)
Land surface temperature, °C		<0.001‡	
<28§	10.75 (10.51–10.99)	–	1.00
28–31	2.62 (2.51–2.73)	<0.001	0.24 (0.23–0.26)
32–34	2.86 (2.79–2.93)	<0.001	0.27 (0.26–0.274)
35–37	4.68 (4.63–4.74)	<0.001	0.43 (0.42–0.45)
>37§	0.98 (0.93–1.03)	<0.001	0.09 (0.09–0.10)

*HFRS, hemorrhagic fever with renal syndrome; CI, confidence interval; NDVI, normalized difference vegetation index.
†Incidence = number of HFRS cases/100,000 population.
‡p value of each landscape element; others are p value of subdivision analyses.
§Reference group.

respectively. Among different cumulative temperature zone, the HFRS incidences were significantly different (df = 4, p<0.001). There was also a significant difference in HFRS incidence regarding LST (df = 4, p<0.001). The highest incidence of 10.8/100,000 was found in areas with LST <28°C. The incidence dropped when the LST value increased to 28°–34°C and increased again to 4.7/100,000 when LST levels reached 35°–37°C (Table 1).

As to the soil types, the univariate logistic regression analysis showed that anthrosols, alfisol, and semihydromorphic soils, which are good for cultivation, had higher risk for HFRS prevalence. All other soils seemed to be less likely to harbor the disease agent (Table 2).

The univariate logistic regression analysis also showed that land for agriculture use, including rice land, irrigated farmland, nonirrigated farmland, and orchard land, were the landscape elements with high risk for HFRS. Other types of land use, except for timber forest land and wetland, were protective against the disease (Table 3).

Multivariate logistic regression analysis indicated that elevation, NDVI, precipitation, and annual cumulative temperature were significantly associated with HFRS incidence. Semihydromorphic soils (OR = 1.53), timber forest land (OR = 2.04), and orchard land (OR = 1.97) were risk factors for HFRS incidence (Table 4).

Table 2. Result of univariate logistic analysis in different soil types in relation to HFRS occurrence in China, 1994–1998*

Soil type	p value	OR (95% CI)
Anthrosol	<0.01	1.36 (1.12–1.64)
Ferralisol	<0.01	0.74 (0.61–0.89)
Alfisol	<0.01	1.88 (1.56–2.25)
Semiluvisol	<0.05	0.80 (0.65–0.99)
Caliche	<0.01	0.16 (0.11–0.23)
Arid	<0.01	0.06 (0.03–0.12)
Desert	<0.01	0.42 (0.33–0.54)
Skeletol primitive	<0.01	0.41 (0.33–0.50)
Semihydromorphic	<0.01	2.41 (2.00–2.90)
Hydromorphic	<0.05	0.60 (0.37–0.95)
Saline	<0.01	0.55 (0.39–0.77)
Alpine	<0.01	0.02 (0.01–0.04)

*HFRS, hemorrhagic fever with renal syndrome; OR, odds ratio; CI, confidence interval.

Table 3. Result of univariate logistic analysis in different land use types in relation to HFRS occurrence in China, 1994–1998*

Land-use type	p value	OR (95% CI)
Rice land	<0.01	1.75 (1.46–2.09)
Irrigated farmland	<0.01	1.49 (1.25–1.77)
Nonirrigated farmland	<0.01	2.39 (1.93–2.97)
Timber forest land	0.63	1.05 (0.86–1.27)
Orchard land	<0.01	2.68 (1.67–4.41)
Sparse woods	<0.01	0.63 (0.51–0.75)
Bush	<0.01	0.52 (0.44–0.62)
Prairie and grassland	<0.01	0.14 (0.11–0.18)
Hilly/mountainous grassland	0.7	0.96 (0.81–1.15)
Desert	<0.01	0.20 (0.14–0.28)
Wetland	<0.05	1.70 (1.02–2.86)
Saline-alkali land	<0.01	0.25 (0.14–0.43)
Bare land	<0.01	0.05 (0.03–0.09)

*OR, odds ratio; CI, confidence interval.

Discussion

In the early 1990s, the spatial distribution of HFRS and its variation regarding to geographic and meteorologic features were well described in China, based on a national investigation (*18*). However, because of the limitation of technique used in the analyses of that study, the HFRS distribution and related environmental factors could be neither displayed at the county level nor visualized on a digital map. Recently, we used GIS-based spatial analysis to elucidate temporal and spatial distribution of HFRS and to highlight geographic areas with a substantially high incidence of the disease (*12*). The results indicated that the application of GIS, together with spatial statistical techniques, provides ways to quantify explicit HFRS and to further identify environmental factors responsible for the increasing disease risk. In the current study, we combined a landscape epidemiologic approach with GIS and remote sensing techniques to increase our understanding of HFRS and its relationship with landscape elements in China.

HTNV and SEOV, the major causative agents of HFRS in mainland China, are associated with 2 distinct rodent hosts, i.e., *A. agrarius* and *R. norvegicus*, respectively. The former thrives in rural areas, while the latter is an anthropophilic urban species. HTNV- and SEOV-related HFRS cases should be differentiated to explore the association between HFRS incidence and landscape elements because each rodent species has its own breeding sites with special landscape attributes. Unfortunately, in China, the reported HFRS cases are not distinguished by causative HV. Since the rodent host (*A. agrarius)* of HTNV usually lives in rural areas, large cities and counties with population density >1,000/km^2 were excluded from the analyses to remove most, if not all, HFRS cases caused by SEOV and to restrict the study to mainly HTNV-type infections.

The reason for the increased risk for HFRS in regions with lower elevation is not clear; population density and human activities are likely explanations. Population density remarkably increases as elevation decreases and most likely facilitates transmission of HV from rodent hosts to human, subsequently leading to increases in HFRS incidence.

HFRS incidence was highest in the frigid-temperate zone, mostly in northeastern China, followed by incidence in the warm-temperate zone. We assume that the HTNV rodent hosts prefer the temperate area. Very few cases occurred in areas that were either extremely cold or extremely hot. The findings of a previous study on rodent surveillance supported our hypothesis, which suggested that the density as well as HTNV infection rate of *A. agarius* in temperate zones was much higher than those in other areas (*8*).

Economic activities are probable reasons for higher HFRS in the areas of particular soil type and land use. In China, semihydromorphic soil is the major cultivated soil type, usually used for growing wheat, corn, and other crops,

Table 4. Result of multivariate logistic regression analysis in relation to HFRS occurrence in China, 1994–1998*

	p value	OR (95% CI)
Elevation, m		
<100	–	1.00
100–200	0.75	0.93 (0.61–1.43)
201–500	0.47	0.87 (0.59–1.28)
501–1,000	<0.01	0.58 (0.39–0.86)
1,001–1,500	<0.01	0.27 (0.17–0.43)
1,501–2,000	<0.01	0.22 (0.12–0.39)
2,001–3,000	<0.01	0.31 (0.16–0.60)
>3,000	<0.01	0.05 (0.01–0.25)
NDVI	<0.01	
<0.1	–	1.00
0.1–0.2	0.73	1.12 (0.60–2.11)
0.2–0.3	0.25	1.44 (0.77–2.69)
>0.3	0.22	0.64 (0.32–1.29)
Precipitation, mm/y	<0.01	
<400	–	1.00
400–800	<0.01	9.94 (3.92–25.23)
801–1,200	<0.01	8.16 (2.97–22.44)
>1,200	<0.01	4.95 (1.70–14.39)
Annual cumulative air temperature, °C	<0.01	
<1,600	–	1.00
1,600–3,400	<0.01	0.47 (0.28–0.79)
3,401–4,500	0.41	1.25 (0.73–2.15)
4,501–8,000	0.17	1.58 (0.82–3.07)
>8,000	0.14	2.76 (0.71–10.72)
Soil or land-use type		
Ferralisol	<0.01	0.65 (0.46–0.90)
Desert	<0.01	0.59 (0.41–0.84)
Skeletol primitive	<0.01	0.66 (0.50–0.88)
Semihydromorphic	<0.01	1.53 (1.14–2.06)
Alpine	<0.01	0.23 (0.07–0.73)
Timber forest	<0.01	2.04 (1.48–2.81)
Orchard	<0.01	1.97 (1.18–3.29)
Sparse woods	<0.01	0.60 (0.46–0.78)
Bare land	0.02	0.45 (0.23–0.87)

*HFRS, hemorrhagic fever with renal syndrome; NVDI, normalized difference vegetation index; OR, odds ratio; CI, confidence interval.

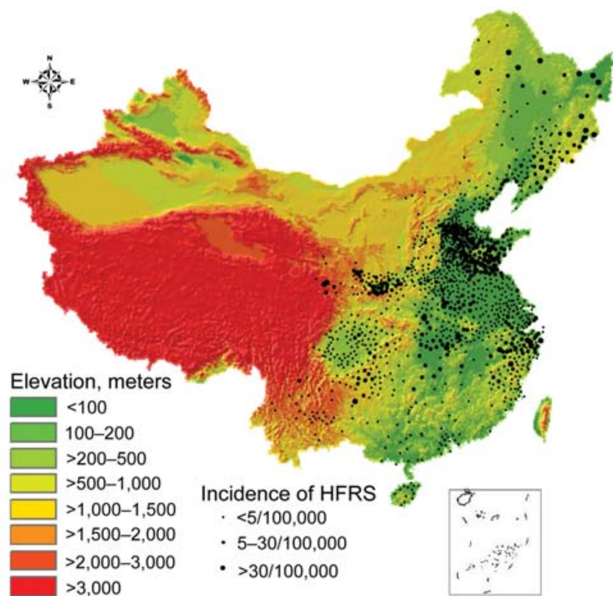

Figure 2. Topographic map of the People's Republic of China, showing relationship between elevation and incidence of hemorrhagic fever with renal syndrome (HFRS).

which can provide adequate food for rodent hosts and subsequently lead to increase rodent density.

Timber forest and orchard land were also appropriate environments for rodent hosts. Forest workers and farmers had more chances to come into contact with contaminated urine and feces of rodents infected with HTNV. An investigation conducted on various land types showed that the highest trap-success rate of *Apodemus* rodents in the country was 28.9% in Heihe County. The county has 38% timber forestland, 16% nonirrigated farmland, and 3% wetland (H. Chen, pers. comm.).

This study characterized the landscape attributes that seem to be favorable for HFRS incidence. Although analyses are still preliminary, the findings can be helpful for generating hypothesis for further investigation. For better analyses, the human and rodent HFRS surveillance in China, including discrimination of HFRS cases due to different HVs, should be enhanced.

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Precautionary Behavior in Response to Perceived Threat of Pandemic Influenza

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Faced with an epidemic of an infectious disease, persons may take precautionary actions to try to reduce their risk. Such actions include avoiding situations that persons perceive to be risky, which can have negative health and economic effects. Therefore, we conducted a population-based survey of persons' precautionary actions in response to a hypothetical influenza pandemic. For the 5 European and 3 Asian regions that had been affected by severe acute respiratory syndrome, the pattern of reported precautionary action was broadly similar across the regions; ≈75% of respondents reported that they would avoid public transportation and 20%–30% would try to stay indoors. Some regional differences were noted; Europeans were more likely than Asians to avoid places of entertainment, and Asians were more likely to avoid seeing physicians. This international survey provides insight into what might be expected during an influenza pandemic.

The risk of acquiring an infectious disease may stimulate persons to take precautionary actions to try to reduce this risk as they perceive it. The potential effect of this perceived risk–induced behavior was apparent during the outbreak of severe acute respiratory syndrome (SARS) in 2003. For example, use of public transportation in affected areas and international flights to these areas were reduced dramatically (*1*,*2*). Precautionary actions, such as avoiding public transportation or avoiding situations in which persons congregate, may have potential epidemiologic effects and would be expected to have economic consequences. The demand for certain goods and services may decline, and output may be reduced if persons avoid work or social interactions and associated purchase of goods. The economic effect of such precautionary actions may be substantial. For instance, the economic effect of SARS has been estimated at US $30–$100 billion (*3*–*5*), although the outbreak was confined to a few months and <10,000 persons were infected. Macroeconomic estimates suggest that the indirect general demand–reducing effect of SARS in non-health sectors was greater than the direct health effect and associated productivity losses to SARS patients and their families (*6*).

The nature and scale of this economic shock have caused concerns that pandemic influenza could have a catastrophic effect on the global economy. Understanding the factors that lead persons to take preventive actions to avoid infection may help forecast the possible course of an epidemic and its economic effect. This information would help decision-makers give appropriate advice to limit individual, community health, and economic effects.

The spread of highly pathogenic avian influenza (H5N1) and the documented illness and deaths of >300 persons in >12 countries (*7*) has heightened concerns that an influenza pandemic may be imminent. The effect of efforts to limit the dramatic health and economic consequences of such a pandemic will depend on how persons react. Research on public reaction to previous outbreaks has shown that persons may take misjudged precautionary actions (e.g., avoid places and activities that bear low risk for infection, avoid healthcare facilities for fear of infection, refuse to comply with quarantine efforts) that may contribute to the pandemic's adverse economic effect (*8*). Therefore, to improve communication efforts by health officials, to enable pandemic containment, and to avoid unwarranted losses to the economy, knowledge of how per-

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sons will respond to the threat of an outbreak is crucial (*6*). However, research in this area is lacking (*9*). To overcome this gap, we conducted a population-based survey in 8 regions (5 European countries and 3 east Asian regions) to estimate whether persons might take precautionary actions during an influenza pandemic, the extent of such behavior, and factors that might influence it.

According to most models of health behavior, perception of being at risk is a prerequisite for behavior change, a supposition supported by empirical studies (*10*,*11*). These models endorse the belief that a high perceived risk of harm encourages persons to take action to reduce their risk. However, the direction of the association between risk perception and behavior in empirical studies varies positively, negatively, or not at all (*11*,*12*). The empirical literature that links risk perception and health behavior is subject to some debate about methods (*6*,*13*). First, the relationship between risk perception and preventive actions may be simultaneous, which makes it difficult to determine causality from observed behavior in cross-sectional data. Second, at least 2 broad methods exist for analyzing the role of risk in social science, and the choice of method is important. The most common approach is the realist approach, in which risk is seen as an objective threat or danger that can be measured independent of the social context within which it occurs (*6*,*13*). The alternative is the social constructionist approach, which describes risk as being based on objective facts about danger and hazard, amenable to rationalistic calculations, which are then mediated, perceived, and responded to in particular ways through social, cultural, and political processes (*13*). We used the social constructionist approach, in which individual and societal level factors can affect the relationship between risk perception and health behavior.

Our study sample was drawn from 2 sources: Asian regions in which SARS cases were reported and European countries in which no SARS cases occurred. Both categories could incur a new SARS or pandemic influenza outbreak. These categories are useful for determining whether previous exposure to a similar type of hazard has had any effect on risk perceptions and associated precautionary actions.

Methods

As a part of a survey of risk perception, knowledge, and sources of information for SARS and influenza, we conducted a study on precautionary actions for a hypothetical influenza pandemic in 5 European countries (Denmark, Spain, Great Britain, the Netherlands, and Poland), and 3 Asian regions (Guangdong [People's Republic of China], Hong Kong [Special Administrative Region, People's Republic of China], and Singapore) that had been affected by SARS. The overall survey, from which risk behavior was analyzed separately, is described in more detail elsewhere (*14*). Brief details of the sample and questionnaire are given below.

Sample

From September 20 through November 22, 2005, interviews were conducted in native languages by native speakers of each region, who used computer-assisted telephone interviewing and random-digit dialing. Unanswered numbers were tried again as many as 5 times; when possible, call-back appointments were made. Persons 18–75 years of age were eligible for participation, and the member of the household with the most recent birthday was invited to participate.

Questionnaire

The questionnaire was based on a SARS and influenza risk perception questionnaire (*14*). The full questionnaire is available from www2.eur.nl/fgg/mgz/sarscontrol/questionnaire_risk_perceptions_survey.htm, and a copy of our questionnaire is available on request.

The questionnaire was translated into local languages and back translated to check the accuracy of these translations. Basic demographic and socioeconomic information about age, sex, education, health, and employment status and the like was sought. Respondents were asked to rate how serious they thought it would be to contract a range of illnesses including a heart attack, common cold, a new strain of influenza, and SARS, and how likely they themselves and the average person would be to contract these diseases in the following year. They were asked a number of questions to ascertain their level of knowledge of SARS and influenza, where they had obtained information on these diseases, and how trustworthy they perceived these information sources to be.

Respondents were then asked to imagine that a global influenza epidemic had reached their country. They were given a list of 6 places (public transportation; entertainment places such as cinemas, restaurants and theaters; shops; work or school; hospital; or home) and asked in which of these they thought they would run the greatest risk for infection. They were then randomly given 1 of 2 scenarios: a high-risk scenario in which over a 5-week period, 10% of their fellow inhabitants of all ages would be seriously ill with influenza and 0.1% would have died of the disease; and a low-risk scenario in which these rates were 2.5% and 0.025%, respectively. These scenarios were presented to the respondents in terms of rounded numbers of cases and deaths, scaled to their jurisdictions' population size (rather than rates). Respondents were next given the following list of 8 precautionary behavior modifications and asked whether they would adopt any of them: avoid public transportation (e.g., trains, buses, airplanes); avoid going out

for entertainment (e.g., bars, restaurants, theaters, cinema); limit shopping to the essentials; take leave from work; keep children out of school, even if school remains open (only adults with children were asked this question); limit physical contact with friends and family; avoid seeing doctors, even when sick from something unrelated to flu; and stay indoors at all times.

To prevent their forgetting the earlier settings on the list and to limit the interview to a maximum of 15 minutes, respondents were not given the full lists of the riskiest places and precautionary actions mentioned above. Instead, 3 places were randomly selected from the list of 6 possible places, and 3 precautionary actions were selected from the 8 possible. Additionally, the 3 places and the 3 precautionary actions were presented in random order. The main limitation of this sampling method is that it effectively reduces our sample size, but we expect sampling bias to be minimal because options (risky places and precautionary actions) were allocated randomly.

Analysis

The analysis was performed by using STATA software version 8 (StataCorp LP, College Station, TX, USA). Simple *t* test was used to compare the differences in means (or proportions) between the 2 broad sources of samples (Europe and Asia) in terms of riskiest place and adopting precautionary behavior. Probit regression was used to assess the effect of individual- and regional-level covariates on each reported precautionary action. The main outcome variable was whether respondents reported that they would avoid the places presented to them. For 8 different specifications, the explanatory variables remain the same and only the outcome of interest (the probability of taking the preventive action) varied. We did not adjust for multiple comparisons, which should be considered when interpreting these results.

For the regression analysis, we controlled for respondents' age, sex, region of residence, educational history, and perceived risk for influenza. Our measure of perceived risk was based on protection motivation theory (*15*), which proposes that the intention to protect oneself depends on 4 factors: 1) perceived severity of a threatened event, 2) perceived probability of the occurrence (vulnerability), 3) perceived efficacy of the recommended preventive behavior (perceived response efficacy), and 4) perceived self-efficacy (level of confidence in one's ability to undertake the recommended preventive behavior). Risk perception (beliefs about potential harm) has many dimensions, but in keeping with nearly all theories, we focused on only 2 (*12*): 1) likelihood and 2) severity of harm if no action is taken.

We also examined the added effect of response efficacy and self-efficacy on precautionary actions. To measure persons' perceived probability of harm/infection (vulnerability) we asked respondents, "How likely do you think it is that you will develop or contract flu from a new flu virus in the case of global flu outbreak?" For severity, we asked, "How serious would it be for you to get the disease in the next year?" In line with protection motivation theory (*14*,*15*), risk perception was constructed by multiplication of severity (scale of 1 to 10) and vulnerability (scale of 1 to 5) scores. To make the severity and likelihood scores comparable, the severity score was first divided by 2. To normalize the skewed distribution of the constructed risk perception variable, a square-root transformation was performed, which resulted in a measure of risk perception on a scale from 1 (low) to 5 (high) (*14*).

Results

Respondents

Of the eligible persons who were contacted by phone, 42% completed the interview and the rest refused to participate, which resulted in a sample size of 3,436. The cooperation rate varied between 21% in the United Kingdom and 81% in Poland (*14*). Unadjusted summary statistics and description of the key variables of interest are given in Table 1. The number of respondents in each participating region ranged from 401 to 502 (Table 2). Table 1 shows that respondents from European countries had a higher perceived risk for influenza, lower perceived risk for SARS, and in general were older than respondents from Asia. Compared with Asians, relatively more Europeans had a secondary education, fewer had a university education, and substantially fewer lived in urban areas.

Riskiest Place

Public transportation was identified as the riskiest place by >54% of persons who were given this option (43% in Singapore to 63% in Spain; Table 2) and by respondents from 6 of the 8 regions. Places of entertainment were generally ranked as the next most risky setting (in China and Singapore the ranking of public transportation and entertainment was reversed), followed by hospitals, shops, then work or school (Figure 1). Respondents from all regions reported the home to be the least risky setting (Table 2, Figure 1).

Precautionary Behavior

Avoidance of public transportation was consistently reported across the region as the most likely precautionary behavior. From 65% (in Singapore) to 85% (in Great Britain) of respondents reported that they would avoid public transportation. Similar proportions of European respondents reported that they would avoid places of entertainment, although a far smaller proportion of Asian respondents said that they would (Figure 2), despite Asians being more like-

Table 1. Variables measured in survey of risk perception for pandemic influenza, Europe and Asia*

Variable	Definition	Mean score (SD)	
		Respondents from Europe (n = 2,196)	Respondents from Asia (n = 1,240)
SARS risk perception	Risk perception score (1–5)	2.47 (0.95)	2.95 (1.13)
Influenza risk perception	Risk perception score (1–5)	2.95 (1.01)	2.83 (1.05)
Influenza severity	Perceived severity of influenza (1–10)	6.94 (2.55)	6.56 (2.69)
Influenza vulnerability	Perceived vulnerability to influenza infection (1–5)	2.81 (1.17)	2.75 (1.24)
Sex	1 if female	0.40 (0.49)	0.45 (0.50)
Age	Age in years	47.46 (14.32)	39.13 (15.03)
Education medium	1 if respondent has more than a secondary but at least a higher secondary education	0.59 (0.49)	0.51 (0.50)
Education high	1 if respondent has university qualification	0.30 (0.46)	0.42 (0.49)
Urban area	1 if respondent's area of residence is city/town	0.61 (0.49)	0.96 (0.20)
European region	1 if respondent is from European region	NA	NA
High-risk scenario	1 if given outbreak scenario is high risk	0.50 (0.50)	0.66 (0.47)
Health	Health status on 1–6 Likert scale	4.28 (1.10)	4.25 (1.08)
Vaccinated	1 if vaccinated against influenza in past year	0.19 (0.39)	0.22 (0.41)
Employed	1 if employed	0.60 (0.49)	0.60 (0.49)

*NA, not available.

ly to report this setting as risky (Figure 1). Approximately 60% of respondents said that they would shop for essentials only, and ≈50% said that they would take leave from work, prevent their children from attending school, or limit contact with friends and family. Approximately 25% of European and 35% of Asian respondents said that they would try to stay indoors or avoid seeing physicians (Figure 2). Univariate analysis results suggested a statistically significant difference between regions in terms of proportions of persons who would adopt precautionary actions in case of a hypothetical influenza outbreak (Table 3).

Multivariate regression was used to test the association between the likelihood of reporting precautionary actions and individual-, country-, and regional-level characteristics (Table 4). The coefficients in Table 4 reflect marginal effects, which can be interpreted as probabilities. For example, the coefficient attached to the European region in the first regression equation (avoiding public transportation) was 0.038, which can be interpreted as Europeans being 3.8% more likely than Asians to avoid public transportation.

In general, individual characteristics such as age, sex, self-reported influenza vaccination, and health status had little effect on reported precautionary measures (although younger persons were less likely to avoid places of entertainment and more likely to take leave from work). Even persons' perceived risk for influenza had little effect except for avoiding public transportation; more respondents with higher risk perceptions reported being likely to avoid this setting (Table 4). The only individual-level variable that appeared to affect many of the precautionary actions was employment status. Fewer employed respondents reported being likely to avoid public transportation, entertainment venues, and work, and less likely to stay at home than those not employed full-time (e.g., homemakers, retirees, students). Although employed respondents were less likely (or perhaps less able) to adopt precautionary measures for themselves, they were more likely than persons who were not employed to report that they would withdraw their children from school (Table 4). The other notable individual-level covariate was education. In general, more respondents with higher educational levels reported being likely to avoid entertainment and shopping than did those with lower educational levels. Those with higher educational levels were generally less likely to report that they would take precautionary measures in other settings, but the effects were not statistically significant (Table 4).

The risk scenario given to the respondents (high vs. lower illness and death rates) did not significantly affect

Table 2. Perceived risk of setting during influenza pandemic, Europe and Asia*

Location	Sample size	Most risky†	Least risky†
Guangdong, PRC	409	Entertainment (56)	Home (0)
Hong Kong, SAR, PRC	401	Public transportation (52)	Home (2)
Singapore	430	Entertainment (48)	Home (12)
Spain	427	Public transportation (63)	Home (3)
Poland	502	Public transportation (60)	Home (1)
Denmark	463	Public transportation (58)	Home (4)
Great Britain	401	Public transportation (49)	Home (4)
The Netherlands	403	Public transportation (48)	Home (5)

*PRC, People's Republic of China; SAR, Special Administrative Region.
†Numbers in parentheses indicate percentage of respondents who were given this option.

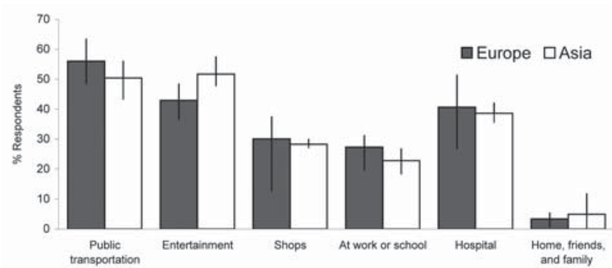

Figure 1. Proportion of respondents who reported each setting as the riskiest for acquiring pandemic influenza, by region. Vertical lines indicate range of means for each region.

the results (Table 4). Region, however, did affect many of the reported precautionary actions. For example, Europeans were 19% more likely than Asians to report that they would keep their children from school, whereas Europeans were 13% less likely to report that they would avoid seeing physicians (Table 4). Respondents who lived in urban areas were less likely than their rural counterparts to report that they would avoid entertainment venues and restrict their shopping to the essentials, although the differences were not large (Table 4).

Discussion

As a part of a large population-based survey of perceptions of pandemic influenza risk, we studied preventive behavior in 8 regions. Conducting comparable surveys in a number of different countries (3 of which had large SARS outbreaks in 2003) made it possible to make intercountry comparisons and assess underlying factors that may lead to precautionary actions. Our results suggest that large numbers of persons would try to take precautionary measures to reduce their risk of acquiring pandemic influenza. Approximately 75% of respondents said that they would avoid public transportation, and similar numbers would avoid places of entertainment and restrict their shopping to the essentials. These reported actions are in agreement with those reported in similar hypothetical studies and recorded behavior in the face of an epidemic. A recent survey of public health professionals in the United States (*16*) indicated that almost half would avoid work, a proportion similar to that reported by the general public in our survey. A survey of the Chinese community in the Netherlands, conducted just after the SARS epidemic, indicated that 84% had avoided travel to SARS-affected areas and 50% had avoided large gatherings of people (unpub. data), results that are comparable to those reported here. Furthermore, data on the use of public transportation and entertainment facilities in SARS-affected regions (*17*) suggest that demand for these services is affected by the public's perceived risk of acquiring disease. The effect of such precautionary measures could be large in the case of pandemic influenza; the east and Southeast Asian economies lost an estimated $60 billion in the SARS outbreak because of reduced demand and business revenues (*18*).

Knowledge of what persons are likely to do can be used to estimate the health and economic effects of various pandemic influenza scenarios. We describe what proportion will take precautionary actions as well as the socioeconomic background of these persons, which would be useful for improving communication efforts by public health officials and clinicians in response to an outbreak.

One of the strengths of our study was its multicountry approach; with few exceptions, the patterns of potential precautionary actions were similar among respondents in each region. Public transportation was generally regarded as the most risky place and most likely to be avoided; home was regarded as the least risky setting. Individual-level characteristics such as age, sex, health, and educational status played little role in reported precautionary actions. Some regional differences were noted; Asian respondents reported that they were less likely to avoid restaurants and other entertainment establishments and more likely to avoid visiting physicians (the latter may have been related to their increased awareness of SARS [Table 3], which was often acquired in a healthcare setting). The identification of shops and hospitals as risky places had the largest variation between countries in Europe but the smallest variation between regions in Asia (Figure 1). The dominant pattern, however, was broadly similar across sociodemographic, health, and geographic strata.

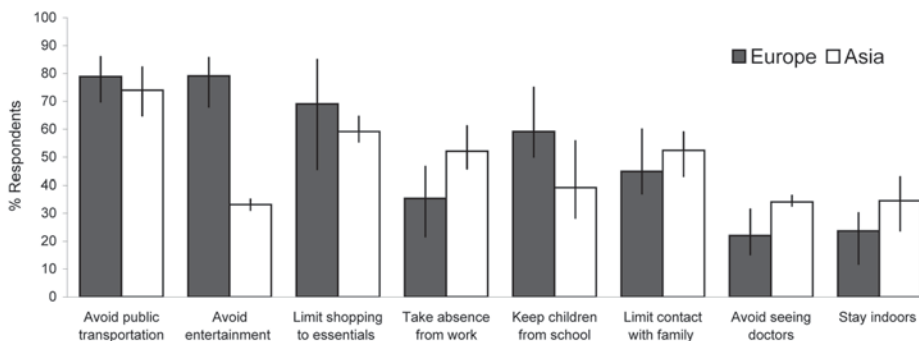

Figure 2. Proportion of respondents who reported that they would take precautionary actions, by region. Vertical lines indicate range of means for each region.

Table 3. Perception of risk for hypothetical pandemic influenza, Europe and Asia

Variable	Sample size*	Europe, % (SD)	Asia, % (SD)	H_0: proportions (*E*) – proportions (*A*) = 0†
Riskiest place				
Public transportation	1,700	56 (0.50)	50 (0.50)	0.0234
Entertainment	1,793	43 (0.50)	52 (0.50)	0.0004
Shop	1,702	30 (0.46)	28 (0.45)	0.4340
Work/school	1,668	27 (0.45)	23 (0.42)	0.0444
Hospital	1,749	41 (0.49)	39 (0.49)	0.4025
Family	1,696	3.4 (0.18)	5 (0.22)	0.1190
Precautionary behavior				
Avoid public transportation	1,341	79 (0.41)	74 (0.44)	0.0418
Avoid entertainment	1,263	79 (0.41)	33 (0.47)	0.0000
Limit shopping to essentials	1,355	69 (0.46)	59 (0.49)	0.0002
Be absent from work	1,307	35 (0.48)	52 (0.50)	0.0000
Keep children from school	349‡	59 (0.49)	39 (0.49)	0.0002
Limit contact with friends/family	1,293	45 (0.50)	52 (0.50)	0.0100
Avoid seeing doctors	1,310	22 (0.42)	34 (0.47)	0.0000
Stay indoors	1,316	24 (0.43)	35 (0.48)	0.0000

*Sample size represents the number of persons randomly chosen to answer each option.
†H_0, null hypothesis that difference between European (*E*) and Asian (*A*) proportions is not statistically significant. Significant differences using p value of *t* test.
‡This question was asked of only the 349 respondents who had a child going to school.

The multivariate analysis provides some useful insights. Employment has emerged as an important determinant of prospective precautionary actions; employed persons were less likely to report that they would take preventive actions.

Our measure of risk perception (combining severity and vulnerability) was not associated with precautionary actions (except avoiding public transportation), and the measures of severity and vulnerability, separately, did not indicate any statistically significant influence. Thus, neither the risk perception score nor its individual components seemed to affect preventative actions, apart from the likelihood of avoiding public transportation. Liu et al. (*19*) found different components of risk perception to be significant in different geographic areas. In the Netherlands, higher risk perception was associated with more self-reported precautionary actions for SARS; however, when other explanatory variables like age, sex, education were included, no significant association between risk perception and precautionary actions was observed (*20*). If risk perceptions really do have little effect on precautionary behavior, public health messages aimed at changing persons' perceptions of risk might be ineffective at changing their behavior. Clearly, this area requires further empirical study.

The main drawback of this type of survey is the difficulty in validating results. First, the participation rate varied 21%–81% among regions. Although a low response brings into question the representativeness of the samples, the similarity in findings between regions suggests that the low participation rate in some regions did not bias the

Table 4. Results of regression analysis (marginal effects) of precautionary behavior for hypothetical influenza pandemic, Europe and Asia

Variable	Avoid public transportation	Avoid entertainment	Limit shopping	Take absence from work	Keep children from school	Limit contact with family/ friends	Avoid seeing doctor	Stay indoors
Sex (male)	0.005	–0.048	–0.054*	0.042	0.017	–0.021	–0.018	0.013
Age	–0.001	0.003*	0.001	–0.003*	–0.004	0.001	–0.001	0.001
Education medium	0.039	0.113*	0.175	–0.023	–0.223*	–0.015	–0.015	–0.033
Education high	0.033	0.139*	0.185*	–0.030	–0.189	0.010	–0.036	–0.055
Urban area	–0.044	–0.071*	–0.108*	0.004	–0.064	0.015	–0.061	0.009
European region	0.038	0.422*	0.072*	–0.139*	0.186*	–0.077*	–0.130*	–0.120*
High-risk scenario	0.000	0.018	0.056*	0.036	–0.056	–0.013	0.040	0.002
Risk for influenza	0.025*	0.004	0.012	0.009	–0.051	0.001	–0.006	0.014
Health	0.017	–0.009	–0.008	–0.013	–0.071*	–0.009	0.016	–0.011
Vaccinated	0.019	0.011	0.041	0.025	0.045	0.002	0.003	0.022
Employed	–0.051*	–0.059	–0.081*	–0.070*	0.053	–0.059	–0.033	–0.113*
Observation	1,341	1,263	1,355	1,307	349	1,293	1,310	1,316
Log likelihood	–710.699	–687.188	–843.241	–863.953	–226.204	–887.342	–744.488	–747.710

*Significant at 0.05.

findings. Second, because of the hypothetical nature of the questionnaire, concluding that persons actually would respond in the way that they have indicated here is not possible; however, the fact that a large section of the Dutch Chinese population did report taking precautionary actions to avoid SARS lends support to our findings (unpub. data).

Although the quantitative nature of the results may be difficult to validate, the qualitative findings are likely to be more robust. A new influenza pandemic would most likely result in persons' limiting their use of public transportation, entertainment, and shopping for nonessentials. Also, although the public may perceive the risk from healthcare facilities to be relatively high, they would not necessarily avoid them.

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Simian Foamy Virus Transmission from Apes to Humans, Rural Cameroon

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Simian virus infections of humans are an increasing public health concern. Simian foamy virus (SFV) infections have been reported in persons occupationally exposed to nonhuman primates and in a few hunters in Cameroon. To better understand this retroviral zoonosis in natural settings, we studied persons who lived in southern Cameroon, near nonhuman primate habitats. First we studied a general population of 1,164 adults; 4 were SFV positive according to serologic and molecular assays. Then we studied 85 persons who reported having been bitten or scratched by nonhuman primates; 7/29 (24.1%) of those who had contact with apes (gorillas or chimpanzees) were SFV positive, compared with only 2/56 (3.6%) of those who had had contact with monkeys. These data demonstrate efficient transmission of SFVs to humans in natural settings in central Africa, specifically following ape bites, and viral persistence in the human host.

A large proportion of viral pathogens that have recently emerged in humans have originated in various animals. After initial interspecies transmission, these viruses have evolved and disseminated into the human population through various distinct mechanisms. However, understanding of the initial steps of the emergence of some viruses and associated diseases remains poor. Microbiologic studies of these high-risk populations are thus necessary to obtain new insights into the early events of this emergence process (*1–4*).

Nonhuman primates represent a potential source of microbes for humans (*1*,*5–12*), e.g., simian immunodeficiency virus and simian T-cell lymphotropic virus (*12–15*). Simian foamy viruses (SFVs) are exogenous complex retroviruses, highly prevalent in several animal species in which they cause persistent infections (*16–26*). Phylogenetic analyses have demonstrated a species-specific distribution of such retroviruses. This species specificity indicates a long-term coevolution of SFVs with their natural hosts (*27*), which could explain their possible lack of pathogenicity observed in vivo and the persistence of the infection (*23*,*24*,*28–31*). Among nonhuman primate populations, SFV seroprevalence can reach 75%–100% in adults, and SFVs appear to be present at high concentrations in the saliva of infected animals (*16–18*,*22*,*29*,*31*).

In humans, SFV infection has been reported in 1%–4% of persons occupationally exposed to nonhuman primates in zoos, primate centers, and laboratories, mainly in North America but also in Europe (*7–10*). More recently, naturally acquired SFV infections were described in a few hunters living in Cameroon (*11*) and in 1 person who had had contact with *Macaca fascicularis* in Indonesia (*32*).

After other studies demonstrated high prevalence and genetic diversity of SFVs in monkeys and apes in Gabon and Cameroon (*16*,*17*), we investigated the presence of SFV infection in humans living in these regions. Our goals were to 1) determine, by using specific serologic and molecular methods, the prevalence of SFV infection in the adult population of different ethnic groups (including Pygmies) who lived in rural areas of Cameroon near natural nonhuman primate habitats and who were thus at risk for cross-species transmission; 2) trace the origin of the SFVs infecting these persons by isolation and molecular characterization of the virus; and 3) gain new insights into the epidemiologic determinants and risk factors linked to such naturally acquired retroviral infections, especially the type of nonhuman primates, the circumstances of the contact leading to the infection, and possible intrafamilial transmission of such viruses.

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Materials and Methods

Populations

The first study, a retrospective study, was based on a large series of samples collected during 1994–2000 for epidemiologic studies on human T-lymphotropic virus (HTLV)-1 and HTLV-2 as well as human herpesvirus 8 (*33*,*34*). The samples originated from adults of 3 ethnic populations: Bakola Pygmies and 2 groups of Bantus, who lived in lowland tropical remote forest areas (Bipindi/Lolodorf and Ntem) in southwestern Cameroon (Figure 1).

The second study, the hunter study, was conducted in 2004–2005 in remote villages near nonhuman primate habitats in the South Province of Cameroon (Figure 1). This study was focused on persons who reported direct and often severe contacts (bites, wounds, scratches, other injuries) with animals, especially nonhuman primates, mainly while hunting.

Both studies received clearance from national and local authorities. All participants received detailed information about the study and gave consent. Blood samples were collected in 5–10 mL EDTA tubes. Plasma was available from all participants in the retrospective study, whereas for some in the hunter study, only a few drops of blood were taken by fingerstick and conserved on filter paper (Whatman samples) as described (*35*). See online Technical Appendix, available from www.cdc.gov/EID/content/13/9/1314-Techapp.pdf, for more details.

Serologic Tests, Virus Isolation, and Molecular Studies

We screened by Western blot (WB) all plasma and Whatman samples for the presence of SFV antibodies as described (*18*,*22*,*26*). Plasma was tested at a 1:100 dilution. For each Whatman sample, a 1-cm punch was diluted in 1 mL of phosphate-buffered saline and tested at a 1:8 dilution (online Technical Appendix). Virus isolation, electron microscopy, and immunofluorescence (IFA) were performed as described (*9*,*21*,*26*,*36;* online Technical Appendix).

For the molecular studies, genomic DNA was extracted from the peripheral blood buffy coat by using the QIAamp DNA Blood Mini Kit (QIAGEN, Courtaboeuf, France). Two SFV proviral genomic regions (465 bp of the *integrase* gene and 109 bp of the long terminal repeat [LTR]) were amplified in nested PCR (*18*,*21*,*37*). *Integrase* PCR products were purified, cloned, and sequenced. The GenBank accession numbers of the 13 new *integrase* sequences are DQ838495–DQ838507. Phylogenetic analyses were performed as described (*18*,*38*,*39*; online Technical Appendix.)

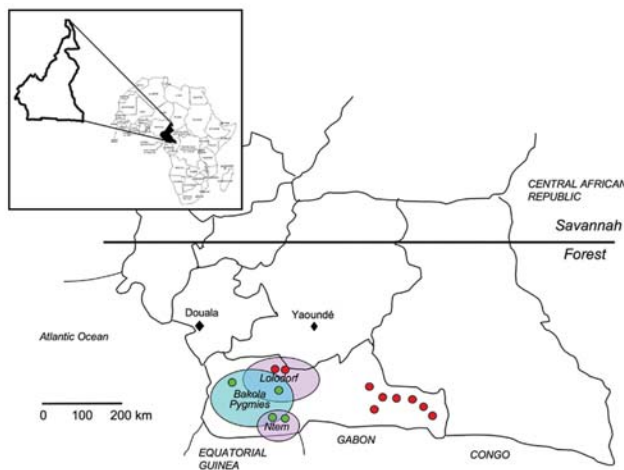

Figure 1. Geographic distribution in Cameroon of the studied populations and the 13 persons infected by simian foamy virus (SFV), according to serologic and molecular results. Red, SFV-positive persons from the hunter study; green, SFV-positive persons from the retrospective study; blue circle, Pygmy area; violet circles, Bantu areas.

Results

Retrospective Study

The retrospective epidemiologic survey was performed among 1,164 adults (mean age 50.6 years) who lived in the Ocean region of Cameroon (Figure 1; Table 1). The studied populations included 478 Bakola Pygmies (mean age 47.6 years) and 686 Bantus (mean age 52.6 years).

Of the 1,164 samples tested by WB assay based on chimpanzee foamy virus antigens, 21 (1.8%) were considered clearly positive (strong reactivity to both p70 and p74 ape proteins, Gag doublet) (Figure 2, panel A), 86 (7.4%) were considered borderline/indeterminate (presence of either a faint gag doublet or of at least a strong band of the right size and 1 or few other bands of often low intensity) (Figure 2, panel C), and the remaining 1,057 samples were considered negative (absence of any band) (Figure 2, panel C; Table 1). The 86 indeterminate samples were then tested by WB assay using antigens from a monkey foamy virus (originating from participant AG16, Figure 2, panel B); all were still indeterminate or negative.

DNA was available from 11 of the 21 persons whose WB assay results were positive and from 52 of 86 whose results were borderline/indeterminate. All 63 DNA samples were amplifiable by PCR for β-globin gene. When *integrase* primers were used, PCR was positive for 4 of 63 samples (Table 2). When LTR primers were used, PCR was positive in 3 of these 4 samples (Table 2).

Field interviews indicated that 3 persons (2 Bakola Pygmies [801001 and 210301] and 1 Bantu [60601]) were frequent hunters and had been severely bitten by gorillas 25–35 years ago (Table 2); all 3 had scars on their legs and fingers (Figure 3). The fourth infected person (A051302) was a Bantu woman who did not recall any bites or injuries from monkeys or apes. However, she had had frequent contact with

Table 1. Serologic results for simian foamy virus retrospective study, rural Cameroon, 1994–2000

Area	Study population					Test results		
	Ethnicity	Age range, y	Total no.	Men	Women	Negative	Borderline, no. (%)	Positive, no. (%)
Bipindi Lolodorf	Bakola Pygmies	30–82	478	214	264	448	16 (3.34)	14 (2.92)
	Bantus	40–83	370	180	190	326	40 (10.81)	4 (1.08)
Ntem	Bantus	20–78	316	144	172	283	30 (9.49)	3 (0.9)
Total		20–83	1,164	538	626	1,057	86 (7.38)	21 (1.8)

wild game meat from nonhuman primates while butchering and preparing meals, as is common in this area (*3*,*4*).

Sequence analyses of the 4 *integrase* gene fragments indicated that the 3 persons bitten by gorillas were infected with a gorilla foamy virus. These 3 sequences were similar to the sequence CAM1083 (96.7%–98.5% identity) reported in a Cameroonian hunter infected by a gorilla foamy virus (*11*) and to known sequences of foamy virus from gorillas living in Cameroon and closely related to each other (97%–99% identity). The Bantu woman had been infected by a chimpanzee belonging to *Pan troglodytes troglodytes*, 1 of 2 chimpanzee subspecies endemic to Cameroon (online Appendix Figure, available from www.cdc.gov/EID/content/13/9/1314-appG.htm).

Hunter Study

Our next step was to not only characterize more cases of such interspecies transmission, looking especially for viral acquisition from other nonhuman primates, but also to assess the frequency of such phenomena and to define the parameters that characterize a risk population. Thus, we focused our work on persons who had regular contact with nonhuman primates, hunters in lowland rain forest regions.

During 2004–2005, field missions were initiated in remote villages of Bantus and Baka Pygmies in different areas of south Cameroon. In each village we specifically asked for persons who had had direct contact and severe bites, scratches, wounds, other injuries from animals, mainly nonhuman primates.

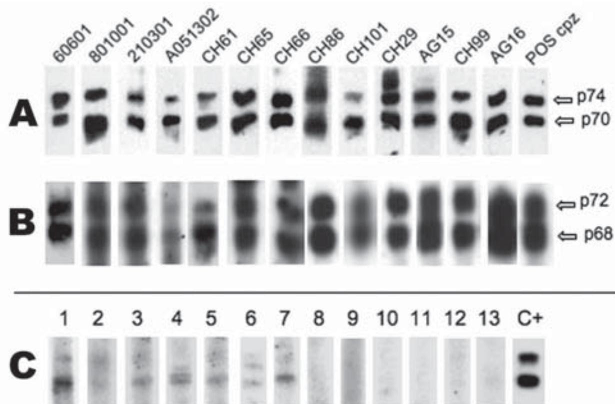

Figure 2. A) Western blot (WB) results based on chimpanzee (cpz) simian foamy virus (SFV) antigens. B) WB results based on monkey simian foamy virus antigens originating from participant AG16. C) Example of sero-indeterminate samples (lanes 1–7) and negative samples (lanes 8–13), detected by cpzSFV WB. Last lane (POS cpz), serum from an SFV-positive chimpanzee.

This study included 102 persons, 84 men and 18 women, most of them adults (mean age 40 years, range 2–80 years). Of these 102, 29 (28.4%) had had contact with apes (gorillas, chimpanzees), and 56 (54.9%) with monkeys (*Cercopithecus nictitans),* mandrills, and a few other small monkeys not precisely identified). Thus, 85 of 102 had been in contact with nonhuman primates. Contact with rats, elephants, warthogs, duikers, squirrels, porcupines, and leopards was reported by 17 (16.6%).

From the 102 persons, we obtained 61 plasma samples and 41 dried blood spots. All samples were tested by WB, and 10 (9.7%) were clearly SFV seropositive (Figure 2). Of 15 specimens that were indeterminate/borderline, WB based on monkey FV antigens (originating from participant AG16) showed them all to be negative or indeterminate.

PCR performed on the available DNA (from the 10 WB-seropositive, 8 sero-indeterminate, and 33 seronegative persons) gave positive results for the *integrase* gene in 9 of the 10 WB-positive samples (Table 2) and negative results for the others. The LTR PCR was positive for 7 of 9 *integrase*-positive samples and none of the 42 others.

All 9 SFV-positive persons belonged to the group of 85 persons who had had known contact and bites or scratches from apes or monkeys. Thus, the subsequent epidemiologic analysis was restricted to these 85 (71 men, 14 women; mean age 39 years). According to univariate analysis, foamy virus–positive serologic results were associated with the type of nonhuman primate encountered (monkeys 3.6% vs. apes 24.1%, $p = 0.003$) and the type of encounter (pets 0% vs. hunting 16.1%, $p = 0.022$) (Table 3). No other studied risk factor (except age at time of contact) was significantly associated with positive results.

Among the 56 persons who had received severe bites or scratches from nonhuman primates while hunting, 7 (36%) of the 19 that had encountered an ape were infected with SFV, in contrast to only 2 (5.4%) of 37 who had had contact with a small monkey ($p<0.05$) (data not shown). To determine possible intrafamilial transmission of SFVs, we tested 4 wives and 1 husband of 5 of the index case-participants as well as 5 of their children (Table 2). All were seronegative according to WB.

Table 2. Demographic and epidemiologic features, serologic and PCR results, 13 SFV-infected inhabitants of rural Cameroon*

Participant code, sex, ethnicity	Year(s) of sample collection	Age at sample collection, at animal contact, y	Type of animal	Wound location	Serologic results (specimen)	LTR/ *Intergase* PCR	*Integrase* sequence	Viral load, copies/500 mg DNA
60601, M, Bantu†‡	1999	67, 30	Gorilla, monkey	Finger	+ (plasma)	+/+	Gorilla	10–100
801001, M, Pygmy†‡§	1996, 1998	60, 35	Gorilla	Leg	+ (plasma)	+/+	Gorilla	100–1,000
210301, M, Pygmy†‡	1996, 2004	68, 35	Gorilla	Leg	+ (plasma)	-/+	Gorilla	1–10
51302, F, Bantu†‡§	1998	40, NK	None?	NK	+ (plasma)	+/+	Chimp.	100–1,000
CH29, M, Pygmy¶	2004, 2005	50, 49	Chimp., gorilla	Finger, foot	+ (DBS, plasma)	+/+	Gorilla	1–10
CH61, M, Bantu*§¶	2004, 2005	65, 52	Gorilla	Hand, arm	+ (plasma)	+/+	Gorilla	10–100
CH65, M, Pygmy¶	2004	58, 26	Gorilla	Head, arm	+ (plasma)	+/+	Gorilla	1–10
CH66, M, Pygmy¶	2004	60, 56	*Cerco. nictitans,* Chimp.	Hand, foot	+ (plasma)	+/+	Chimp.	ND
CH86, M, Bantu†¶	2004	62, 47	Gorilla	Hand	+ (plasma)	+/+	Gorilla	1–10
CH99, M, Bantu¶	2004	26, 25	Monkey, species?	Hand	+ (plasma)	–/+	Mandrill	1–10
CH101, M, Bantu¶	2004	76, 65	Gorilla	Hand	+ (plasma)	+/+	Gorilla	1–10
AG15, M, Bantu¶	2004, 2005	71, 28	Chimp.	Hand, foot	+ (DBS, plasma)	+/+	Chimp.	100–1,000
AG16, M, Bantu¶	2004, 2005	43, 23	Monkey *Cerco.*	Foot	+ (DBS, plasma)	–/+	*Cerco.*	1–10

*SFV, simian foamy virus; LTR, long terminal repeat; +, positive; –, negative; DBS, dried blood spot; NK, not known; Chimp., chimpanzee; ND, not determined; *Cerco., Cercopithecus.* The age at contact with nonhuman primate and the type of animal contact concern the results of the field interviews performed for each person found to be infected by SFV.
†Wife or husband also tested.
‡Retrospective study participant.
§1 (A051302) or 2 (CH61 and 801001) children also tested.
¶Hunter study participant.

Of the 9 SFV-positive persons, 7 had been severely bitten by a gorilla (4 persons) or chimpanzee (1 person) 1–53 years ago while hunting (Table 2); some displayed large scars on the legs, arms, feet, or fingers (Figure 3). Hunters CH66 and CH29 had been severely bitten by 2 different animals in 2 separate hunting incidents. The 2 other SFV-positive persons were adult men who had been bitten by a small monkey, including a mandrill and a *C. nictitans* (Table 2).

Phylogenetic analyses of the 9 *integrase* products indicated that all belonged to the large clade of the African SFVs with 5 strains from gorilla, 2 from chimpanzee, 1 from mandrill, and 1 closely related to *Cercopithecus* strains (online Appendix Figure). The 2 hunters who had been bitten by 2 different animals were infected with chimpanzee (CH66) and gorilla (CH29) foamy viruses, respectively.

Thus, for each of the 9 case-participants, the match was nearly perfect between the history of contact with a given nonhuman primate species (mainly through severe bites that had occurred decades ago) and the simian virus sequence that was found in the infected person (Table 2).

In Vivo Virus Persistence

Because each of the 6 persons from whom we obtained 2 samples (plasma, dried blood spots, or both) at different times was SFV positive by WB, persistent infection was evident for each person. The duration of this persistent infection was 1–8 years.

Isolation of 2 New Foamy Virus Strains

SFV was assayed for 2 persons (AG15 and AG16) from whom blood was available for culture. Giant-cell formation and syncytia were first observed for AG15's sample after 26 days of coculturing, whereas cytopathic effect (CPE) was detected only after 33 days for AG16's sample. The destruction of the monolayer of BHK-21 was quite rapid (2–4 days) after the first appearance of the CPE. Syncytia and giant cells showed a strong and clear specific immunofluorescence (Figure 4).

Electron microscopic analyses of cultured cells with a strong CPE demonstrated the presence of multinucleated giant cells. Typical foamy virus particles (diameter 100–110 nm) were frequently observed with several envelope

Table 3. Univariate analysis results for risk factors for simian foamy virus, 85 persons, rural Cameroon*

Risk factor	Total no. tested	Positive, no. (%)	p value
Age at contact, y			
≥45	65	4 (6.2)	
<45	20	5 (25)	0.017
Sex			
Male	71	9 (12.7)	
Female	14	0	0.159
Ethnicity			
Bantu	72	6 (8.3)	
Pygmy	13	3 (23.1)	0.112
Type of animal interaction			
Pet†	29	0	
Hunted	56	9 (16.1)	0.022
Type of nonhuman primate			
Monkey	56	2 (3.6)	
Ape	29	7 (24.1)	0.003
Wound type			
Scratches	9	0	
Bites	76	9 (11.8)	0.275
Wound location			
Upper body	31	2 (6.5)	
Lower body	54	7 (13)	0.348
Scars			
Absent	12	0	
Present	73	9 (12.3)	0.198

*Only the 85 persons with nown severe bites or scratches from a nonhuman primate. Univariate analyses were performed by using STATA (StataCorp LP, College Station, TX, USA) software with the χ^2 and Fisher exact tests with critical p value = 0.05.
†Most pets were *Cercopithecus nictitans* and mandrills; some were small chimpanzees.

spikes and a spherical central core (Figure 4). Budding of such virus particles was observed, mainly from the membrane surface of the endoplasmic reticulum.

PCR was performed on DNA extracted from the viral isolates after 2 months of culture. Comparative sequence analyses of the *integrase* product showed 100% nucleotide identity for AG16 (*Cercopithecus* strain) and 99.8% for AG15 (chimpanzee strain) between the SFV sequences from the peripheral blood mononuclear cell uncultured DNA and the cultured viral isolate.

Foamy Virus Load in Buffy Coat

To determine the peripheral blood viral load in persons infected by SFVs and to check whether the discrepancies in the results between the 2 PCR assays (*integrase* and LTR) could be related to a low viral load (reaching the limits of our PCR sensitivity), we used a semiquantitative PCR assay (*18*). Of the 13 infected persons, 7 (Table 2) had a very low viral load, 1–10 copies in 500 ng of total DNA. For only 4 (all of them positive for both nested PCRs), the viral load was higher, 100–1,000 copies in 500 ng of total DNA (Figure 5; Table 2).

Discussion

Animal reservoirs are one of the most important sources of emerging infectious diseases that threaten humans. Recent zoonotic transmission of retroviruses has led to the emergence of HIV-1 and HIV-2 in humans (*13*). Nonhuman primates are natural hosts for other retroviruses. Although SFVs have been recently shown to infect persons occupationally exposed to nonhuman primates in zoos and primate centers, little is known about modes of cross-species transmission of these viruses in their natural habitat.

In the current study of adults living in central African regions with high nonhuman primate diversity, ≈2% of 1,164 persons showed clear seroreactivity to SFVs and at least 4 were persistently infected with SFV, with detectable viral sequences in their peripheral leucocyte DNA. These results confirm and extend to other areas of Cameroon the original findings published by Wolfe et al., who found that 10 (1%) of 1,099 of a comparable population had antibodies to SFV with a positive PCR for only 3 of them (*11*). These data, combined with the findings of our hunter study, which identified 9 more SFV-infected persons, demonstrate infection by a large diversity of SFVs in persons from geographically isolated areas. Such retroviral zoonosis is thus widespread and occurs in diverse villages where hunters are frequently in contact with nonhuman primates (*3*,*4*). In another context, a model has predicted that in Bali, Indonesia, for every 1,000 visitors to a monkey temple, approximately 6 will be infected with SFV (*40*).

Our study demonstrates efficient transmission of SFVs to persons in natural settings in central Africa, specifically after the persons had been bitten while hunting, and a viral persistence in the human host. Indeed, >35% of the hunters bitten severely (often with soft tissue crushing, tearing, and bleeding) by a gorilla or a chimpanzee were SFV infected. This strongly suggests that in a natural situation, contact of human blood with the saliva of an adult ape or monkey is the key factor for SFV transmission to humans. This situation is similar to that of persons occupationally exposed to nonhuman primates in zoos and primate centers, as nearly all of them reported having been bitten by monkeys or apes (*5*,*9*,*10*). Some studies have shown that SFVs are present at

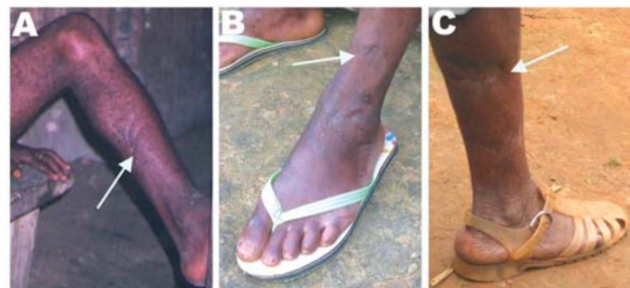

Figure 3. Wounds resulting from bites or scratches from a nonhuman primate. A) Participant no. 801001. B) Participant no. AG16. C) Participant no. 210301.

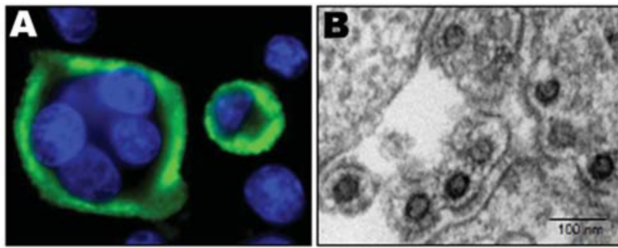

Figure 4. Immunofluorescence and electron microscopy results. A) Typical multinucleated giant cells with a clear seroreactivity of AG16 antigens, determined by using an immunofluorescence assay with positive anti–foamy virus serum, on BHK-21–infected cells cocultivated with stimulated peripheral blood mononuclear cells. B) Electron microscopy of ultrathin sections from cells infected by AG16 foamy virus.

high concentration in the saliva (with viral replication) of infected animals (*29*,*31*). We recently provided evidence that *Macaca tonkeana* mostly acquire SFVs through severe bites, mainly young adults when they compete for sex partners (*18*). In our study, contact with pets was not found to be associated with SFV infection. This might be because pet bites mainly cause superficial tissue damage and rarely cause serious wounds and because some of the animals are probably not SFV infected due to their young age when captured.

In our study, SFV *integrase* or LTR sequences were not detected in several of the persons who were confirmed seropositive by WB. Although the presence of divergent SFVs could explain such discrepancy, low viral load in the blood samples is more likely, because our PCR primers have been shown to amplify a large variety of African SFVs but also several rather divergent Asian SFVs (*16*–*18*,*22*). This lack of detection of FV sequences by PCR may also indicate nonspecific reactivity with SFV Gag antigens. Lack of SFV sequences has also been recently reported in the peripheral blood mononuclear cell DNA of 7 of 10 African hunters who were SFV seropositive according to WB (*11*).

We provide the first data, to our knowledge, on the quantification of viral load of SFVs in humans. Our results, based on 13 infected persons, indicate a low viral load in most persons but a large range (1–1,000 copies in 500 ng of

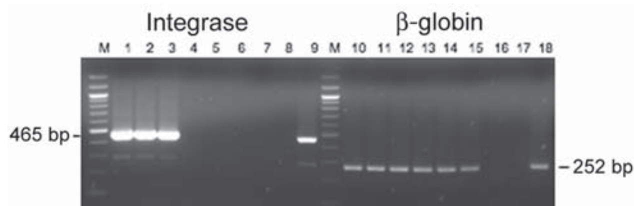

Figure 5. Semiquantitative PCR for *integrase* and β-globin genes using AG15 peripheral blood buffy-coat DNA. Lanes 1–7 and 10–16, serial dilutions of the DNA from 500 ng to 0.5 pg; lanes 8 and 17, negative controls; lanes 9 and 18, positive controls; M, 100-bp ladder.

total peripheral blood leukocytes DNA). These viral loads are comparable to those in wild-born chimpanzees (*16*) and captive *M. tonkeana* (*18*).

Our work did not demonstrate the presence of SFV in the spouses of 5 index case-participants and in 5 of their children. Combined with the scarce published findings on this topic, these results suggest that SFV transmission among humans does not occur easily by sexual contact or saliva exposure (*8*–*10*).

Another concern is the illness and death that might be associated with these retroviral persistent infections after interspecies transmission. The apparent lack of pathogenicity of SFV infection in humans, which is still based on a limited number of cases, contrasts strongly with the massive in vitro lytic properties of these viruses in monkey and human cells (*8*–*10*,*18*,*30*). The selection bias inherent in the enrollment of healthy persons in our study, as well as in those enrolled by Wolfe et al. (*11*) and Switzer et al. (*10*), greatly limits the ability to identify any potential acute or severe associated diseases. A case-control study based on a larger number of SFV-infected persons would help shed light on possible chronic diseases or biological abnormalities associated with human SFV infection. SFV infection in immunocompromised persons, especially those with HIV infection, could also heighten public health concerns because such coinfection is probable in central African areas where HIV-1 is highly endemic.

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Dr Calattini is a researcher whose primary interest is the molecular epidemiology of retroviruses, especially spumaviruses and human T-lymphotropic viruses. She is studying the interspecies transmission of such retroviruses from nonhuman primates to humans in central Africa.

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Family Clustering of Viliuisk Encephalomyelitis in Traditional and New Geographic Regions

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Viliuisk encephalomyelitis is an acute, often fatal, meningoencephalitis that tends to develop into a prolonged chronically progressive panencephalitis. Clinical, neuropathologic, and epidemiologic data argue for an infectious cause, although multiple attempts at pathogen isolation have been unsuccessful. To assess mechanisms of disease transmission and spread, we studied 6 multiplex families. Secondary cases occurred among genetically related and unrelated persons in a setting of prolonged intrahousehold contact with a patient manifesting the disease. Transmission to unrelated persons was documented in a densely populated region around the city of Yakutsk in which Viliuisk encephalomyelitis had not been previously known. Initially identified in a small Yakut-Evenk population on the Viliui River of eastern Siberia, the disease subsequently spread through human contacts to new geographic areas, thus characterizing Viliuisk encephalomyelitis as an emerging infectious disease.

Viliuisk encephalomyelitis (VE) is clinically and pathologically defined as an acute meningoencephalitis that progresses to a more prolonged panencephalitic syndrome (*1–3*). In a small number of patients, the initial acute phase had gone undetected. Cerebrospinal fluid (CSF) pleocytosis is present during the acute phase and subsequent progressive stages of illness. Postmortem examination identifies diffuse infiltration of the meninges with mononuclear and plasma cells; multiple micronecrotic lesions in the brain parenchyma are surrounded by T and B lymphocytes and reactive astrocytes (*4*,*5*). These disease characteristics suggest that VE is an infectious disease, although the causative agent has not been identified and the mechanisms of disease transmission and spread remain unknown.

Strong evidence exists that VE is disseminating through migration of affected persons from villages along the Viliui River in Eastern Siberia, where VE has been endemic for at least a century, to densely populated regions around the city of Yakutsk (*6*), located ≈4° (450 km) below the Arctic Circle (*7*,*8*). This characterizes VE as an emerging infectious disease. From 1950 to 1959, all known VE patients were born in villages in the Viliui valley (*1*), whereas in 1970–1979, 32% of VE patients were identified in previously unaffected regions of Lena and Aldan valleys in the vicinity of Yakutsk (*7*). VE prevalence in these newly affected regions remained stable at 35% in the decade1980–1989 and has been slowly declining since the mid-1990s (*9*).

A preliminary study of familial aggregation of VE patients was based on data collected in the 1950s, 1960s, and 1970s (*7*,*8*). Of 194 VE-affected families, 27 had multiple VE cases, 2 per family in 24 families and 3 per family in 3. Secondary cases occurred in 11 full siblings, 3 children of the index case-patient, a niece, half-sibling, a cousin, 3 spouses, and 10 adopted children or other genetically unrelated persons living in the same household (*7*). The phenomenon of VE clustering in the affected households was verified by statistical analysis. Statistical analyses indicated that clustering of ≥2 cases per family occurred more frequently than should be expected on the basis of family size and VE prevalence rates (χ^2 test, p = 0.00036) (*7*,*8*).

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We sought to document and better characterize the phenomenon of VE clustering in the affected households. We conducted a detailed study of 5 families living in villages along the Viliui River and of 1 family in the region surrounding the city of Yakutsk, to which the disease has recently spread (Figure).

Patients and Methods

VE patients were identified by physicians from neurology services established in the 1950s in Viliuisk (*1*,*3*) and Yakutsk (*6*). Early detection and frequent follow-up of VE patients were accomplished by village-to-village searches and periodic hospitalizations. VE diagnoses were made according to established clinical (*2*,*3*) and pathologic (*4*) criteria; standardized neurologic assessment was performed on all patients, and neuropathologic examination was performed by 3 independent groups (*4*,*5*,*10*). In the 50-year period between 1950 and 2000, 301 patients were identified. Families with 2 or more patients were repeatedly studied in the villages by visiting epidemiologist/neurologist teams, which collected epidemiologic data and reviewed medical histories. Six well-characterized VE-affected families with 2 or 3 patients were included in the current study; neuropathologic examination was performed on 1 patient from each family.

Studies were conducted under clinical protocols approved by the Institutional Review Boards of the Institute of Health, Sakha (Yakut) Republic, and the US National Institutes of Health. The protocol was subsequently reviewed and approved by the Office of Protection from Research Risks, US Department of Health and Human Services (OPRR-S-16078–01). Informed consent was obtained for each element of this study.

Results

Five families originated from settlements in a high-incidence middle Viliui region with a rural population of 10,000, composed of remnants of the indigenous Tungus (Evenk) tribes that have been largely assimilated by the dominant Yakut (Sakha) people. Families 1, 3, and 4 were identified in villages around Lake Mastakh, the peak VE-endemic area; family 2 was from a village near the town of Viliuisk; family 5 was identified in a settlement 100 km down the Viliui River; and family 6 was studied near the capital city of Yakutsk, ≈400 km southeast of Lake Mastakh (Figure). The population in this central region is ethnic Yakut (Sakha).

Family 1

Family 1 consisted of 2 parents and 5 siblings (3 daughters and 2 sons).VE developed in each daughter; no other VE patients were identified in the extended pedigree of this family. In the index case-patient (patient 1-1, Table 1), the

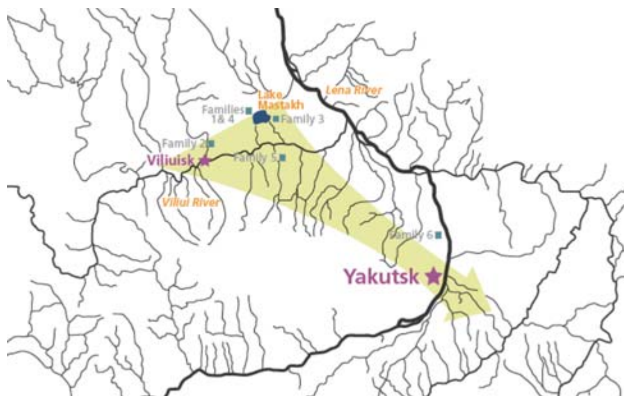

Figure. Location of families with Viliuisk encephalomyelitis characterized in this report. Arrow indicates the general direction of Viliuisk encephalomyelitis dispersion from traditional disease-endemic areas on the Viliui River to densely populated regions of the Sakha (Yakut) Republic around the capital city of Yakutsk.

second-born daughter, high fever, severe headache, myalgia, chills, and double vision developed when she was 16 years of age in 1954. She was admitted to the hospital in a coma and remained drowsy and mentally and physically slow for 6 weeks. Six months later, she had a relapse of a febrile illness with persistent neck stiffness, Kernig sign, and repetitive generalized seizures. After the acute phenomena subsided, she showed evident cognitive impairment, upper motor neuron pattern of weakness in limb muscles, hyperactive deep tendon reflexes, bilateral Babinski sign, dysarthric speech, and spastic gait. Ten months after disease onset, she exhibited dementia dysarthria and spastic quadriparesis. The disease progressed relentlessly, and the patient died 18 months after the onset of symptoms. Cell count and protein concentration level were consistently abnormal during the entire course of illness (the results of CSF testing and neuropathologic findings are listed in Table 2).

The eldest sister (patient 1-2) became acutely ill at 38 years of age, 15 years after the death of patient 1-1. She exhibited high fever, chills, headache, myalgia, nausea, and frequent vomiting and was comatose when admitted to the hospital; examination findings included dysphagia, neck stiffness, and Kernig sign. The patient's condition rapidly deteriorated, and she died on day 75 of illness. On postmortem examination, the meninges were thickened and infiltrated with mononuclear, plasma, and polymorphonuclear cells. Inflammatory changes were especially pronounced in the meninges overlying the affected cortical areas. In the brain, multiple widespread micronecrotic foci surrounded by inflammatory infiltrates were observed throughout the cerebral cortex, basal ganglia, cerebellum, and brain stem. The infiltrates were composed of lymphocytes (predominantly T-cells with occasional B-cells), microglial cells, macrophages, and reactive astrocytes. Perivascular cuffs

Table 1. Clinical features of Viliuisk encephalomyelitis in affected members of 6 families*

	Family 1			Family 2		Family 3		Family 4			Family 5		Family 6		
Feature	1-1	1-2	1-3	2-1	2-2	3-1	3-2	4-1	4-2	4-3	5-1	5-2	6-1	6-2	6-3
Sex	F	F	F	F	F	F	M	M	F	F	F	F	M	F	F
Age at onset, y	16	38	26	16	44	31	21	51	47	36	20	34	46	37	53
Febrile stage of illness															
Duration, wk	7	11	2	2	10	ND	2	ND	ND	4	ND	1	ND	6	26
Maximum temperature, °C	40.5	39	38	37.9	39.8		39.0			39.0		ND		38.5	38.8
Reduced level of consciousness	++	+++		+	+++		+			++				+	+++
Generalized seizures	++			+						+					
Neck rigidity and Kernig sign	+	++		+	++		+			+					+
Nausea, vomiting	+	++	+		++									++	+
Upper motor neuron pattern of muscle weakness	+				+					+				+	
Relapse of febrile illness	+	+								+					+
Outcome	a	d	a	a	d	a	a	a	a	a	a	a	a	a	d
Advanced disease															
Cognitive decline	++		+	++		m.i.	++	++	++	++	++	++	+	++	
Apathy	+			+			++		+	+		+		+	
Dysarthria	+++		++	++		++	+	++	+	++			+	+	
Dysphagia							+	+		+		+			
Brisk deep tendon reflexes	++		++	++		++	++	++	+	++	+	++	+	++	
Spastic quadri/paraparesis	+++		+	++		+	++	+	++	+++	–	+	+	–	
Increased muscle tone	+		+	+		+	++	+	+	++	–	+		+	
Babinski sign	+		+	+			+	+	+	+		+			
Spastic gait	+++		++	++		+	+++	+	+	+	+	+	+	+	
Falls	+			++			+			+			+		
Sphincter dysfunction	+			++			++					+			
Extrapyramidal rigidity	+		+	++		+	++	++	+	+			+	+	
Obesity	+		+	+										+	
Cachexia		+			+					++					+
Overall duration of illness	18 mo	11 wk	A	48 mo	10 wk	17 y	24 mo	9 y	6 y	11 mo	11 y	23 mo	13 y	27 mo	26 wk

*ND, not documented; m.i., memory impairment; +, mild; ++, moderate; +++, strong; –, not expressed; d, died within acute phase; a, advanced disease developed; A, alive.

consisting of mononuclear cells were widespread in the affected areas. Diffuse neuronal loss was observed within and outside the affected areas. Mild fibrillary gliosis in its early stages was present in the affected areas of pontine nuclei and inferior olives (*5*).

The youngest sister (patient 1-3) had a 2-week febrile illness with headache, chills, and insomnia at the age of 22, 11 years after the death of patient 1-1. Subsequently, cognitive decline, muscle stiffness, dysarthria, and spastic gait developed. Disease progression slowed 10 years after the onset, and she is still alive in her 39th year of illness.

Family 2

In family 2, which consisted of parents and 6 siblings (3 sisters and 3 brothers), an acute febrile illness developed in the second-born sister (patient 2-1) when she was 16 years of age. She recovered, but 3 months after the onset became slow mentally and clumsy physically, with spastic gait and frequent falls. Seven months after the disease onset, she was admitted to the hospital with recurrent generalized tonic-clonic seizures, cognitive decline, dysarthria, brisk tendon reflexes throughout, flexor and extensor pathologic reflexes, increased muscle tone, and spastic gait. The disease relentlessly progressed; 28 months after the disease onset, she was obese, demented, dysarthric, with muscle weakness and spastic gait. She died 48 months after symptom onset. CSF tests suggested inflammatory brain disease (Table 2).

In the oldest sister (patient 2-2) a sudden febrile illness developed when she was 44 years of age, 16 years after her younger sister's death. She sought treatment for severe headache, confusion, nausea, and vomiting and had meningeal signs on examination. During hospitalization, she remained febrile, and hyperactive tendon reflexes developed along with ankle clonus, bilateral Babinski sign, progressive dysphagia, and respiratory failure. The patient died 72 days after disease onset. Results of postmortem examination were consistent with the diagnosis of acute meningoencephalitic form of VE (Table 2). No other VE patients are known among the immediate or distant family members.

Table 2. Laboratory investigations and postmortem findings in Viliuisk encephalomyelitis patients from 6 families*

Laboratory and postmortem findings	Family 1			Family 2		Family 3		Family 4			Family 5		Family 6		
	1-1	1-2	1-3	2-1	2-2	3-1	3-2	4-1	4-2	4-3	5-1	5-2	6-1	6-2	6-3
Cerebrospinal fluid															
Cell count (cells/μL)	20–102	8	3	45–62	15–44	2	11–58	25	17	4–18	NA	12	NA	17–65	11–27
Predominant cell type	Lym			Lym	Lym		Lym							Lym	Lym
Protein (mg/dL)	150	166	23	480	99	16	99	33	66	132		66		66	99
Bacterial culture	Neg	Neg	NA	Neg	Neg	NA	Neg	NA	NA	NA		NA		Neg	Neg
Postmortem															
Inflamed meninges		+			+		+		+			+			+
Micronecrotic lesions in the brain parenchyma		+			+		+		+			+			+

*NA, data not available; Lym, lymphocytes; Neg, negative; +, observed.

Family 3

Family 3 comprised an affected mother (patient 3-1) and her 3 sons; VE developed in the mother in 1953 and in her oldest son (patient 3-2) in 1973. Patient 3-1 had an insidious onset of muscle stiffness, gait disturbance, dysarthria, and memory impairment at the age of 31. On examination, she had severe dysarthria, moderate muscle weakness in the lower limbs, brisk tendon reflexes, flexor pathologic signs, spastic gait, and bilateral mild muscle atrophy of the hand and forearm muscles. The disease progressed slowly, and the patient died 17 years after onset.

In patient 3-2, a 2-week febrile illness developed when he was 21 years of age, with severe headache and dizziness, 2 years after his mother's death. He recovered but was unable to do any work on his family's farm. The following year, rapidly progressive dementia, dysarthria, muscle stiffness, and spasticity developed. On examination, he had brisk tendon reflexes with ankle clonus, spastic quadriparesis, and decreased proprioceptive sensation in the lower limbs with preserved vibration sense. Later he had bulbar symptoms and atrophy of small muscles of the hands and feet and died in respiratory distress 24 months after disease onset. A cranial computed tomographic scan showed cortical atrophy and diffuse leptomeningeal enhancement over the parietal and occipital lobes.

Study of the neuropathologic features of patient 3-2 showed thickened and cloudy meninges and microscopically diffuse infiltration with mononuclear, plasma, and polymorphonuclear cells. Organized necrotic foci with central lysis of tissue and adjacent reactive gliosis were abundant in the brain parenchyma. Small vessels within and adjacent to these foci showed endothelial cell proliferation and perivascular cuffs of T-lymphocytes. Some areas showed confluence of many recent and old necrotic lesions, leading to an extensive destruction in all cortical laminae, reactive fibrillary gliosis, and secondary demyelination in the underlying white matter. Organizing and active inflammatory foci were present in the putamen, the globus pallidus, and claustrum. Examination of the spinal cord showed gross degeneration of corticospinal tracts and less pronounced degeneration in Goll columns. Perivascular cuffs of mononuclear cells were frequently found in the degenerating tracts and the adjacent areas (*5*,*11*).

Family 4

Family 4 comprised a woman with 3 sons and a daughter from consecutive marriages; VE developed in the eldest son (patient 4-1), his half-sibling (patient 4-2), and 1 of his 4 children (patient 4-3). Patient 4-1 had a short febrile episode in 1952 at the age of 51 years; the following year, muscle stiffness and speech and gait abnormalities developed. Two years later, he had overt dementia, characteristic dysarthria, and spastic gait. He died 9 years after the onset of neurologic symptoms. The CSF specimen analyzed during the second year of illness showed pleocytosis (Table 2).

Patient 4-2 became visibly affected in 1965, shortly before the age of 47, 4 years after her half-brother's death. The illness had an insidious onset, beginning with clumsiness and decreasing muscle strength in the lower limbs. Further progression led to intellectual decline and slurred dysarthric speech. On examination, she was mute and moved slowly due to spasticity predominantly in the lower limbs. She died 6 years after the disease onset. Postmortem examination showed characteristic necrotic lesions with marked central lysis of tissue, reactive gliosis, and secondary demyelination in the underlying white matter. T-lymphocytes and rod-shaped microglia were present within the gray and white matter (*5*).

Sudden acute disease developed in patient 4-3 in 1971 when she was 36 years of age, 10 years after her father's death. At hospital admission, she was unresponsive, febrile, and had neck stiffness and Kernig sign. She remained confused and lethargic for 4 weeks and thereafter showed significant memory loss and signs of spasticity. Nine weeks later, her condition worsened; she remained unconscious on life support and died 11 months after disease onset.

Family 5

Family 5 consisted of parents and 5 siblings (3 sisters and 2 brothers); VE developed in 2 sisters. The younger of

the 2 affected sisters (patient 5-1) was the first to exhibit a gradual cognitive decline, spastic gait, dysphonia, and slurred speech, but not overt dysarthria, at the age of 20 years in 1957. The disease progressed very slowly; she became globally demented and died 11 years after the onset.

Her older sister (patient 5-2) had a short flulike illness at the age of 34 years, while the index patient was still alive. This was followed by cognitive decline and slowness of movements. Examination during the second year of illness showed deepening dementia, moderate muscle weakness in the upper and lower limbs, increasing spasticity, and urine incontinence. The disease rapidly progressed, and the patient died 23 months after the onset of flulike symptoms. Postmortem study showed marked brain atrophy and widened ventricles and multiple fresh and organized necrotic foci surrounded by spongiform degeneration and reactive gliosis. Perivascular cuffs consisting of lymphocytes, plasma, and polymorphonuclear cells were widespread in the affected portions of the cerebral cortex, subcortical ganglia, cerebellum, and inferior olives (*11*). No other members of the extended family were known to be affected.

Family 6

Family 6 included a man and his 2 consecutive wives; VE developed in all. The man (patient 6-1) was born in a disease-endemic region on the Viliui River near the village where family 5 was identified. At the age of 25 years, he moved to a small settlement in the suburbs of the capital city of Yakutsk (Figure). This migrant worker lived for many years with a local Yakut family and married a young member of the adopting family (patient 6-2). They had a healthy child. Around the time of symptom onset in 1959, patient 6-1 moved to another village in this same area where his second wife, a local woman (patient 6-3) took care of him during his illness. At this time (late 1950s), no VE cases were known in this part of the country; neither the local people nor the practicing physicians in the region had ever seen or heard of VE.

The illness in patient 6-1 had an insidious onset when the patient was 46 years of age; he exhibited increasing clumsiness and loss of muscle strength in the lower limbs. A year later, his speech became dysarthric and his gait slow and spastic. He lost the ability to walk and speak around the 10th year of illness and died 13 years after the disease onset. The patient was repeatedly studied at the regional hospital.

In patient 6-2, an acute disease developed when she was 37 years of age, 17 years after her relationship with patient 6-1 ended. She sought treatment for fever, headache, dizziness, chills, nausea and frequent vomiting, diplopia, and abnormal behavior. Ten weeks later, she showed a substantial intellectual decline, slowness of movements, spasticity, limb ataxia, and slow abnormal gait with frequent falls. Six months after the disease onset, she gained weight, became globally demented, and exhibited dysarthria, muscle weakness in the upper and lower limbs, and spastic gait. She died 27 months after the onset of symptoms. CSF studies during the illness showed inflammatory response (Table 2).

Patient 6-3 became acutely ill at the age of 53 years, 2 years after the death of patient 6-1. She experienced severe headache, chills, and nausea and vomiting. Six weeks after symptom onset, she had a second episode of febrile illness and was admitted to the hospital with high temperature; she was also disoriented and aggressive. Her condition worsened and she died 26 weeks after disease onset. On postmortem examination, the meninges were infiltrated; the cerebral cortex and other gray matter structures contained widespread micronecrotic foci surrounded by inflammatory infiltrates with a tendency for these lesions to be replaced with gliofibrotic scars (*5* [Case 1], *10*).

Discussion

We studied 6 families that included 15 patients with a definitive diagnosis of VE, according to published clinical and neuropathologic criteria (*3*,*4*). The abrupt febrile disease onset in most of the studied patients, the developing meningoencephalitis with CSF pleocytosis, and inflammatory changes systematically found in the brain tissue strongly suggest that VE is an infectious disease. Furthermore, VE patients show evidence for intrathecal immunoglobulin G synthesis, which correlates with the clinical manifestations (*12*). The prolonged occurrence of increased cell count and elevated protein concentrations in the CSF, up to 5–6 years from the disease onset, and the development of chronically progressive dementia and movement abnormalities, suggest that the pathogen is an unconventional organism, which may explain the failure of its isolation and identification (*7*).

Although the occurrence of VE exclusively in the Yakut (Sakha) population may suggest a genetically determined susceptibility of the indigenous Viliui population, segregation analysis excluded Mendelian inheritance (*7*,*8*). A recent case-control study discovered allelic associations ($p<0.05$) between interferon-γ (IFN-γ) gene polymorphisms and VE susceptibility. Notably, allelic association was found only in older patients who survived the acute disease phase, which suggests that IFN-γ variants may be predisposing to the development of chronic VE (T. Oleksyk, pers. comm.). The spread of VE to new geographic regions argues against the view that some Siberian subpopulations are more susceptible to VE than others.

Five families with >1 VE patient were identified and studied in a high-incidence mid-Viliui region, but the most interesting data were obtained from studies of family 6 in a region located 400 km away from the peak VE-endem-

ic region around Lake Mastakh, where VE has not been previously known. Transmission to unrelated persons in a new environment confirms that prolonged intrahousehold contact is a significant risk factor. VE transmission to unrelated persons was observed in several other families, but the clinical, pathologic, or epidemiologic documentation is insufficient.

Our analysis shows that the most severe disease resulting in death after an acute illness occurred in secondary but not primary cases (families 1, 2, 4, and 6). This discordance between the primary and secondary cases within a family suggests that transmission of infection in the setting of close intrahousehold contact may result in shorter incubation times and faster progression of the illness. Variability of VE phenotypic manifestations has been reported (*7*), but it was instructive to observe extremely diverse outcomes in members of the same family.

In summary, VE is a unique meningoencephalitis occurring in the Yakut (Sakha) population of Eastern Siberia. Although the pathogen has not been identified, clinical and pathologic phenomena described here indicate that the only plausible explanation is underlying infection. Analysis of case clustering in 6 families supports the view that VE can be transmitted in a setting of a prolonged intrahousehold contact with a patient manifesting the disease. To our knowledge, this is the first report of VE transmission to unrelated persons, occurring in a region in which VE has not been previously known. The spread from high-incidence foci along the Viliui Valley to new geographic areas strongly indicates that VE is an emerging infectious disease.

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HIV, Hepatitis C, and Hepatitis B Infections and Associated Risk Behavior in Injection Drug Users, Kabul, Afghanistan

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Limited prevalence data for HIV, hepatitis B surface antigen (HBsAg), and hepatitis C virus (HCV) exist for Afghanistan. We studied a cross-sectional sample of adult injection drug users (IDUs) in Kabul, Afghanistan, from June 2005 through June 2006. Study participants completed interviewer-administered questionnaires and underwent testing for HIV, antibody to HCV, and HBsAg. Overall prevalences of HIV, HCV, and HBsAg were 3.0% (95% confidence interval [CI] 1.7%–5.1%), 36.6% (95% CI 32.2%–41.0%), and 6.5% (95% CI 4.2%–8.7%), respectively (N = 464). Among male IDUs (n = 463), risky behavior, including sharing syringes (50.4%), paying women for sex (76.2%), and having sex with men or boys (28.3%), were common. Needle sharing, injecting for ≥3 years, and receiving injections from nonmedical providers were independently associated with increased risk for HCV infection. The high prevalence of risky behavior indicate that Kabul is at risk for an HIV epidemic. Scale-up of harm-reducing interventions is urgently needed.

Injection drug use has become increasingly common in Central and South Asia, fostered by readily available opium and heroin (*1*,*2*). Many countries in this region are experiencing HIV epidemics driven by injection drug use that is extending to other populations (*3*,*4*). Four countries bordering Afghanistan (Pakistan, Tajikistan, Uzbekistan, and Iran), which provided refuge to many Afghans during the extended period of civil war, are experiencing HIV epidemics among injection drug users (IDUs) (*1*,*3*,*5*,*6*). The population of Kabul, the capital of Afghanistan, has increased to ≈3 million since 2001 because of returning refugees (*7*,*8*). Refugees may have acquired high-risk bebavior, such as injection drug use, that may place communities at risk upon their return.

Afghanistan provided 88% of the world's opium supply in 2005 (*9*). Although noninjection use of opium (smoking, vaporization, or oral ingestion) is traditional in Afghanistan, injecting likely represents a new behavior (*10*). This behavior may be learned in countries of refuge during times of political unrest, as indicated by the participants in a United Nations Office on Drugs and Crime study in 2003, in which 50% (n = 34) of participants had started using heroin in either Pakistan or Iran (*11*). A prior study in the border city of Quetta, Pakistan, reported that Afghan IDUs were more likely than their Pakistani counterparts to engage in risky behavior (*12*). These observations raised concern that injection drug use and accompanying high-risk behavior are increasing in Afghanistan and that a concentrated HIV epidemic may soon ensue (*13*).

There were an estimated 470 IDUs in Kabul in 2003, although the United Nations Office on Drugs and Crime Afghanistan survey in 2005 estimated that there were 50,000 heroin users in Afghanistan, of whom 14% reported injecting drugs (*10*,*11*). The same study also estimated that most IDUs reside in Kabul and, of all heroin-using IDUs interviewed, 70% stated they had shared needles (*10*). Of IDUs interviewed, all were men, but anecdotal evidence from harm-reduction programs indicated that a few IDUs in Kabul were female (*10*). Although drug use is illegal in Afghanistan and warrants either rehabilitation for a first offense or imprisonment for recurrent offenses (*14*) the

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motivating factor stated for initiating injection was constant pain that was not relieved by smoking (*10*). However, little is currently known about other aspects of injection drug use in Kabul, such as syringe sources or harm-reducing programs.

Little data are available on HIV, hepatitis B surface antigen (HBsAg), or hepatitis C virus (HCV) prevalence and associated risk behavior in Afghanistan. As of October, 2005, only 41 cases of HIV had been reported, although this is believed to underestimate the potential problem (*15*). We assessed prevalence of HIV, HCV, HBsAg, and associated risk behavior among IDUs in Kabul.

Methods

Study Design and Participants

We conducted a cross-sectional study of IDUs in Kabul, Afghanistan, from June 2005 through June 2006, through the Voluntary Counseling and Testing (VCT) Center at the Central Polyclinic, an Afghan Ministry of Public Health facility. At the time of this study, there were 3 harm-reduction programs in Kabul, of which 1 had on-site syringe exchange.

Eligible participants were those ≥18 years of age who reported having injected drugs within the past 6 months (confirmed through injection marks) and were able to provide informed consent. Before data collection, this study was reviewed and approved by the investigational review boards of the University of California, San Diego; the US Naval Medical Research Unit No. 3; the Walter Reed Army Institute of Research; and the Ministry of Public Health of Afghanistan.

Procedures

Potential participants were approached by an experienced outreach worker known to them. If participants were interested in entering the study, they accompanied the outreach worker to the VCT Center. At the VCT, a study representative explained the study in a confidential setting and obtained informed consent. The participant was assigned a unique study number, the sole identifier, which was required for receiving test results as needed. Participants were interviewed by a trained study representative matched to the participant's sex. The questionnaire included sociodemographics, travel and medical histories, past and current drug use and sexual behavior, and knowledge of bloodborne and sexually transmitted infections. No data were recorded from those declining participation or ineligible to enter the study.

Pretest and posttest counseling were given, and rapid antibody testing was performed by using the Abbott Determine HIV 1/2 test, the Abbott Determine HBsAg test (both from Abbott Diagnostics Japan, Tokyo, Japan), and the Standard Diagnostics HCV test (Standard Diagnostics Laboratories, Yongin-si Gyeonggi-do, Republic of Korea) for HCV. Participants with a positive HIV test result underwent sequential testing with the OraSure OraQuick HIV 1/2 test (OraSure Technologies, Bethlehem, PA, USA). Repeatedly positive rapid HIV test results were confirmed by Western blot (HIV BLOT 2.2; GeneLabs Diagnostics, Singapore). Hepatitis B was confirmed with a second, serum-based rapid test (Standard Diagnostics HBV; Standard Diagnostics Laboratories) because nucleic acid testing was not available in Kabul. The Abbott and Standard Diagnostics HBsAg rapid tests had sensitivities of 99.0% and 99.0% and specificities of 99.0% and 100.0%, respectively, and a positive predictive value of 99.9%, assuming a baseline HBsAg prevalence of 5.0% (*16*,*17*). The presence of antibody to HCV was confirmed with a recombinant immunoblot assay (RIBA) test (RIBA 3.0 SIA; Chiron Corporation, Emeryville, CA, USA).

All confirmatory testing was performed at the VCT Center in Kabul by trained laboratory personnel. All participants received a small nonmonetary gift and risk-reduction counseling, with referrals for detoxification and needle and syringe programs upon request.

Statistical Analysis

Prevalence of infection was calculated with confidence intervals (CIs) based on Poisson distribution for HIV and binomial distribution for HBsAg and HCV. The only female participant was excluded from remaining analyses. Correlates of HIV, HBsAg, and HCV infection were assessed with univariate and multivariate logistic regression analyses. Variables were entered into a multivariate model if they were significantly associated with HIV, HBsAg, or HCV infection at the 5% level in univariate analysis or showed epidemiologic relationships. A multivariate model was generated to identify factors independently associated with HIV, HBsAg, and HCV infections by using the likelihood ratio test to determine which variables were retained.

Results

Sociodemographic Data and Prevalence of Infection

A total of 464 participants were enrolled; 463 were male. Fourteen participants (3.0%, 95% CI 1.7%–5.1%) were infected with HIV, 30 (6.5%, 95% CI 4.2%–8.7%) were positive for HBsAg, 170 (36.6%, 95% CI 32.2%–41.0%) were infected with HCV, and 7 (1.5%, 95% CI 0.6%–3.1%) were coinfected with HIV and HCV.

Among male participants, most were Afghan, had traveled outside Afghanistan in the previous 10 years, and reported heroin as their most frequently used drug in the past 6 months, either alone (42.4%) or with pheniramine

maleate (56.0%) (online Appendix Table, available from www.cdc.gov/EID/content/13/9/1327-appT.htm).

Risk Behavior

High-risk injection and sexual behavior were common. Sharing needles or syringes (50.4%) and difficulty obtaining new syringes (43.6%) were frequently reported. Patronizing female sex workers and having sexual relations with men or boys were also common. More than half the participants had been incarcerated; of these, nearly one third injected drugs while in prison. A total of 23.1% had received a therapeutic injection in the past 6 months, and 5.2% had sold or donated blood (online Appendix Table).

Correlates of HIV, HBsAg, and HCV Infection

No sociodemographic variables were significantly associated with HIV or HBsAg infection. Sharing needles and injecting drugs while in prison were associated with HBsAg by univariate logistic regression analysis (online Appendix Table). Multivariate analysis showed that HBsAg remained associated with injecting drugs in prison (adjusted odds ratio 3.23, 95% CI 1.16–9.00). Univariate analysis showed that those with HIV infections were more likely to report needle or syringe sharing and injecting drugs for ≥3 years (online Appendix Table). No variables were independently associated with HIV infection by multivariate logistic regression analysis (results not shown).

Participants with HCV infection were less likely to be educated or married and had higher incomes (online Appendix Table). HCV infection was associated with needle or syringe sharing, injecting drugs for ≥3 years, having sex with men or boys, and receiving injections from a nonmedical provider (online Appendix Table). Adjustment by demographic factors did not appreciably change these relationships. Multivariate logistic regression showed that needle or syringe sharing, injecting drugs for >3 years, and receiving injections from a nonmedical provider were independently associated with HCV infection, and inverse associations persisted for higher education level and for being married (Table).

Discussion

This report is among the first to describe HIV, HBsAg, and HCV prevalence and risk behavior in Afghanistan. The low HIV prevalence among IDUs in Kabul is not surprising given the short median duration of injection drug use. Although opium has been used for centuries in Afghanistan, our data are consistent with the suggestion that injection drug use is a relatively new behavior in this setting (*10*). Although HIV prevalence was low, 37% were HCV infected, a finding that potentially foreshadows an HIV epidemic caused by risk factors shared by these infections.

Table. Factors independently associated with HCV infection (n = 170) by multivariable analysis in 463 male injection drug users, Kabul, Afghanistan*

Factor	Value
HCV prevalence	107 (36.8)
Demographic factors	
Married	0.60 (0.40–0.92)
Higher educational level	0.51 (0.29–0.88)
Drug practices	
Ever shared needle or syringe	2.60 (1.71–3.96)
Duration injection drug use >3 y	3.28 (2.17–4.96)
Medical encounters	
Injections by a nonmedical provider	2.71 (1.26–5.82)

*HCV, hepatitis C virus. Values are no. (%) or adjusted odds ratio (95% confidence interval). Analysis was adjusted for marital status, educational level, duration of injecting, sharing needles or syringes, and injections by a nonmedical provider.

Injecting drugs in prison was related to HBsAg and marginally to HIV by univariate analysis, which are similar to findings in Iran and other settings (*3*,*18*,*19*). In Iran, Zamani et al. reported that that multiple incarcerations increased likelihood of HIV infection (*20*). We did not observe this relationship, but this result may be due to low statistical power. However, 47% of the Iranian prison population is incarcerated because of drug-related offenses (*18*). Afghan law allows an addict, as diagnosed by a medical professional, to enter a detoxification facility, which may reduce the number and exposure of IDUs to prison settings. Because one third of Afghan IDUs who had been incarcerated reported drug injection in prisons, prisons should remain a priority site for needle and syringe programs.

The established risk factors of needle sharing and duration of injection use were strongly related to both HIV and HCV infections. The prevalence of HBsAg was relatively low among the IDUs and close to that reported by International Committee of the Red Cross/Crescent–supported blood banks in 2004. While only those with acute or active hepatitis B would have circulating antigen, the prevalence of hepatitis B in this group still seems comparatively low, given the prevalence of risky behavior. The prevalence of HIV and hepatitis B infection may not have not reached sufficient levels to result in a self-sustaining epidemic; further surveillance is warranted. The prevalence of HBsAg likely underestimates the number of IDUs exposed to hepatitis B. Because hepatitis B infection resolves after the acute phase in 90%–95% of those infected as adults, the actual proportion of IDUs infected may approach or exceed 64.7% of participants (*21*). A more reliable number might be accessed through testing for antibody to HbsAg, which might be used to create a cost-effective vaccination program for this high-risk group.

Although donating blood was not associated with any of the 3 infections, risk for bloodborne infection through iatrogenic routes deserves emphasis because 8.3% of

participants who reported donating or selling blood were infected with HIV. A prior report estimated that only 30% of blood donations were screened in Afghanistan (*22*). Furthermore, those infected with HCV were more likely to have had injections from nonmedical providers, which has been linked to a high prevalence of HCV and hepatitis B in neighboring Pakistan (*23*).

Our study has some limitations. Respondent-driven sampling was not possible because of concerns of compromising the identities of IDUs; participants were enrolled by convenience sampling, which may not be representative of IDUs in Kabul. Because risky behavior was assessed by self-reporting, socially desirable responses may have been made. Analysis of factors associated with HIV and HBsAg had low power because of low prevalence of these infections, which potentially masks some associations. Additionally, testing for surface antigen may have underestimated the true prevalence of hepatitis B infection because only those with acute or chronic infections would be detected. Another approach for future studies would be screening for both surface antigen and antibody to HbsAg and offering vaccination to IDUs negative for this antibody.

In summary, although prevalence of HIV and HBsAg is low among IDUs in Kabul, the prevalence of HCV and high-risk behavior are alarmingly high. Political instability, poverty, mobility, and low literacy may also increase vulnerability of IDUs to HIV and other bloodborne or sexually transmitted infections (*13*). During the study, 1 needle and syringe program and 3 drug rehabilitation and counseling programs were operating in Kabul; opioid substitution treatment was not available. Initiation or scale-up of interventions, particularly needle and syringe programs and opioid substitution therapy, are urgently needed to prevent an HIV epidemic among Afghan IDUs. Attempts to prevent or control HIV and other bloodborne infections among IDUs without adequate coverage of IDUs by harm-reduction programs have been unsuccessful (*24*,*25*). However, settings with outreach programs that linked VCT, needle and syringe programs, and opiate substitution therapies have stabilized HIV prevalence among IDUs at low levels (*25*,*26*). Political support for harm-reduction and HIV awareness campaigns among the Ministries of Counter Narcotics, Public Health, and Religious Affairs is present in Afghanistan; donor attention is urgently needed to expand these efforts to avert an HIV epidemic.

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Spectrum of Infection and Risk Factors for Human Monkeypox, United States, 2003

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For the 2003 monkeypox virus (MPXV) outbreak in the United States, interhuman transmission was not documented and all case-patients were near or handled MPXV-infected prairie dogs. We initiated a case–control study to evaluate risk factors for animal-to-human MPXV transmission. Participants completed a questionnaire requesting exposure, clinical, and demographic information. Serum samples were obtained for analysis of immunoglobulin G (IgG) and IgM to orthopoxvirus. When data were adjusted for smallpox vaccination, case-patients were more likely than controls to have had daily exposure to a sick animal (odds ratio [OR] 4.0, 95% confidence interval [CI] 1.2–13.4), cleaned cages and bedding of a sick animal (OR 5.3, 95% CI 1.4–20.7), or touched a sick animal (OR 4.0, 95% CI 1.2–13.4). These findings demonstrate that human MPXV infection is associated with handling of MPXV-infected animals and suggest that exposure to excretions and secretions of infected animals can result in infection.

Monkeypox (MPX) is a smallpox-like, but zoonotic, disease endemic to regions of West and Central Africa. This disease results from infection with *Monkeypox virus* (MPXV) (*1*), a member of the genus *Orthopoxvirus* within the family *Poxviridae* (*2*). The principal animal reservoir of MPXV is unknown, but varied sylvan species are susceptible to MPXV infection (*3–6*), and multiple species have been implicated in virus transmission to humans (*7*,*8*).

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Identification of specific human activities (e.g., animal trapping, hunting, or skinning) or types of exposure to animals (e.g., bites; exposure to feces, urine, or respiratory droplets) that could result in MPXV transmission from animals to humans has been difficult to study because of the remote geographic locations involved and the retrospective nature of case reporting and investigation.

The introduction of MPXV into the United States in 2003 in a consignment of wild-caught animals (exotic pets) from Ghana led to the occurrence and recognition of the first human infections outside Africa (*8*). Although MPXV can be transmitted from person to person, no instances of interhuman transmission were documented during the US outbreak (*3*,*9*), which ultimately resulted in 47 confirmed and probable human cases (*10*). Manifestations of human illness seen during this outbreak were considered to have been mild relative to those observed for persons infected with Central African variants of the virus (*11*). All human case-patients were documented to have handled or to have been near infected prairie dogs that had been temporarily housed in the same complex as MPXV-infected, imported rodents. (One case-patient recalled no specific exposure but had ample opportunity for exposure.) The infected prairie dogs were sold as pets, and most ended up in private homes. In this environment, persons had diverse opportunities for exposure to infected animals, varying from being in the same room with an infected animal to cleaning the cage of an infected animal. In addition, several animals were transported to veterinary clinics for evaluation after they became ill, which increased the potential range of human exposures to infected animals. The US outbreak provides a means to identify activities or types of direct or indirect

exposure to infected animals that may have been associated with increased risk for MPXV transmission from animals to humans.

A case–control study was conducted to evaluate risk factors for infection among persons exposed to MPXV-infected prairie dogs during the US outbreak in 2003. This study constituted a single arm of a multiobjective investigation exploring questions related to development of immune responses, infection sequelae, and the relationship between route of infection to MPXV and clinical outcomes. The purposes of this study were to identify independent risk factors associated with MPXV infection and disease in humans and to examine the role of previous smallpox vaccination and age in shaping human susceptibility to MPXV infection.

Patients and Methods

Human Study Participants

The protocol for this study (part of the multi-objective study) was reviewed and approved by the institutional review board for human subjects research at the Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA). Approval was received (by deferral) from partner institutions in Wisconsin (Wisconsin Department of Health and Family Services), Illinois (Illinois Department of Public Health), Indiana (Indiana State Department of Health), Kansas (Department of Health and Environment), and Georgia (Emory University). No monetary incentives or other forms of compensation were provided to study participants.

Definitions and Participant Enrollment

The 47 persons who met criteria for confirmed or probable MPXV infection on the basis of a combination of clinical symptoms, exposure information, and laboratory criteria (*12–14*) (www.cdc.gov/ncidod/monkeypox/casedefinition.htm), and who had exposure to an infected prairie dog, were considered eligible for enrollment as study case-patients. Standardized case definitions were used to assign confirmed and probable case status (Table 1). Persons defined as having probable cases were those who had illness onset ≤21 days of exposure to MPXV who experienced fever (≥37.4°C) and vesicular pustular rash, or rash (potentially uncharacterized) plus had immunoglobulin (IgM) to orthopoxvirus. MPX cases were confirmed on the basis of any of the following laboratory findings from clinically derived specimens: MPXV isolation, detection of MPXV-specific nucleic acid signatures, positive electron microscopy findings, or positive immunohistochemical findings (the last 2 in the absence of other orthopoxvirus virus exposures).

Persons eligible for enrollment as study controls were those who 1) had exposure to an infected prairie dog either in a household or workplace setting, 2) did not acquire MPX disease (i.e., did not meet the clinical criteria for MPX disease as outlined in the case definition), and 3) did not show signs of infection in the absence of disease (i.e., were negative for IgM to orthopoxvirus).

Local health department personnel contacted potential study case-patients by telephone to ascertain their willingness to participate in the study and to request the names of potential family or social contacts. Persons who volunteered were then enrolled in person at the time of interview, and written consent was obtained. Thirty case-patients and 35 potential controls ultimately consented to participate in the study.

Three persons enrolled as potential controls were found to have elevated levels of IgM to orthopoxvirus.

Table 1. Criteria used to define categories of study participants, monkeypox virus outbreak, United States, 2003*

Study classification	Classification by case definition†	Criteria met		
		Epidemiologic‡	Clinical§	Laboratory¶
Case	Confirmed	Yes	Yes	Yes#
	Probable	Yes	Yes (fever with vesicular pustular rash, or rash of unspecified type plus IgM)	No (if rash type unspecified, IgM 7–56 d after rash onset)
Not included	Suspect	Yes	No (fever or rash of unspecified type)	No
Control**	Not a case	Yes	No	No
Infected but not diseased	Unclassified	Yes	No	No (IgM detected at time of study)

*IgM, immunoglobulin M.
†Available from www.cdc.gov/ncidod/monkeypox/casedefinition.htm, January 2004 (*3,12,13*).
‡Epidemiologic criteria for classification of monkeypox cases included exposure to an exotic or wild mammalian pet (obtained on or after the known importation event) exhibiting signs of illness (e.g., conjunctivitis, respiratory symptoms, and rash); exposure to an exotic or wild mammalian pet that had been exposed to an animal infected with monkeypox; or exposure to a suspected, probable, or confirmed human case of monkeypox.
§Clinical criteria were rash (macular, papular, vesicular, or pustular; generalized or localized; discrete or confluent) plus fever (subjective or measured temperature ≥99.3°F [≥37.4°C]), plus ≥2 other signs and symptoms (chills and/or sweats, headache, backache, lymphadenopathy, sore throat, cough, shortness of breath), all beginning <21 d after last possible exposure.
¶Laboratory criteria included culture of monkeypox virus, or demonstration of monkeypox virus DNA by PCR from patient clinical specimens, or demonstration of virus morphologically consistent with an orthopoxvirus by electron microscopy or immunohistochemical testing methods (in the absence of exposure to another orthopoxvirus).
#Positive laboratory findings were sufficient to confirm a monkeypox case in the absence of complete clinical or epidemiologic history.
**Persons investigated but ruled out as having monkeypox virus infections were eligible to enroll in the study as controls; additional controls were identified as in Patients and Methods.

These persons were excluded as controls and were classified as MPXV infected without clinical disease. These 3 infected but not diseased case-patients were retained in a separate category to allow assessment of MPXV infection, as well as MPX disease, as an outcome measure.

MPXV-infected Prairie Dogs

A prairie dog was defined as being MPXV infected if tissues obtained postmortem were positive for MPXV DNA by culture or PCR (*15*). Alternatively, in the absence of laboratory testing, the animal was defined as being infected if it had signs and symptoms of MPXV infection and was housed at a facility where other animals known to have been MPXV infected were also kept (*5*,*16*).

Data Collection

Beginning in late July 2003 (≈40 days after illness onset of the last known human MPX case), face-to-face interviews were conducted with case-patients and controls. A standardized questionnaire was used to obtain information regarding demographic profile, exposure to potentially infected animals and infected humans, signs and symptoms of illness, and factors that might affect susceptibility, including smallpox vaccination history. The data used in this study pertaining to type of animal exposure have been summarized (*10*).

Blood Collection and Serologic Analysis

At the time of interview, a blood sample was requested from all study participants for detection of antibodies to orthopoxvirus and investigation of cellular immunity. Donation of a blood specimen was not required for enrollment in this study, but those specimens that were obtained were assayed for IgG and IgM reactive with orthopoxvirus antigen (vaccinia) according to the methods of Karem et al. (*12*).

Data Analysis

Demographic and medical history data were analyzed as dichotomous categorical variables (i.e., present vs. absent). Two classification schemes were used for age groups; one grouped participants according to whether they were adults (>18 years of age) or children (≤18 years of age) and the other grouped participants according to whether they would have had an opportunity to have received smallpox vaccine as a routine childhood immunization (≥31 and <31 years of age, respectively). Routine smallpox vaccinations stopped in the United States in 1972. Regarding the setting in which the exposure occurred, if a participant indicated he or she was exposed at home and an additional setting, home was noted as the principle setting, otherwise the response was left as recorded. Occupation was stratified into nonanimal-related versus animal-related. Animal-related occupations were veterinarian, veterinarian technician or assistant, or pet store employee. Responses from participants who indicated exposure to ≥1 infected prairie dog were combined into 1 response; affirmative responses were selected over negative responses when exposure to different animals was not uniform.

Odds ratio (OR) with 95% confidence interval (CI) was calculated to examine associations between disease status and exposure variables (e.g., age and smallpox vaccination status). Multiple logistic regression analysis was used to obtain ORs adjusted for the effect of smallpox vaccination (*17*) and to examine the risk for disease or infection associated with given exposures. The level of statistical significance established for the analysis was $p \leq 0.05$. The small size of the study population precluded further assessment of independent risk factors. All statistical analyses were performed by using SAS version 9.1 (SAS Institute, Cary, NC, USA).

Results

Characteristics of Case-Patients and Controls

Sixty-one persons from 4 states affected during the MPX outbreak were enrolled in the study (Table 2). The 2 categories of MPX case-patients evaluated were persons who met the epidemiologic case definition for confirmed or probable MPX (case-patients, n = 30) and persons who did not have illness meeting the case inclusion criteria but who had elevated levels of IgM to orthopoxvirus at the time of enrollment and sampling (infected but not diseased [IBND] patients, n = 3). Twenty-eight study controls were identified from the pool of enrollees. All case-patients and controls had exposure to ill or infected prairie dogs; 4 persons reported exposure to other potentially infected animals (i.e., African rodents).

Most persons enrolled were >18 years of age at the time of study initiation (Table 2). The mean age of case-patients at study enrollment (25.0 years) was significantly lower than that of controls (33.3 years; 2-sided p = 0.013, by Student *t* test); the median ages of the 2 groups were 27.0 and 36.5 years, respectively. Only 6 case-patients (20%), 3 IBND case-patients (100%), and 15 controls (53.6%) reported having had ≥1 smallpox vaccinations (Figure 1). All but one of the smallpox-vaccinated participants was ≥31 years of age at the time of the study, which is consistent with their having received routine vaccination before the 1972 abandonment of this practice in the United States (*18*). The only vaccinated study participant <31 years of age, a control, had been vaccinated as a child as a precaution for international travel.

Characteristics of IBND Patients

Three persons classified as MPX IBND patients were adults (mean age 40.7 years, median age 38.0 years), and all

Table 2. Characteristics of monkeypox case-patients and controls selected from the population of persons exposed, monkeypox virus outbreak, United States, 2003

Characteristic	Study classifications, no. (%)		
	Case-patients	Case-patients infected but not diseased	Controls
State			
Illinois	8 (26.7)	1 (33.3)	8 (28.6)
Indiana	10 (33.3)	1 (33.3)	17 (60.7)
Kansas	1 (3.3)	0	0
Wisconsin	11 (36.7)	1 (33.3)	3 (10.7)
Age, y			
≤18	10 (33.3)	0	7 (25.0)
>18	20 (66.7)	3 (100)	21 (75.0)
History of smallpox vaccination			
Yes	6 (20.0)	3 (100)	15 (53.6)
No	24 (80.0)	0	13 (46.4)
Setting in which exposure occurred*			
Home	18 (60.0)	1 (33.3)	17 (60.7)
Neighbor's home	3 (10.0)	0	6 (21.4)
Pet store	2 (6.7)	1 (33.3)	1 (3.6)
Veterinary clinic	7 (23.3)	1 (33.3)	4 (14.3)
Exposure source†‡			
Prairie dog (PD)	30 (100)	3 (100)	28 (100)
PD and African animal	1 (3.3)	1 (33.3)	2 (7.1)
PD and other animal	1 (3.3)	0	2 (7.1)
Only other animal	0	0	0
Symptoms postexposure			
Fever	28§ (93.3)	2 (66.7)	4 (14.3)
Rash	30 (100)	1 (33.3)	7 (25.0)
Lymphadenopathy	20 (66.7)	1 (33.3)	4 (14.3)
Mouth sores	8 (26.7)	0	1 (3.6)
Conjunctivitis	4 (13.3)	0	0
Cough	17 (56.7)	0	2 (7.1)
Total	30	3	28

*Indicates principal setting for exposure.
†Reported exposures pertain to animals with laboratory-confirmed monkeypox infections (*15*) or ones that had lesion-producing illnesses plus prior exposure to a confirmed infected animal. African animals included dormice, giant Gambian rats, jerboas, hedgehogs, striped mice, and pygmy mice.
‡Includes all reported exposures; categories not mutually exclusive.
§Self-reported information for fever was indicated as unknown or was missing for 2 persons who had laboratory-confirmed monkeypox infections.

reported having had ≥1 smallpox vaccination before 1972; 2 had visible vaccination scars. Symptoms of systemic illness (chills, sweats, myalgias) developed in all 3 patients in the 21 days after last known exposure to an MPX-infected prairie dog (Table 2), but classic MPX disease (1 reported papular rash without fever and 2 had fever without lesions or rash) did not develop. Two persons reported having touched infected prairie dogs (handled ≥1 infected animal), and the third had only indirect exposure to an infected animal (touched soiled bedding near an infected animal); none reported a bite or scratch from an ill prairie dog. The settings in which these persons were exposed differed for each person.

Risk Factors for Acquiring MPX

Evaluation of associations between exposure variables and MPX disease outcomes with inclusion of unadjusted OR, adjusted OR (aOR, adjusted for history of smallpox vaccination), and 95% CI is shown in Table 3. Results relating to MPX disease as an outcome measure (i.e., that excluded the 3 IBND patients from the case group) are shown, unless results were qualitatively different when the 3 IBND patients were included. In those instances, results are reported for the diseased plus the 3 IBND patients.

When not considering participant age or exposure history, a history of remote smallpox vaccination (>25 years previous) was protective against acquisition of MPX illness (OR 0.2, 95% CI 0.1–0.7). However, smallpox vaccination status was highly correlated with age (Spearman rank-order correlation $r^2 = 0.809$, $p<0.001$), and its effect independent of age could not be assessed. Age group and sex did not differ between case-patients and controls.

Several types of direct exposure (touching or receiving a bite or scratch sufficient to break the skin) and indirect exposure (nontactile) with infected animals were associated with risk for MPX. Regarding direct exposures,

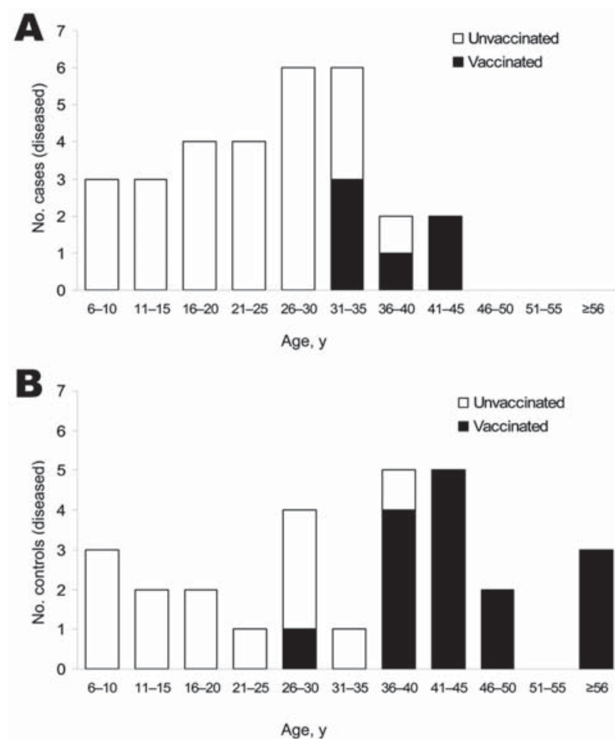

Figure 1. Age distribution of monkeypox virus–infected case-patients (A) and controls (B) and smallpox vaccination status. No study participants reported having received a smallpox vaccination within 25 years of August 2003.

having touched a sick animal was significantly associated with development of MPX (OR 3.8, 95% CI 1.2–11.7) regardless of smallpox vaccination status (aOR 4.0, 95% CI 1.2–13.4). Having received a scratch from an infected animal was significantly associated with disease but only when not adjusted for smallpox vaccination (OR 5.6, 95% CI 1.1–28.6; aOR 3.9, 95% CI 0.7–21.1). Among 9 persons who were scratched by an infected prairie dog, 1 had had prior smallpox vaccination. Only 1 MPX case-patient (a previously vaccinated person) reported a prairie dog bite; no controls reported having been bitten.

Among indirect exposures, having been near an infected animal (defined as having come within 6 feet of the animal without touching it) was not associated with development of MPX. Having cleaned the cage or touched used bedding of an infected animal was significantly associated with MPX development, both with and without adjustment for prior smallpox vaccination (OR 5.3, 95% CI 1.5–18.9; aOR 5.3, 95% CI 1.4–20.7). Also, regardless whether exposure was direct or indirect, having had daily exposure to the animal while it was ill was significantly associated with MPX developing (OR 3.8, 95% CI 1.2–11.7; aOR 4.0, 95% CI 1.2–13.4).

When MPX infection (n = 33, 30 patients and 3 IBND patients), rather than MPX disease, was chosen as the outcome measure, only direct (touch) exposure (OR 5.0, 95% CI 1.4–17.6; aOR 4.9, 95% CI 1.3–17.9) and having cleaned the cage or touched used bedding of an infected animal (OR 3.6, 95% CI 1.2–10.7; aOR 3.6, 95% CI 1.2–11.4) were significantly associated with infection. Again, regardless of age or exposure characteristics, reported history of prior, remote smallpox vaccination was protective against MPX infection (OR 0.3, 95% CI 0.1–0.9).

Exposure Characteristics of Vaccinated and Unvaccinated Adults and Children

Because the study population was too small to support multivariate analysis, we were unable to evaluate associations between disease outcomes and individual exposures while controlling for other potentially contributing factors. Therefore, we evaluated types of exposures to prairie dogs among different study subgroups (smallpox-vaccinated persons vs. unvaccinated persons, and adults vs. children) to examine whether differences in proportions exposed could have biased observed associations between disease acquisition and previous smallpox vaccination or age.

The proportion of smallpox-vaccinated and unvaccinated study participants who reported various exposures to infected prairie dogs is shown in Figure 2, panel A. For all but 1 category of exposure, a smaller proportion of smallpox-vaccinated persons reported exposure than unvaccinated persons, which potentially introduced bias in interpreting lack of prior vaccination as a risk factor for disease. The exception, having been bitten by an infected animal, was reported by only 1 person, a smallpox-vaccinated person in whom relatively benign MPX developed. This person was not hospitalized and had <25 lesions. Unvaccinated persons had a higher frequency of scratches and breaks in the skin (10 [27.0%]) than did smallpox-vaccinated persons (1 [4.8%]; p = 0.044, by 2-tailed Fisher exact test). A similar evaluation of exposure characteristics in children and adults indicated that while certain exposures were more common in children than in adults (e.g., having had an infected animal as a pet, having had daily or direct exposure to an infected animal) (Figure 2, panel B), neither age category uniformly had higher proportionate exposure.

Risk Factors for Vaccinated and Unvaccinated Participants

To evaluate potential differences in risks for MPX disease acquisition between smallpox-vaccinated and unvaccinated persons, case-patients and controls were stratified by vaccination status and exposures were examined. No exposure or demographic variables were associated with

Table 3. Smallpox vaccination status, demographic characteristics, and potential exposures to infected prairie dogs among case-patients and controls, monkeypox virus outbreak, United States, 2003*

Characteristic	Case-patients, no. (%)	Controls, no. (%)	OR (95% CI)	aOR (95% CI)
Smallpox vaccination				
Yes	6 (20.0)	15 (53.6)	0.2 (0.1–0.7)	
No	24 (80.0)	13 (46.7)		
Age, y†				
≤18	10 (33.3)	7 (25.0)	1.5 (0.5–4.7)	0.6 (0.2–2.4)
>18	20 (66.7)	21 (75.0)		
Sex				
Female	17 (56.7)	16 (57.1)	1.0 (0.3–2.8)	0.9 (0.3–2.7)
Male	13 (43.3)	12 (42.9)		
Type of exposure to prairie dog(s)				
Animal as pet				
Yes	17 (56.7)	9 (32.1)	2.8 (0.9–8.1)	2.4 (0.8–7.4)
No	13 (43.3)	19 (67.9)		
Daily exposure while animal was ill				
Yes	23 (76.7)	13 (46.4)	3.8 (1.2–11.7)	4.0 (1.2–13.4)
No	7 (23.3)	15 (53.6)		
Touched rash or eye crusts				
Yes	18 (60.0)	11 (39.3)	2.3 (0.8–6.6)	2.2 (0.7–6.7)
No	12 (40.0)	17 (60.7)		
Scratched‡				
Yes	9 (30.0)	2 (7.1)	5.6 (1.1–28.6)	3.9 (0.7–21.1)
No	21 (70.0)	26 (92.9)		
Cleaned cage/touched bedding				
Yes	14 (46.7)	4 (14.3)	5.3 (1.5–18.9)	5.3 (1.4–20.7)
No	16 (53.3)	24 (85.7)		
Proximity, no touching§				
Yes	12 (40.0)	7 (25.0)	2.0 (0.6–6.2)	2.0 (0.6–6.5)
No	18 (60.0)	21 (75.0)		
Direct exposure¶				
Yes	23 (76.7)	13 (46.4)	3.8 (1.2–11.7)	4.0 (1.2–13.4)
No	7 (23.3)	15 (53.6)		

*OR, odds ratio; CI, confidence interval; aOR, adjusted OR. The aORs and 95% CIs were adjusted for history of (≥1) smallpox vaccination at any time during case-patient's life.
†Routine immunization was stopped in the United States in 1972 (*18*), persons <33 years of age at the time of the outbreak were unlikely to have received prior smallpox vaccination.
‡Scratched with a break in the skin.
§Came within 6 feet of the prairie dog but never touched it.
¶Direct exposure to an infected animal included having touched the animal or having received a bite or scratch from it sufficient to break the skin.

infection or disease status when participants were stratified by smallpox vaccination status. However, power to detect statistically significant associations was limited because of small numbers.

Discussion

Although complexity of exposures varied among persons affected during the 2003 outbreak of MPX in the United States, prairie dogs were the common vehicle implicated in virus transmission to humans (*3*,*8*,*19*,*20*). The goal of this study was to identify host characteristics and specific types of exposure to infected animals that were associated with increased risk for infection or disease. Results demonstrated that several types of direct and indirect exposures to infected animals (including being scratched by an infected animal, handling one, or cleaning a soiled cage) were associated with increased risk of acquiring MPX. The observation that daily exposure to infected prairie dogs was associated principally with disease, and not merely with infection, suggests that increasing the intensity or duration of exposure to viral inoculum may increase the probability for overt illness.

Insights into potential transmission mechanisms by which handling or exposure to excretions or secretions of an infected animal could result in human infection come from pathology of MPXV infection in prairie dogs. Immunohistochemical evaluation of lesion and organ tissue obtained from prairie dogs that became ill during the US outbreak showed abundant viral antigen in surface epithelial cells at ulcerated sites on the tongue, conjunctiva, and throughout the lungs (*5*). Results of experimental infection studies of prairie dogs with MPX mirrored these observations (*16*), which suggests that respiratory, mucocutaneous, and transdermal routes of virus transmission are plausible,

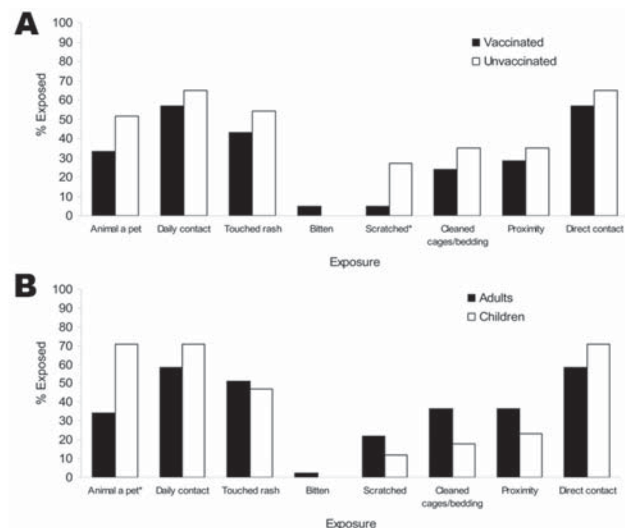

Figure 2. Characteristics of exposure to infected prairie dogs of A) vaccinated and unvaccinated monkeypox case-patients and controls and B) case-patients (adults and children) and controls. *Denotes statistically significant differences in exposure between groups.

all of which are consistent with types of exposures implicated in this study.

Although a history of smallpox vaccination was associated with diminished risk for MPXV infection in this study, accurately interpreting this finding is difficult in the absence of consideration of age or exposure history. In human epidemiologic studies, age has been shown to affect susceptibility to MPX. Additionally, studies conducted in the 1980s in the Democratic Republic of the Congo (former Zaire) highlighted both age and smallpox vaccination status as key determinants of MPX acquisition in humans; unvaccinated children 5–15 years of age, those old enough for autonomous interaction with animals, had the highest rates of MPXV infection (*21–23*). In these studies, a prior history of smallpox vaccination within 3–19 years before MPXV exposure provided up to 85% protection against MPXV infection across various age classes. In our study, no study participants received smallpox vaccination within 25 years of MPX exposure, and the role of vaccination could not be evaluated independent of age because there were few unvaccinated older persons in the study population and no vaccinated children. Lack of adequate comparison groups needed to discriminate between age- and vaccination-related effects could emphasize potential protective benefits of remote smallpox vaccination more than older age at disease acquisition.

Also complicating interpretation of the role of vaccination in influencing susceptibility is our observation that vaccinated participants had fewer overall exposures to infected animals than did unvaccinated participants. Vaccinated persons had fewer invasive (scratch) exposures, which were associated with the highest case conversion rate (81.8%) among all measured exposures (except bite exposure, which was recorded only 1 time). Thus, discerning whether vaccinated participants benefited from having had fewer exposures to infected animals or from residual smallpox vaccine–derived immunity was difficult. The disproportionate enrollment of controls in different states (e.g., less for Wisconsin, where many exposures occurred in veterinary care settings), may have contributed to this effect. Because size and composition of the study population were insufficient to support multivariate modeling approaches for assessment of vaccination status, age, and exposure as independent risk factors, we were unable to clearly define a protective benefit of remote vaccination against acquisition of MPXV infection.

Although suggested in 1 report (*24*), systematically collected data from patients infected with MPXV of the West African genetic clade during the outbreak in the United States have not supported a role for remote smallpox vaccination in mitigation of MPX disease severity (*25*). In our study, mild subclinical MPXV infections occurred in 3 adult study participants, all of whom were previously vaccinated. Whether the diminished severity was a consequence of the manner of exposure, the age of the person, the person's vaccination status, or some other factor is unknown. No common exposure was identified among these persons, and each reported symptoms of viral illness. All had evidence of a specific immunologic response after exposure to MPXV. Subclinical and asymptomatic MPXV infections have been suggested in both children and adults in other studies (*21*,*22*,*24*), but we provide evidence of mild, systemic, nonspecific symptoms in the context of an acute (IgM) antibody response. The epidemiologic role of such cases remains to be determined, but their identification here supports the need for investigation of persons with nonspecific symptoms during future MPX outbreak investigations.

The route of MPXV infection (bite, mucocutaneous) influences the time course and manifestations of illness (*10*). We have assessed overall risk for infection associated with types of exposures. It is arguable that behavioral interactions between prairie dogs and pet owners or veterinarians have no direct parallels with types of animal-human interactions that result in MPXV infections in African settings. However, results of this study highlight the role of direct physical contact and potential exposure to excretions and secretions of a sick animal (urine, feces, saliva). These results also provide insights into how natural infections might occur in Africa (e.g., not only during preparation of carcasses, as has traditionally been suggested, but also through exposure to animal nests or excreta). There are no smallpox vaccination programs ongoing in Africa,

and smallpox vaccines currently licensed are not considered suitable for use in populations with high prevalences of immunocompromised persons (*26*,*27*). Behavioral interventions offer the best opportunity to prevent introduction of MPXV into human communities.

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Dr Reynolds is an epidemiologist in the Poxvirus and Rabies Branch at CDC. Her research interests include the epidemiology and natural history of orthopox and parapox viruses.

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Effect of Interventions on Influenza A (H9N2) Isolation in Hong Kong's Live Poultry Markets, 1999–2005

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Live poultry markets (LPMs) are a recognized source of influenza viruses. Since 2001 and 2003, respectively, a first and second monthly "rest-day" has been implemented in Hong Kong's LPMs, when stalls are cleared of unsold poultry and disinfected. We assessed the incremental effectiveness of each rest-day and the banning of live quail sales in 2002 in reducing (H9N2) subtype isolation rates for chickens and minor poultry, by using a multivariable Poisson generalized linear model. There was a 58% reduction (p = 0.001) in virus isolation after 1 monthly rest-day in minor poultry compared with 27% (p = 0.22) in chickens. Combining 1 rest-day with the removal of quails further reduced virus isolation in chickens but not in minor poultry. However, an additional rest-day each month did not appear to affect isolation rates for either species.

The abundance and diversity of avian influenza viruses in live poultry markets (LPMs) have been recognized since the 1970s (*1*), and avian influenza viruses are recognized as key to the emergence of pandemic influenza (*2*). More recently, there has been increasing recognition of their pivotal role in the amplification and maintenance of avian influenza viruses, in introduction of infection to poultry farms (*3–5*), and in zoonotic transmission of avian influenza viruses to humans (*2*,*6*). Nevertheless, LPMs proliferate throughout south Asia and Southeast Asia as well as in other parts of the world, including parts of the United States.

In Hong Kong, LPMs were identified as a major risk for human influenza (H5N1) disease in 1997, when 6 of 18 infected persons died of this highly pathogenic novel strain (*6*). The territory-wide depopulation of all poultry stopped the outbreak. Again in 2001, early detection of multiple new genotypes of highly pathogenic influenza (H5N1) subtype led to another mass culling of poultry in markets before any zoonotic infection occurred. Since July 2001, a compulsory "rest-day" in these poultry markets has been imposed on day 25 of each month. The previous day, all remaining birds are sold or slaughtered, and the next day the stalls, free of poultry, are cleaned and disinfected. This rest-day has been synchronized with 1 of 3 standing monthly rest-days in the wholesale market (Cheung Sha Wan; Figure 1) (*7*). Since influenza A (H9N2) viruses found in quail were identified as the donor of the internal genes of the (H5N1) virus that caused human disease in Hong Kong in 1997 and because isolation rates of these viruses from quail were particularly high (*8*), the sale of live quails, together with any other live poultry at the same premises, was banned effective February 2002. Episodic reappearance of (H5N1) viruses in the LPMs in late 2002 and early 2003 led authorities to introduce a second rest-day on day 10 of each month beginning March 2003 (*7*).

The effects of the first rest-day on avian influenza virus carriage in LPMs were previously demonstrated by showing that the virus isolation rates of (H9N2) virus (a subtype endemic in poultry in southern China) within poultry markets were significantly reduced soon after the market rest-day (*3*). Here we assessed the impact of the first and second monthly market rest-days. We also addressed the question of the marginal effect of the second rest-day on the isolation rates of H9N2 virus, after adjusting for other important covariables such as temperature, relative humidity, market ventilation system, importation, and sales of poultry strati-

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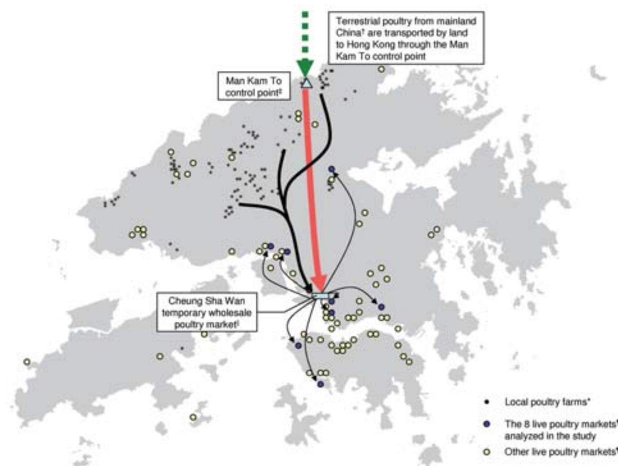

Figure 1. Supply chain of live terrestrial poultry in Hong Kong. During the study period, 110–140 local poultry farms were registered in Hong Kong with ≈2 million chickens. Weekly inspection, flock laboratory surveillance, and universal vaccination of chickens with inactivated influenza (H5N2) vaccine have been routine since June 1, 2003. All terrestrial poultry (e.g., chickens and minor poultry such as quail, pheasant, chukar, guinea fowl) are transported to Cheung Sha Wan temporary wholesale poultry market by trucks before redistribution to live poultry markets (LPMs). *Ducks and geese were imported by sea to a separate wholesale poultry market (not shown), where they were centrally slaughtered, and the chilled carcasses were sold to market. †Approximately 100 registered mainland farms supplied ≈40% of live chickens in 2006. Since January 15, 2004, all such birds have been vaccinated against H5 influenza. They are transported by trucks inside labeled cages to Man Kam To control point. ‡Imported birds must be accompanied by valid veterinary health certificates issued by a recognized veterinary authority. Samples are also taken for laboratory testing. The birds are then transported to Cheung Sha Wan temporary wholesale poultry market by lorry in which water and land birds are segregated. §Birds are transported to LPMs after confirmation of negative test results from the laboratory. Regular laboratory surveillance and monthly rest-days in the wholesale market synchronized with those in LPMs are also routinely carried out. ¶Birds are distributed to live poultry stalls and kept in cages made of stainless steel or nonabsorbent materials. A ban of live waterfowl sales had been imposed since December 1997. Since December 2001, quails had also been removed from being sold alongside chickens and by March 2002, live quails were completely banned in LPMs. There were 60–80 LPMs (light circles) in Hong Kong; 8 of these (dark circles) were analyzed in this study.

fied by type. Additionally, we tested for the effects of the removal of quails from poultry markets in February 2002.

Low-pathogenic influenza A (H9N2) is endemic in poultry across Asia (*8*,*9*) and can serve as an indicator of the dynamics of influenza transmission in poultry. Because (H5N1) virus infection is uncommon in Hong Kong, especially after universal inoculation with inactivated (H5N2) subtype vaccines of all local and imported chickens was introduced in June 2003 and January 2004, respectively, we analyzed the dynamics of (H9N2) virus activity to provide an indicator of influenza virus transmission in LPMs. In addition to being an indicator of influenza virus (including [H5N1] subtype) transmission within poultry in general, (H9N2) viruses have been transmitted to humans and are themselves regarded as viruses with pandemic potential (*10*).

Materials and Methods

Sources of Data

Since September 1999, we have collected fecal samples from 8 LPMs, from a total of 60–80 regulated by the Food and Environmental Hygiene Department in Hong Kong. (The exact number varied, and mostly decreased, during the period of observation.) We tested these samples for influenza viruses as part of an ongoing longitudinal epizootic surveillance program. The 8 selected sites were a convenience sample with geographic representativeness (Figure 1); each site services a large catchment area covering major regions of Hong Kong. Data from 76 consecutive months, September 1999–December 2005, comprising periods with 0, 1, and 2 monthly rest-days, were analyzed.

Live terrestrial poultry from local farms or those imported from mainland China are collected initially at a single wholesale market and redistributed to retail LPMs (Figure 1). In the 8 selected LPMs, the number of stalls in each market was 3–24 in 2000 and was down to 1–16 in 2006. The number of poultry cages in each stall was 20–50. We selected 1 stall from each market for intensive sampling, in which 1 random fresh fecal sample was swabbed from each cage. Approximately 50%–60% of the cages in the stall were sampled. For the other stalls in each market, ≈10%–20% of cages were randomly sampled, 1 swab per cage.

We sampled chickens, which comprise most (80%) poultry consumption in Hong Kong, and other avian species collectively termed "minor poultry." These included pigeons, pheasants, silkie chickens, guinea fowls, and chukar partridges. Quails were not included in the analysis because live sales of these birds have been banned since 2002. The number of samples specific to quails was also very small (≈3% of all samples), precluding separate analyses due to lack of statistical power. Quails sold in markets were raised locally or imported from farms in mainland China. Isolation rates of influenza (H9N2) subtype in quail at the wholesale market before their entry into retail markets were compared for 6 months. The (H9N2) subtype isolation rate from cloacal swabs in the wholesale market was ≈3% compared to an isolation rate of 17% from fecal droppings in retail markets at the corresponding periods (unpub. data). This finding suggests that virus transmission was amplified within the retail markets in quail. In view of the common practice of stacking cages with different poultry species one above the other or placing cages side by side,

transmission of virus across and within species in the market through the fecal-oral route was highly possible. Hence, we also examined the effect of removing quails from LPMs on isolation rates in other species. Waterfowl, ducks, and geese are recognized as the natural reservoirs of influenza viruses (*1*,*2*); ducks yield especially high virus isolation rates. Because of this, since 1998, after the 1997 (H5N1) zoonotic incident in Hong Kong, ducks and geese had been removed from LPMs in Hong Kong, imported separately, and sold already slaughtered and chilled. Figure 1 shows the live poultry supply chain, and Figure 2 summarizes our sampling procedure.

In addition, we collected potential confounding covariable data, including the total sales of chickens and minor poultry, proportion of chickens imported as a ratio to the total (all minor poultry analyzed were imported from mainland China except for some locally raised pigeons), temperature and relative humidity, and type of ventilation system used in LPMs. Weekly average temperature and relative humidity were obtained from the Hong Kong Observatory (*11*). Older LPMs are naturally ventilated, whereas the newer markets have installed either a market economic air treatment (MEAT) system, which lowers the temperature by 3°C when it rises to >25°C, or an air-conditioning system, which operates on a thermostat maintaining ambient temperature at 23°C.

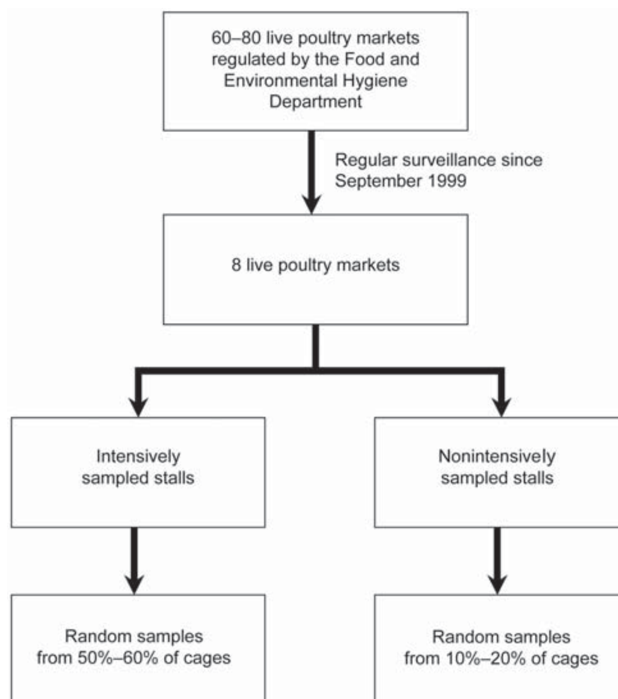

Figure 2. Sampling procedures from live poultry markets (LPMs) in Hong Kong.

Laboratory Procedures

Fecal swabs were collected and transported in vials containing 2.0 mL transport medium containing M199 (9.5 g/L), penicillin G (2× 10^6 U/L), polymyxin B (10× 10^6 U/L), gentamicin (2,500 mg/L), nystatin (0.5× 10^6 U/L), ofloxacin HCl (100 mg/L), and sulfamethoxazole (1 g/L). An aliquot of 200 µL from each swab sample was injected into the allantoic cavity of a 9- to 11-day-old chicken embryo egg and incubated for 3 days at 35°C. Positive isolates were subtyped by hemagglutination-inhibition tests and neuraminidase inhibition test with standard antisera (*1*,*8*).

Statistical Analysis

We fitted a Poisson generalized linear model (*12*) for the outcome variable influenza (H9N2) subtype weekly isolation counts adjusted for the proportion of chickens imported; total sales of chickens and minor poultry; period with 0, 1 (with and without live quails being sold in the markets) and 2 monthly rest-days; ventilation system; weekly average temperature; relative humidity; seasonal variations; and sample size. The antilog of the estimated parameters corresponds to the relative risk (RR) for that factor.

The full Poisson regression model for the number of isolations in a particular week can be represented as log(no. of positive isolates) = log (no. of samples) + β_0 + β_1 (indicator of period II) + β_2 (indicator of period III) + β_3 (indicator of period IV) + β_4 (chicken imported ratio) + β_5 (total sales of chicken) + β_6 (total sales of minor poultry) + β_7 (indicator of MEAT system) + β_8 (indicator of air-conditioned market) + β_9 (temperature) + β_9 (relative humidity) + S(t) + interaction + residual error.

Weekly isolation counts were analyzed from September 22, 1999–December 20, 2005. The indicator variables for periods II, III, and IV take the value 1 within the period with 1 rest-day with quail sales, 1 rest-day without quail sales, and the period with 2 rest-days, respectively, and otherwise take the value 0. S(t) represents a seasonal term and comprises harmonic terms, which are linear combinations of sine and cosine terms similar to Serfling regression (*13*). We investigated second-order interaction terms between periods, chicken imported ratios, total sales of chicken and minor poultry, temperature, and humidity. We only retained statistically significant interaction terms in the final model.

We fitted separate models for chickens and minor poultry to explore the potential heterogeneity of effects across poultry species strata. To verify model goodness-of-fit, 2 coauthors independently viewed residual plots and verified that the model fit was adequate. All analyses were implemented in R version 2.3.1 (*14*).

Results

Figure 3 shows the overall isolation rates by week for chickens and minor poultry from 1999 through 2005. Large

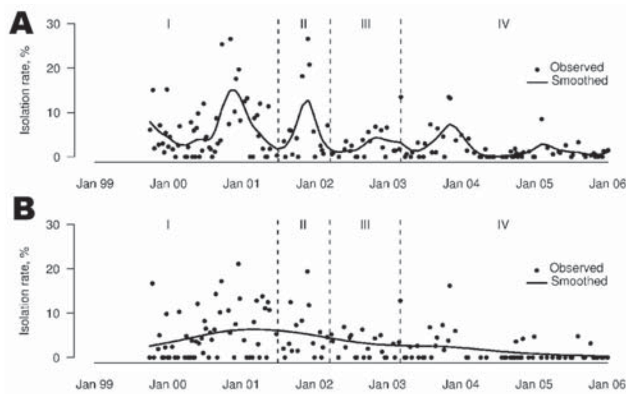

Figure 3. Weekly influenza A (H9N2) isolation rates for chickens (A) and minor poultry (B) in Hong Kong, September 1999–December 2005. Dotted lines denote the different periods: I, no rest-day; II, 1 rest-day with quails sold in live poultry markets; III, 1 rest-day with quails removed from live poultry markets; IV, 2 rest-days.

fluctuations are most likely attributable to seasonality and stochasticity.

Figure 4 shows average isolation rates by calendar day of the month and by number of days after the rest-day(s) for chickens and minor poultry. Overall, mean crude isolation rates for chickens and minor poultry for the period before the introduction of the rest-day were 5.9% and 6.0%, respectively. Similarly, the crude isolation rates for the period in which 1 rest-day per month was implemented were 5.8% and 4.8% before quails were removed and 3.2% and 3.1% afterwards, and when 2 rest-days per month were enforced, they were 2.0% and 2.0%, respectively. The timing of the sample collection in relation to the rest-days is summarized in the online Technical Appendix (available from www.cdc.gov/EID/content/13/9/1340-Techapp.pdf).

In the period before market rest-days were introduced, there was no obvious secular trend over calendar days. During the period with 1 rest-day before live quails sales were banned, a substantial reduction in virus isolation for both chickens and minor poultry occurred immediately after the rest-day, followed by a drift back up to the period-specific baseline 1–3 weeks later. Precisely describing the time course of virus isolation is difficult, given the lack of samples during the intervening period. After quails were removed with 1 rest-day, the average isolation rates for both species groups declined further. In particular, when comparing the isolation rates in weeks 3 and 4 after the rest-day, levels were substantially lower in the period without quails than that with quails. Again, the lack of samples during the first week or so after the rest-day precluded any direct inference about the time trend of virus isolation. However, extrapolating the near-zero isolation rates observed in the period with 1 rest-day in the presence of live quails sales, we might speculate that the average isolation rates were overestimated during the period of 1-rest-day without quails. When there were 2 rest-days, isolation rates were relatively constant throughout each day of the month, with slightly higher isolation prevalence during the week immediately preceding the rest-days. Of note, the

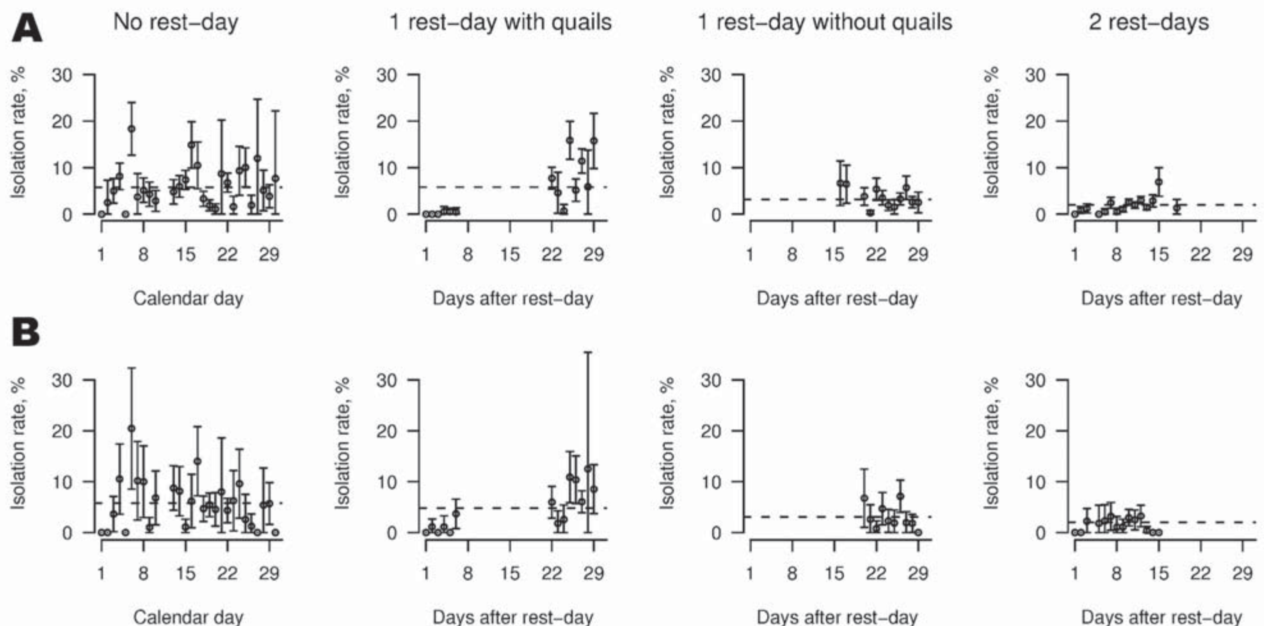

Figure 4. Average influenza A (H9N2) isolation rates by calendar day during the period with no rest-day and by days after a rest-day during the period with rest-days, for chickens (A) and minor poultry (B). Circles denote the isolation rates on each calendar day averaged over the entire period, with 95% confidence intervals. Overall mean isolation rates for each period and poultry type are indicated by the dotted horizontal lines.

95% confidence intervals were fairly wide during the first period with no rest-days because of the smaller number of specimens available (see online Technical Appendix) for the number of samples tested.

The proportion of chickens that was imported declined from 90% to 40% during the period of observation, with a short-lived complete ban in February 2004 due to highly publicized avian influenza outbreaks in Guangdong, Anhui, and Shanghai. The total sales of chickens and particularly minor poultry also showed a downward trend: they decreased ≈50% overall from 1999 to the end of 2005 (online Appendix Figure 1, available from www.cdc.gov/EID/content/13/9/1340-appG1.htm). In terms of the other covariables, there are clear seasonal patterns in temperature and relative humidity (online Appendix Figure 2, available from www.cdc.gov/EID/content/13/9/1340-appG2.htm).

The Table gives the parameter estimates of the final fitted models, which were adjusted for the effect of a number of potential co-variables that may affect virus isolation rates. No second-order interaction terms, except that between total sales of chickens and minor poultry, were found to be statistically significant. For chickens, compared to the reference category of no rest-day, the period with 1 rest-day with quails was associated with an insignificantly lower average isolation rate of (H9N2) virus. With quails removed, the isolation rate showed a 39% decline from baseline (i.e., no rest-day) that was of borderline significance (p = 0.06). However, the later period with 2 rest-days demonstrated little additional effect (p = 0.74, comparing the additional effect of 2 rest-days vs. the effect of 1 rest-day without quails). Naturally ventilated LPMs and those with MEAT system had similar isolation rates; the air-conditioned LPM had a lower rate, albeit with borderline significance.

Findings from the minor poultry model were generally similar. Compared to baseline, the effect of the first rest-day (with or without quails) was significantly more marked. However, there appeared to be little change in the average isolation rate (adjusted RR 0.42 vs. 0.40, p = 0.88) associated with banning live quail sales. The additional effect of the second rest-day was also marginal (p = 0.80, comparing the additional effect of 2 rest-days vs. the effect of 1 rest-day without quails). The isolation rates were not significantly associated with the type of ventilation system used.

For both models, although the proportion of chickens imported was not associated with isolation rates, the number of chickens and minor poultry sold (a reflection of the composite of imported and domestically raised poultry entering the LPMs) appeared to be important determinants of H9 isolation rates. There was significant statistical interaction between chicken sales and minor poultry sales as the 2 trends closely tracked each other (online Appendix Figure 1).

Of note, the abrupt cessation of chicken and minor poultry imports in early-2004 (online Appendix Figure 1) could have introduced a considerable amount of additional variability in the import and total sales parameters, which might have affected the model results. We tested model sensitivity to this effect by omitting those 4 months (Febru-

Table. Adjusted RR, associated 95% CI, and p values of Poisson generalized linear models for influenza (H9N2) virus isolation rates by poultry type*

Characteristic	Chickens			Minor poultry		
	Adjusted RR	95% CI	p value	Adjusted RR	95% CI	p value
Time period						
No rest-day	1.00	Reference		1.00	Reference	
1 rest-day with quails	0.73	0.44–1.20	0.22	0.42	0.25–0.71	0.001
1 rest-day without quails	0.61	0.37–1.02	0.061	0.40	0.24–0.68	0.001
2 rest-days	0.56	0.29–1.09	0.09	0.37	0.16–0.82	0.01
Proportion of chickens imported, per 10% increase	0.93	0.74–1.17	0.54	1.06	0.77–1.45	0.73
Total sales of						
Chicken per 100,000	1.09	1.01–1.17	0.02	1.08	0.99–1.18	0.07
Minor poultry per 100,000	2.98	1.52–5.87	0.002	3.20	1.42–7.22	0.005
Chicken × minor poultry, interaction term	0.97	0.95–0.99	0.01	0.97	0.94–0.99	0.01
Ventilation system						
Natural ventilation	1.00	Reference		1.00	Reference	
MEAT system	1.02	0.77–1.34	0.91	1.12	0.83–1.51	0.48
Air-conditioned	0.57	0.30–1.08	0.09	0.93	0.50–1.72	0.82
Temperature, ºC	1.00	0.92–1.09	0.99	1.11	1.00–1.24	0.06
Relative humidity, %	0.99	0.97–1.00	0.12	0.98	0.97–1.00	0.12
Seasonality term†						
α (cosine component)	0.25	–0.21–0.71	0.29	–0.23	–0.75–0.29	0.38
β (sine component)	0.31	–0.19–0.81	0.23	0.72	0.13–1.30	0.02

*RR, relative risk; CI, confidence interval; MEAT, market economic air treatment.
†The seasonality coefficients α and β contribute to the estimated isolation rate in week t via the terms $\alpha\cos(2\pi t/52) + \beta\sin(2\pi t/52)$.

ary–May 2004) with exceptionally low (or zero) total imports. All the estimates remained robust and did not change appreciably (data not shown).

Discussion

We found that isolations in chickens and minor poultry showed different time-dependent contours and variance, indicating that transmission dynamics may differ between the types of poultry examined in this study (*15*), independent of the confounding effects of quails and waterfowl. Such findings reinforce the importance of examining different bird species separately. The scatter plots of isolation rates show large stochastic fluctuations, in addition to strong seasonal variability, which suggest that intervention effects must be studied with statistical methods that can take into account such variability and the confounding influence of relevant environmental covariables. To the best of our knowledge, the present analysis is the first to have implemented both of these principles.

There was a significant 58% reduction (adjusted RR 0.42, $p<0.01$) in virus isolation in minor poultry after the first monthly rest-day (with live quail sales) was introduced, compared to only 27% (adjusted RR 0.73) in chickens (not significant). With the removal of quails, the effect size became larger in chickens (adjusted RR 0.61) and almost reached significance at the 0.05 level, but not in minor poultry (adjusted RR 0.40). However, an additional rest-day every 2 weeks did not appear to be effective in further reducing isolation rates significantly for either species group, after other contributing factors were adjusted for. A previous study in which (H9N2) virus isolation rates in individual markets immediately before and after the market rest-day were compared demonstrated that the rest-day was associated with a reduction in virus isolation rates (*3*).

Total sales of live birds in LPMs were also a major determinant of transmission, where the effects of chickens and minor poultry were different. In addition, minority poultry, although fewer in numbers (by a whole order of magnitude), appeared to have exerted a greater effect on positive isolation frequencies. These 2 observations suggest that influenza virus transmission in minor poultry within LPMs is more sensitive to changes in environmental conditions than virus transmission in chickens. This could be due to interspecies biologic differences or different market practices. For instance, minor poultry, because of their higher price and lower popularity compared with chickens, tend to have an increased market life and remain in cages longer than chickens, which typically have a more rapid turnover (1–2 days). Also, minor poultry, which tend to come to market at a younger age, may have higher levels of virus carriage.

In any case, from a policy perspective, perhaps an appropriate response could be to separate the sales of live chicken and minor poultry. This would have the additional benefit of preventing cross-species infection to chickens, which are the main poultry consumed (*16*). Indeed, we observed that the effect of 1 rest-day in chickens became larger and more significant statistically after quails were removed. The sales of live ducks and geese in LPMs had already been banned since 1998 in Hong Kong, and the sales of live quails were banned in 2002 (*7*).

The data also suggest that reducing the volume of sales in LPMs reduces virus isolation rates. This finding may be counter-intuitive to the extent that a high turnover is likely to be associated with shorter holding time of the poultry within the market, and one would expect this to be associated with reduced virus isolation rates. On the other hand, decreased volume of sales decreases the risk for introduction of virus into a market, and therefore the risk of establishing transmission within the market.

Analysis of the data by calendar day (Figure 4) confirmed earlier results (*3*,*17*) that rest-days led to an immediate decline in positive isolates by interrupting the amplification cycle. Our findings further suggest that the effect of very low isolation rates can likely be sustained for up to 2 weeks, although we caution that we had little data during the second week to provide definitive support to this observation (online Technical Appendix and Figure 4). Moreover, the analysis in Figure 4 is unadjusted for other covariables and therefore cannot be directly compared with the multivariable model results, which suggest the second rest-day had marginal effects on further reducing virus isolation.

This finding does not necessarily imply that the number of market rest-days in Hong Kong should be reduced in frequency from twice to once per month. In addition to the impact on overall viral load, the frequency in rest-days is predicated on minimizing the duration of the circulation of a potentially pathogenic avian virus (e.g., [H5N1]) within markets after its occasional introduction. Meanwhile, the diminishing marginal effectiveness for each additional rest-day may prompt the implementation of centrally slaughtering of all live poultry for further reduction in transmission risk. This is probably a more important intervention to aim for than removal of another species of poultry from the poultry markets.

The study was conducted on low pathogenic avian influenza (H9N2) viruses because they are endemic in poultry in the region. While the (H9N2) subtype is itself important as a zoonotic pathogen and may be a candidate for the next pandemic virus, the transmission dynamics of this virus may also provide insights into the transmission and control of the highly pathogenic avian influenza (HPAI) (H5N1) viruses that are currently a major threat to animal and human health across Asia. Therefore, our results suggest that for countries confronting endemic (H5N1) subtype infection in poultry, introducing even 1 rest-day per month in these

LPMs is likely to provide definite benefit. Interventions designed to interrupt virus transmission in poultry markets may have even greater effects in retail poultry markets in mainland China and elsewhere in Asia, where aquatic and terrestrial poultry are both present within the same markets, because aquatic poultry appear to be the more important carriers of HPAI (H5N1) viruses.

The winter increase in (H9N2) virus isolation rate and strong seasonal forcing observed parallel those seen for (H5N1) viruses in poultry markets in southern China (*18*,*19*). The reasons for this increase in virus carriage rates in the winter are unclear. However, the lower temperature and humidity may increase virus survival in the environment, thereby increasing virus transmission.

The lack of association with the proportion of chickens that were imported likely reflects the progressive culmination of an effective package of biosecurity measures (e.g., including universal vaccination with sentinel flocks, stringent surveillance from farm to market, segregation of species during transport) further up the supply chain, as detailed in Figure 1, panel A, such that there is little difference in risk for virus introduction into the LPMs between locally farmed and imported chickens.

We did not have information on some parameters that could affect transmission efficacy, such as market and stall designs, poultry density, and proximity of different species. Nevertheless, unless these changed substantially during the time series, which we do not believe to be the case, they should not have had a large effect on the results.

Future research should explore optimizing the number and timing of market rest-days and other interventions by using mathematical and statistical models, with parameters determined by empirical data. Field experiments studying the contextual effects of market conditions could also further inform the transmission dynamics of influenza in LPMs.

These findings and other studies documenting that LPMs can serve as a source of infection for farms (*5*) confirm that these markets maintain, amplify, and disseminate influenza viruses. Thus, while retail poultry markets are a dead-end for the poultry that are slaughtered there, they are not a dead-end for the virus. Indeed, these markets possibly help maintain infection in poultry flocks and provide a potential site for intervening to control virus transmission (*16*). Studies to address their role in maintaining influenza virus circulation in countries where (H5N1) HPAI is endemic are urgently needed. The LPM practice may differ between countries, and these differences may greatly affect the role of these markets in amplifying and disseminating avian influenza viruses. For example, poultry markets where unsold poultry are not held over to the next day are less likely to contribute to amplification of virus load. However, establishing that LPMs play a role in maintaining and disseminating virus in these environments (as they do in Hong Kong) would prove a focal point for strategic intervention to interrupt transmission of this virus.

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Detecting Human-to-Human Transmission of Avian Influenza A (H5N1)

Yang Yang,* M. Elizabeth Halloran,*† Jonathan D. Sugimoto,*† and Ira M. Longini, Jr.*†

Highly pathogenic avian influenza A (HPAI) subtype H5N1 has caused family case clusters, mostly in Southeast Asia, that could be due to human-to-human transmission. Should this virus, or another zoonotic influenza virus, gain the ability of sustained human-to-human transmission, an influenza pandemic could result. We used statistical methods to test whether observed clusters of HPAI (H5N1) illnesses in families in northern Sumatra, Indonesia, and eastern Turkey were due to human-to-human transmission. Given that human-to-human transmission occurs, we estimate the infection secondary attack rates (SARs) and the local basic reproductive number, R_0. We find statistical evidence of human-to-human transmission (p = 0.009) in Sumatra but not in Turkey (p = 0.114). For Sumatra, the estimated household SAR was 29% (95% confidence interval [CI] 15%–51%). The estimated lower limit on the local R_0 was 1.14 (95% CI 0.61–2.14). Effective HPAI (H5N1) surveillance, containment response, and field evaluation are essential to monitor and contain potential pandemic strains.

Highly pathogenic avian influenza A (HPAI) subtype H5N1 is repeatedly crossing the species barrier to humans. Since December 2003, a total of 291 cases of HPAI (H5N1) have been reported in humans, resulting in 172 deaths (i.e., 59% case-fatality ratio) in 12 countries, mostly in Southeast Asia (*1*). Among these cases, 31 family clusters have been documented, ranging in size from 2 to 8 family members. How many of these clusters are due to a common avian source and how many are due to human-to-human transmission are important facts to determine. Should one of these HPAI (H5N1) strains gain the capacity for sustained human-to-human transmission, the resulting outbreak, if not contained, would spread worldwide through the global transportation network more rapidly than adequate supplies of vaccine matched to the new variant could be manufactured and distributed (*2*,*3*). We analyzed data from 2 of the largest of the familial clusters to ascertain if human-to-human transmission took place, and if so, how transmissible the strain was.

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Methods

May 2006 Human Avian Influenza Family Cluster, Indonesia

During late April and early May 2006, a cluster of 8 cases of HPAI (H5N1) was detected and investigated by the Indonesian public health surveillance system in northern Sumatra (*4*–*6*). All case-patients were members of the same extended family. Seven of them resided within 3 adjacent houses in the village of Kubu Sembilang. The remaining patient resided with his immediate family in the village of Kabanjahe (≈10 km away).

The index patient was a 37-year-old woman, thought to have been exposed to dead poultry and chicken fecal material before onset of illness. She also reportedly maintained a market stall that sold live chickens. Although her illness was not confirmed to have been caused by avian influenza (H5N1), her death on May 5, 2006, is suspected to be the result of HPAI (H5N1) infection because of her reported symptoms, illness progression, and prior contact with diseased or dead poultry.

Twenty members of her extended family are suspected to have been in contact with her, many during a family gathering on April 29, 2006 (*7*). At that time, she was manifesting symptoms (i.e., she had a heavy cough, was severely ill, and was prostrate). That night, 9 of these members slept in the same small room as she did (indicated by a black triangle in online Appendix Figure 1, available from www.cdc.

gov/EID/content/13/9/1348-appG1.htm). Of these 9 family members, 2 of her sons (15 and 17 years of age) and her 25-year-old brother, who lived in Kabanjhe, became ill in the next 3 weeks. The sons died. The brother was the only person from this family cluster to recover.

Of the remaining 11 family members, 4 became ill and died. The 29-year-old sister of the index patient, who lived in an adjacent house, became ill after she provided direct personal care to her ill sister (*7*). The 18-month-old daughter of this sister also became ill after she was in the presence of the index patient with her mother. The 10-year-old nephew of the index patient, who lived in the other house adjacent to hers, became ill after he attended the family gathering and frequently visited his aunt's house. The nephew's father became ill after he personally cared for his son. The possibility that HPAI (H5N1) was transmitted from the nephew to his father is also supported by genetic sequencing data (*4*). Though symptoms did not develop in the mother of the nephew, she was directly exposed to her husband during his illness. All case-patients, except for the index patient, were confirmed as influenza (H5N1) positive by PCR. The nephew's mother was confirmed as influenza (H5N1) negative. As an intervention, 54 surviving relatives and close contacts were identified and placed under voluntary quarantine (*7*). All of these persons, except for pregnant women and infants, received oseltamivir prophylactically.

December 2005 Human Avian Influenza Family Cluster, Eastern Turkey

From December 18, 2005, (*8*) to January 15, 2006 (*9*), a cluster of 8 confirmed influenza (H5N1) cases was detected in Dogubayazit District in eastern Turkey (online Appendix Figure 2, available from www.cdc.gov/EID/content/13/9/1348-appG2.htm) (*10–13*). These case-patients were among 21 members of 3 households located within 1.5 km of each other (*14*). All confirmed case-patients were hospitalized after onset of symptoms (*9*). Four of the confirmed case-patients died; the other 4 recovered (*9*). Ten of the remaining 14 household residents were hospitalized with avian influenza-like symptoms but were never confirmed to be infected with influenza (H5N1) (*9*). All but one of the hospitalized residents were children (6–15 years of age) (*9*).

Before onset of symptoms, 4 children from 1 household, 3 of whom had confirmed cases (including the index patient), were reported to have had close contact with the dead bodies of sick chickens (*15*). The 2 confirmed case-patients in the second household reportedly slaughtered a duck together on January 1, 2006, at the beginning of a die-off in the household's flock (*14*). Two of the remaining confirmed case-patients lived in the third household and had no history of contact with sick or dying poultry. The remaining confirmed case occurred in a fourth residence located near the first household (*10*), but because we lacked information on the number of household members and the case-patient's exposure history, we excluded it from these analyses. Most, if not all, of the 21 residents attended a dinner hosted by the family of the index patient on December 24, 2006, while he was symptomatic (*8*).

Statistical Methods

We used a previously developed statistical transmission model (*16*,*17*) to test whether human-to-human transmission occurred, and if it did, to estimate transmission parameters. In the model, persons mix with one another in households and between households. In addition, we include a common source of infection due to zoonotic exposure. Mathematical and statistical details are given in the online Technical Appendix (available from www.cdc.gov/EID/content/13/7/1348-Techapp.pdf).

Model of Probability of Transmission

We define p_1 as the probability that an infectious household member infects another household member in 1 day. If the distribution of the infectious period is known, we can obtain the household secondary attack rate (SAR_1) from p_1, defined as the probability that an infectious household member infects another household member over his or her infectious period. Similarly, we define the daily transmission probability (p_2) and the community SAR (SAR_2) for between household spread. Finally, we define the daily probability (b) that any person is infected from a zoonotic source. The contact structure used for parameter estimation is shown in the Figure. We assume that the distributions of the incubation and infectious periods are predetermined by the investigator.

We establish the likelihood function for each person and then for the whole population for statistical inference. The likelihood function for a person is equivalent to the probability of observing the realized data on that person throughout the outbreak. The likelihood function for a person labeled i is built with the following steps: 1) Obtain the probability that person i is infected by an infectious source labeled j on day t, given person i is not infected up to day $t - 1$. If source j is a person, this probability is p_1, for the same household, or p_2 for exposure in the community, multiplied by the probability of person j being infectious on day t. The probability of person j being infectious on day t is derived from the symptom-onset day of person j and the distribution of the infectious period. If source j is zoonotic, the infection probability is b. The probability of escaping infection is simply 1 minus the corresponding probability of infection. 2) Take the product of the probabilities obtained in step 1 over all humans and zoonotic sources j to obtain the probability of person i escaping infection by any

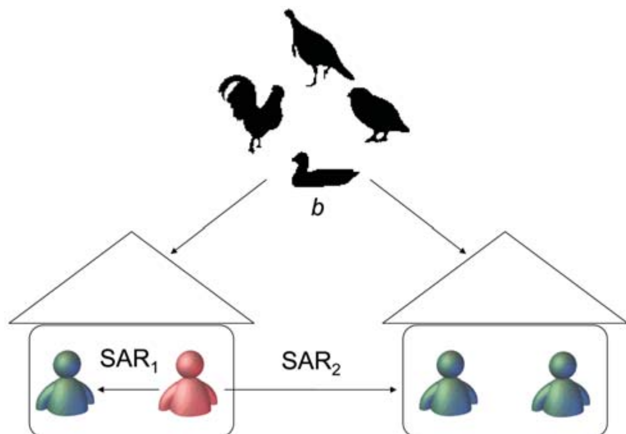

Figure. Schematic of estimation method. An infectious person (in red) infects a susceptible person (in green) in the same household with probability of household secondary attack rate (SAR_1) and infects a susceptible person in a different household with probability SAR_2. The common infectious source (i.e., avian hosts) infects a susceptible person with probability *b* per day. The likelihood function is constructed from symptom-onset dates and exposure information to estimate the above parameters

infectious source on day *t*. 3) Take the product of the probabilities obtained in step 2 over all days before and including day *t* to obtain the probability of person *i* escaping infection up to day *t*. 4) If person *i* is not infected by the end of the outbreak, the likelihood function for person *i* is the product of the probabilities of person *i* escaping infection up to the last day of observation. 5) If person *i* is observed to have symptom onset on day $\tilde{t}$ and the infection time is known to be *t*, the probability of the data regarding person *i* is the product of 3 pieces of information: a) the probability of person *i* escaping infection up to day $t - 1$, b) the probability that person *i* is infected on day *t,* and c) the probability that the duration of the incubation period is $\tilde{t} - t$. Because we do not observe the infection time, the likelihood function for person *i* is obtained by summing the above product, a – c, over all potential values of *t*.

The likelihood function for the whole population is the product of all the individual likelihood functions. In the event that human-to-human transmission occurs, SAR estimates are used to estimate the local basic reproductive number (R_0), which is defined as the average number of secondary cases infected by a typical index case-patient in the beginning of the outbreak (online Technical Appendix). There is potential for sustained transmission if R_0 is >1. If human-to-human transmission is determined to be occurring, then the above parameters are estimated from the symptom dates and contact information from the population under study. Data on exposed persons who do not become ill form an important component of the inference procedure.

Statistical Test

We set up a statistical test with the null hypothesis being that no human-to-human transmission occurs, that is, $p_1 = p_2 = 0$. The alternative hypothesis is either p_1 or p_2 is not equal to 0, or both are not equal to zero. The test statistic we use is proportional to the ratio of the maximum value of the likelihood function assuming the null hypothesis is true (null likelihood) and the maximum value of the likelihood function at the estimated parameter values (full likelihood).

Specifically, we define the likelihood ratio test statistic as –2 log (the null likelihood function divided by the full likelihood function). If no human-to-human transmission occurs, the 2 likelihood functions would be roughly equal, and we expect to see a likelihood ratio close to 1, and, thus, a likelihood ratio statistic close to 0. A large value of the likelihood ratio statistic is evidence of deviation from the null hypothesis. The question is how to obtain a reference set of the likelihood ratio statistic values that we would see under the null hypothesis. Given no human-to-human transmission, all the observed case-patients must have been infected by the zoonotic source. Since the exposure to the zoonotic source is assumed constant for each person on each day, the null likelihood function will not change if we reassign the infection and symptom status of the observed case-patients to a different group of people in the population. By performing such reassignment many times, we obtained a collection of datasets that were each equally likely to have been observed had there been no human-to-human transmission. The values of the likelihood ratio statistic calculated from these datasets form the null distribution for statistical testing. This method is referred to as a permutation test. The p value is given by the proportion of the reference values that are equal to or larger than the observed likelihood ratio statistic value. More technical details are given in the online Technical Appendix.

The probability of infection by the zoonotic source may not be estimable together with SAR_1 or SAR_2 from an observed cluster. In such a situation, a statistical test of the occurrence of human-to-human transmission is still meaningful because the likelihood ratio test statistic is still estimable from the permuted datasets.

Data Required

A list of the inputs that are required for estimation and statistical testing are listed in the Table. Three categories of input parameters are required for this estimation model: outbreak-wide, individual level, and analysis parameters. The duration of the outbreak, the duration of the incubation period for the pathogen, and the minimum and maximum durations of the infectious period for the pathogen are the required outbreak-wide inputs. For each person, their residential location (neighborhood and household), their de-

Table. Parameters and data used in analysis

Category	Parameter/data	Required*
Entire outbreak	Outbreak begin date	X
	Outbreak end date	X
	Latent/incubation period, d†	X
	Infectious period, d†	X
All persons	Neighborhood of residence	X
	Household of residence	X
	Sex	X
	Age, y	X
	Case status (yes or no)	X
Case-patients	Whether outbreak index case-patient (yes or no)	X
	Date of illness onset	X
	Outcome (recovered, died, or don't know/still ill)	X
	Date of outcome	X
	Dates of hospitalization	O
	Period of receiving treatment (dates)	O
Non–case-patients	Dates of hospitalization	O
	Period of prophylactic treatment (dates)	O
Inter-residence visits	Identifier for visiting person	X
	Neighborhood visited	X
	Household visited	X
	Dates of the visit	X
Analysis parameters	End of exposure to the common source of infection (date)	X
	Final day of observation (date)	X
R_0 estimation	Mean no. residents per household	X‡
	Mean no. community contacts per person/d	X‡

*X, required; O, optional; R_0, basic reproduction number.
†The user defines the distribution of this period, including the minimum and maximum length of the period.
‡Required to estimate R_0.

mographic characteristics (sex and age), and whether they were a case-patient or not are required input parameters. Case-patients require additional input of their illness-onset dates, types of outcome, outcome dates, and whether or not they are the index patient in the outbreak. Hospitalization and treatment dates (considered prophylactic for nonpatients) are optional input parameters for each person. For each person who visits another residence during the outbreak period, his or her identifiers, the neighborhood and household visited, and the start and end dates of the visit are required inputs. Analysis-related inputs include the last date of community exposure to potential common sources of infection, the last date of observation, and inputs for R_0 estimation (mean number of residents per household and mean number of out-of-residence contacts per person per day). An expanded version of the model will require the input of other exposure information such as from schools or hospitals.

Results

For the outbreak in Indonesia, online Appendix Figure 1 shows that the incubation period had a probable range of 3–7 days and the infectious period, a probable range of 5–13 days. Thus, we let the incubation period have a uniform distribution of 3–7 days (mean 5 days) and the infectious period a uniform distribution of 5–13 days (mean 9 days). For the data shown in online Appendix Figure 1, only the household SAR (SAR_1) can be estimated. We determine that human-to-human spread did occur by rejecting the null hypothesis of no human-to-human transmission (p = 0.009). The estimated household SAR is 0.29 (95% confidence interval [CI] 0.15–0.51). Thus, a single infected person in a household infected another household member with the probability of 0.29. The average household size for rural Indonesia is ≈5 people. Because we do not have an estimate of the community SAR, we have an estimate of the lower limit of the local R_0, i.e., 1.14 with a 95% CI of 0.61–2.14. A sensitivity analysis on the distribution of the incubation and infectious period shows that the test and estimates for SAR_1 and R_0 are insensitive to uncertainty about these distributions within plausible ranges.

For the outbreak in Turkey, all the parameters are estimable, but we do not reject the null hypothesis of no human-to-human transmission (p = 0.114). Our estimate of the daily probability of infection from the common source is 0.011 (95% CI 0.005–0.025).

Discussion

We have presented statistical evidence that the strain of HPAI (H5N1) that caused the family cluster of human cases in northern Sumatra was spread from human to human and that the household SAR was 29%. This household

SAR is similar to statistical estimates for interpandemic influenza A in the United States (12.7%–30.6%) (*18*,*19*). The mean incubation period of this strain appears to have been ≈5 days, nearly twice as long as for past pandemic strains and current interpandemic strains of influenza. The CI for the estimated lower bound for the local R_0 covers 1. Therefore, even though we determined that human-to-human transmission probably occurred, whether the virus was capable of sustained human-to-human transmission is not clear. This virus may have required very close human contact to be transmitted. Even with no intervention, the finding that $R_0 = 1.14$ indicates that the chance that a single introduction would result in any further spread is ≈12%. In addition, the reported prophylactic use of oseltamivir may have played some role in limiting further spread. We did not find statistical evidence of human-to-human spread for the outbreak in eastern Turkey. This does not mean that no low-level human-to-human spread occurred in this outbreak, only that we lack statistical evidence of such spread. The power would be too low to detect such spread for an outbreak with 7 total cases and small SARs (*17*).

We did not consider the role of heterogeneity—such as age, sex, treatment status, or quarantine—in transmission. The parameters could be made to be functions of time-dependent covariates, as we have done with similar models (*16*,*19*,*20*). We can easily extend the model used here for covariates; however, we must have sufficient data to support such models.

Computer simulations have shown that the targeted use of influenza antiviral agents could be effective in containing a potential pandemic strain of influenza at the source (*21*,*22*), if initiated within 3 weeks of the initial case in the community, and if the R_0 is <1.8. This strategy, known as targeted antiviral prophylaxis, involves treating identified index patients in a mixing group and offering a single course of prophylaxis to the contacts of these index patients in predefined close contact groups, i.e., households at a minimum but also possibly neighborhood clusters, preschool groups, schools, and workplaces. In addition, the voluntary household quarantine of suspected close contacts of case-patients was recommended. Targeted antiviral prophylaxis at the household and neighborhood cluster level was carried out for the outbreak in Sumatra.

Ascertaining whether a potential pandemic strain of influenza is capable of sustained human-to-human transmission and estimating key transmission parameters are important. To estimate more than the household SAR, more detailed community data need to be collected. This would include a complete census of potentially exposed households and persons in the area where immediate transmission could occur from both potential zoonotic and human sources. Such data would enable estimation of important parameters and a more complete estimate of the R_0 rather than just the lower limit.

We have developed a software application, TRANSTAT, for implementing these analyses. This application provides a stand-alone environment for the entry, storage, and analysis of data from outbreaks of acute infectious diseases. A partial list of the input information is given in the Table. The statistical methods presented here can be applied to the data along with several standard epidemiologic tools. This information system would allow for real-time analysis and evaluation of control measures for an outbreak. We would encourage outbreak investigators to use this tool, taking care to input data on the exposed nonpatients as well as case-patients. The authors will provide a link to this software upon request.

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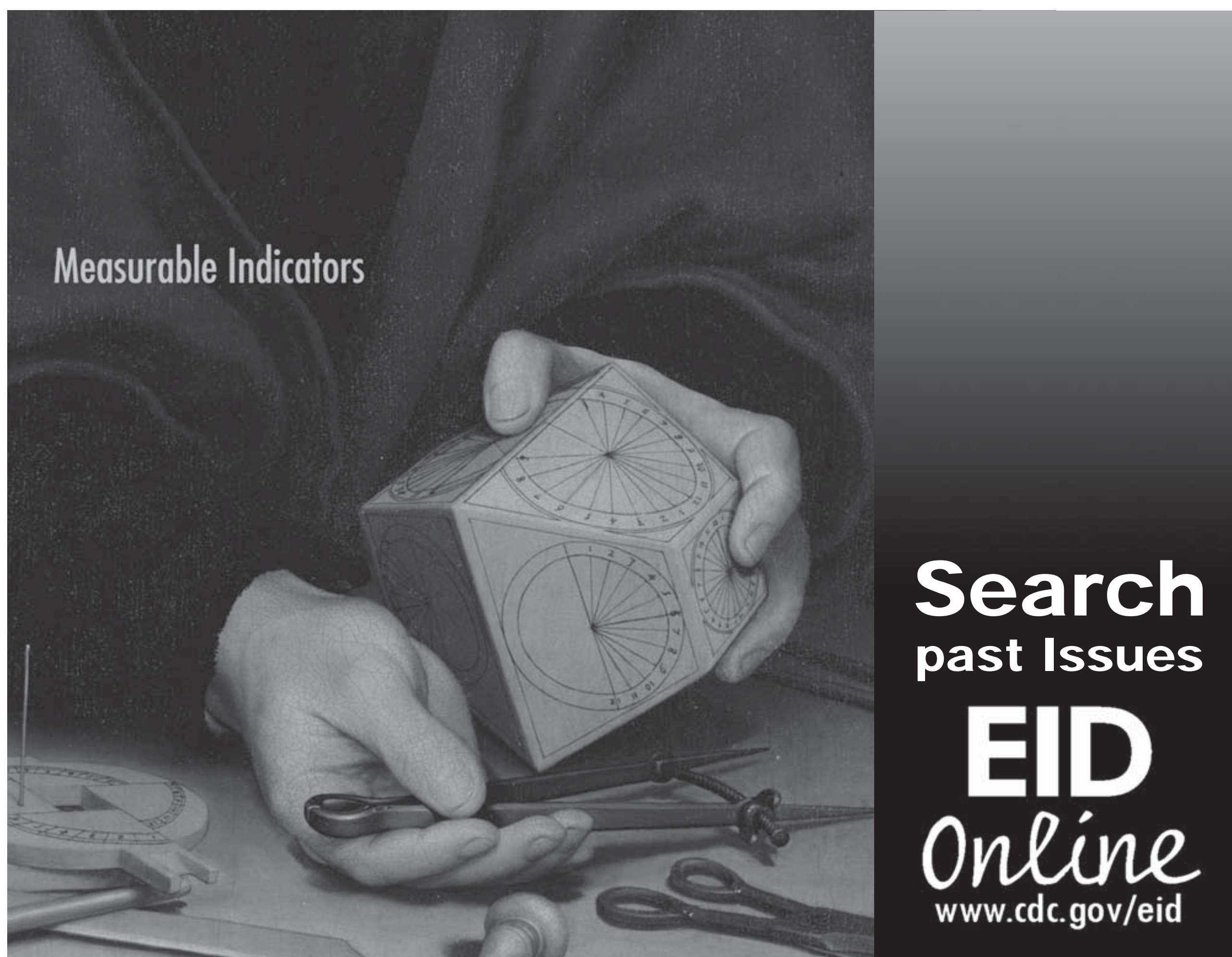

Reduced Sensitivity of Influenza A (H5N1) to Oseltamivir

Jennifer L. McKimm-Breschkin,* Paul W. Selleck,† Tri Bhakti Usman,‡ and Michael A. Johnson†

We tested the neuraminidase drug sensitivity of clade 1 and clade 2 influenza A (H5N1). All viruses demonstrated similar sensitivity to zanamivir, but compared with the 2004 clade 1 viruses, the Cambodian 2005 viruses were 6-fold less sensitive and the Indonesian clade 2 viruses were up to 30-fold less sensitive to oseltamivir.

Two different strains of highly pathogenic avian influenza A (H5N1) have been circulating since 2003. Clade 1 has been found in Vietnam, Thailand, Cambodia, Lao People's Democratic Republic, and Malaysia. Clade 2 subsequently emerged and spread from People's Republic of China to Indonesia, Europe, and Africa in 2004–2005. Because of its systemic availability, oseltamivir is the drug of choice for treating infected persons (*1*).

The Study

We tested the drug sensitivity of neuraminidases (NAs) (*2*) from influenza (H5N1) from chickens, ducks, geese, and quail[1] from 2004 from Vietnam and Malaysia (provided by N. Long, Regional Animal Health Centre, Ho Chi Minh City, Vietnam; and S. Hassan, Veterinary Research Institute, Ipoh, Malaysia), from 2004–05 from Cambodia (provided by S. San, National Animal Health Production and Investigation Center, Phnom Penh, Cambodia), and from 2005 from Indonesia (provided by T. Usman). In the absence of a validated cell culture assay, the 50% inhibitory concentration measured in the NA enzyme inhibition assay is used as the benchmark for measuring drug sensitivity (*3*).

Despite their origins in different countries and different avian species, all clade 1 and clade 2 viruses had a similar sensitivity to zanamivir as the reference influenza (H1N1) NA, (Table 1, Figure, panel A). However, sensitivities of the NAs to oseltamivir (oseltamivir carboxylate) fell into 3 groups when compared to the NA of a reference human influenza (H1N1) strain (Table 1, Figure, panel B). The clade 1 isolates from 2004 were all more sensitive to oseltamivir than was the human influenza (H1N1) control, consistent with recent findings of Rameix-Welti et al. (*4*). However, the NAs of our 2005 Cambodian viruses showed a 6- to 7-fold decrease specifically in oseltamivir sensitivity in comparison to our 2004 Cambodian isolates. These 2005 isolates came from the same area as 1 of the more sensitive 2004 isolates (Kandal), which suggested that at least regional evolution had occurred.

Of more concern was the third group. The NAs from all the clade 2 2005 Indonesian viruses demonstrated a 15- to 30-fold decrease in sensitivity specifically to oseltamivir compared with clade 1 viruses (Table 1, Figure, panel B). Govorkova et al. (*5*) also recently showed that the A/Turkey/15/2006 clade 2 virus was almost 60-fold less sensitive to oseltamivir in a plaque reduction assay than was the clade 1 A/Vietnam/1203/2004 virus. Both these results and those of Rameix-Welti et al. (*4*) contrast to those recently published by Hurt et al., who found no difference between the sensitivities of clade 1 and clade 2 isolates to oseltamivir in the enzyme assay (*6*), although many of the isolates were the same as those tested here. The reason for the discrepancy is not known. The decrease in sensitivity is comparable to that conferred by the N294S recently detected in Egypt (*7*), known to be selected for by oseltamivir treatment (*8*).

Although both drugs are based on the transition state analog of sialic acid, zanamivir has a single substitution of a guanidinium group at the 4′ position on the sugar ring, whereas oseltamivir has an amino group at the 4′ position and, more importantly, a bulky hydrophobic pentyl ether group replacing the glycerol side chain at the 6′ position. Reorientation of E276 in the active site is required to create a hydrophobic pocket necessary to accommodate this pentyl ether group. Mutations that prevent this reorientation from occurring lead to high levels of specific oseltamivir resistance (H274Y, R292K). Decreased binding

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[1]Isolates tested were the following: A/Mississippi/3/2001 wt, A/Mississippi/3/2001 H274Y; A/quail/Kelantan/6309/2004, A/chicken/Kelantan/5858/2004, A/quail/Vietnam/007B/2004, A/duck/Vietnam/007A/2004, A/chicken/Vietnam/0024/2004, A/chicken/Vietnam/0023/2004 A/chicken/Vietnam/0018/2004, A/chicken/Vietnam/0015/2004, A/chicken/Vietnam/0010/2004, A/chicken/Vietnam/008/2004; A/chicken/Cambodia/Siem Reap/77B/2004, A/chicken/Cambodia/Siem Reap/76/2004, A/chicken/Cambodia/Takeo/45/2004, A/chicken/Cambodia/Kandal/23/2004, A/chicken/Cambodia/Pong Peay/1B/2004, A/chicken/Cambodia/Pong Peay/1A/2004, A/goose/Cambodia/Kandal/2005, A/chicken/Cambodia/Kandal/3/2005, A/chicken/Cambodia/Kandal/2/2005, A/chicken/Cambodia/Kandal/1/2005, A/chicken/Indonesia/Wates/130/2005, A/chicken/Indonesia/Wates/126/2005, A/chicken/Indonesia/Wates/83/2005, A/chicken/Indonesia/Wates/80/2005, A/chicken/Indonesia/Wates/77/2005, A/chicken/Indonesia/Wates/1/2005.

Table 1. Mean IC_{50}s of NA sensitivities in MUNANA*-based enzyme inhibition assay for influenza (H5N1) isolates from each region compared with a human influenza (H1N1) control and known resistant H274Y isolate†

Isolate	Zanamivir,‡§ mean IC_{50}, nmol/L	Oseltamivir,‡§ mean IC_{50}, nmol/L	4-Amino-Neu5Ac2en,‡§ mean IC_{50}, μmol/L
Subtype H1N1			
A/Mississippi/3/2001 [2]¶ wt	1.18 (0.24)	2.16 (0.31)	1.12†
A/Mississippi/3/2001 H274Y [4]¶	1.41(0.26)	475.1 (344)	1.31†
Clade 1 subtype H5N1 2004			
Malaysia 2004 [2]	1.21 (0.13)	0.47 (0.07)	2.82 (0.77)
Vietnam 2004 [8]	1.40 (0.44)	0.55 (0.26)	2.47 (0.42)
Cambodia 2004 [6]	1.96 (0.56)	0.41 (0.24)	2.59 (0.15)
Clade 1 subtype H5N1 2005			
Cambodia 2005 [4]	1.53 (0.4)	2.88 (0.58)	2.56 (0.53)
Clade 2 subtype H5N1			
Indonesia 2005 [6]	1.42 (0.63)	11.45 (4.32)	2.00 (0.77)

*Sigma, Saint Louis, MO, USA.
†IC_{50}, drug concentration for 50% inhibition of enzyme activity; NA, neuraminidase; wt, wild type.
‡Drugs provided by GlaxoSmithKline (Stevenage, Hertfordshire, UK).
§Nos. in brackets indicate the nos. of isolates tested against zanamivir and oseltamivir in the same assay. Nineteen influenza (H5N1) isolates were retested in an independent assay against oseltamivir and 4-amino-Neu5Ac2en. Means for oseltamivir are the results of the 2 independent experiments. IC_{50}s for each isolate were calculated by using the Sigmaplot nonlinear curve-fitting function (Systat Software Inc., London, UK). Values in parentheses are standard deviations for the means of the individual IC_{50}s for the isolates in each group.
¶Several plaques were tested from the A/Mississippi/3/2001 against zanamivir and oseltamivir, but only 1 plaque of each was tested against 4-amino-Neu5Ac2en.

to oseltamivir can also be due to altered interactions with its 4-amino group (E119V) (*9*). We therefore tested several viruses from each group for inhibition by a drug that shares the 4-amino substitution. The clade 1 and 2 viruses had similar sensitivity to 4-amino-Neu5Ac2en (Table 1, Figure, panel C), indicating the decreased binding to oseltamivir was specifically due to altered interactions around the 6-pentyl ether group, thus explaining why no altered binding to zanamivir was seen.

Because of the potentially important implications of our findings for public health and stockpiling strategies, and because of the apparent discrepancy with the results of Hurt et al. (*6*), we retested 2 viruses from each group against an independent source of oseltamivir carboxylate (provided by Biota, Notting Hill, Victoria, Australia). We chose A/Indonesia/Wates/77/2005, which had a further slight decrease in sensitivity compared to other Indonesian isolates, as well as one that was in the same range as the remaining Indonesian isolates. Results shown in Table 2 confirm this decreased sensitivity of the clade 2 Indonesian isolates and a small further decrease in sensitivity of A/Indonesia/Wates/77/2005.

Because we only had access to clade 2 isolates from Indonesia, we do not know whether this decreased sensitivity occurs only in the Indonesian clade 2 isolates or globally with all clade 2 isolates. Comparisons of the sequences in the public databases show several mutations in the stalk region, which vary between clade 1 and clade 2 NAs, but 1 mutation in the globular head, H252Y, varies between all clade 1 and clade 2 isolates. A further 3-aa variation occurs between most clade 1 and clade 2 NAs, S343P, E387G, and G459S (N2 numbering), including the Indonesian isolates studied here. An additional V338M variation is found in most Indonesian isolates, including the ones studied here (sequences submitted by N. Komadina). Whether this additional variation confers this decreased oseltamivir sensitivity remains to be elucidated. The A/Indonesia/Wates/77/2005 NA has an additional unique I117V variation, which is adjacent to one of the catalytic arginines, R118 (which would be consistent with a further effect on drug sensitivity to oseltamivir), and a slight decrease in sensitivity to zanamivir (2.3 nmol/L vs mean of 1.4 nmol/L for other Indonesian isolates).

Conclusions

We have shown here that, compared with clade 1 isolates from 2004, some clade 1 Cambodian isolates and clade 2 Indonesian isolates from 2005 demonstrate reduced sensitivity to oseltamivir. Because none of the sequence variations in the public databases correlates with any mutation known to confer oseltamivir resistance, and none of the variations are in the active site (*10*), this suggests that the decrease in sensitivities may be due to drift mutations rather than from exposure to oseltamivir. Recent results show that human isolates can also demonstrate decreased sensitivity to oseltamivir and zanamivir with drift mutations in the NA remote from the active site (*11*). The difference in the amino acid at position 252 may partially account for the altered binding between the clade 1 and clade 2 NAs as other researchers have suggested that the amino acid at position 252 can affect reorientation of the E276 (*4,10*). In clade 1 NAs, this is normally H252, but all clade 2 NAs have Y252. In the recently published N1 structure, although it is from a clade 1 influenza (H5N1) NA, the authors had mutated the NA to Y252 (*10*), which is also found in human NAs. The E276 in this structure does not

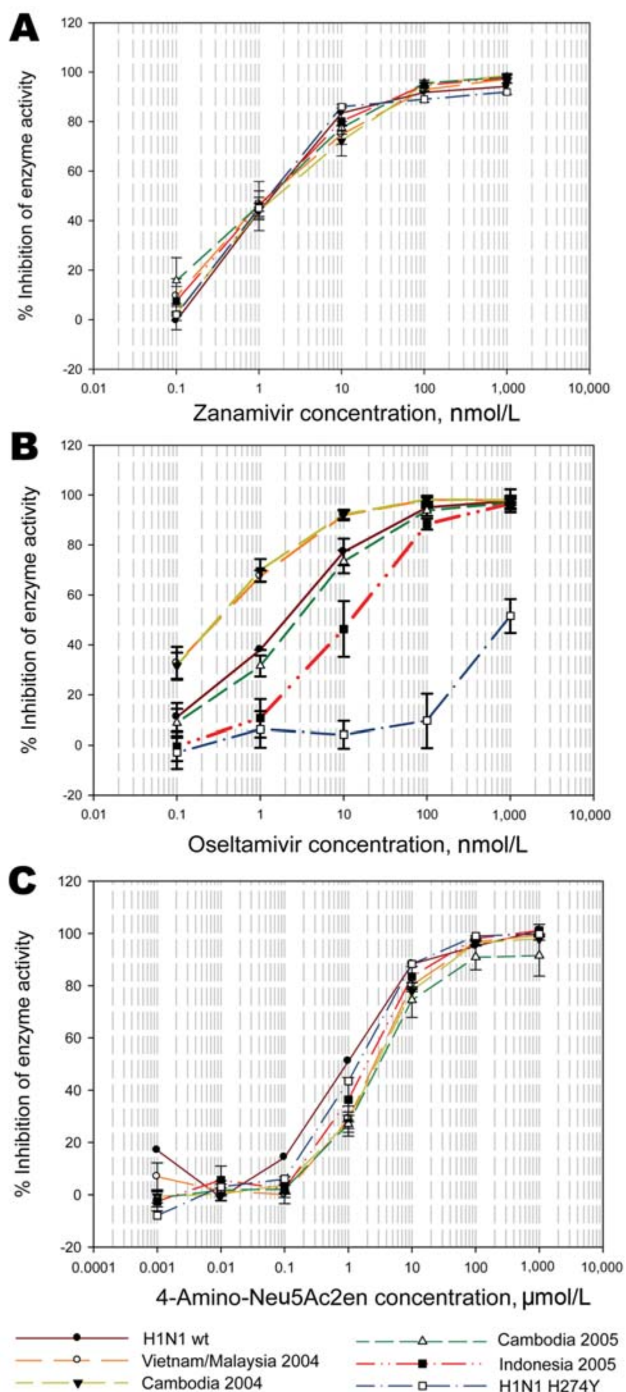

Figure. Sensitivity of clade 1 and clade 2 influenza A (H5N1) viruses to zanamivir, oseltamivir, and 4-amino-Neu5Ac2en in a MUNANA-based enzyme inhibition assay (Sigma, Saint Louis, MO, USA). Viruses were grown in allantoic fluid and irradiated for testing sensitivities of their neuraminidases. Plots are the mean values for inhibition of enzyme activity for each drug concentration of all isolates from that country and year; bars represent standard deviations of values for all isolates from that group. A) Sensitivity to zanamivir; B) sensitivity to oseltamivir; C) sensitivity to 4-amino-Neu5Ac2en.

appear to have undergone full reorientation, which could contribute the reduced oseltamivir binding found in to the Indonesian clade 2 isolates here. Testing the sensitivities of both NAs would provide valuable information on the role of H252. However, mutational analysis of the Indonesian and Cambodian isolates and structure determinations of the different NAs are necessary to fully understand the mechanisms of altered binding.

The specific decrease in sensitivity to oseltamivir of both 2005 Cambodian clade 1 and especially the Indonesian clade 2 influenza (H5N1) isolates is disturbing, especially because they maintain their pathogenicity and transmissibility in birds and are clearly pathogenic in humans, since Indonesia has the highest death rate from influenza (H5N1) infections of any country. This finding is in contrast to recent observations that mutations conferring zanamivir resistance in human strains have poor viability and are not genetically stable (*12*). Such a decrease in oseltamivir sensitivity could lead to suboptimal drug dosing in treating persons infected with these isolates, which is thought to facilitate selection of viruses with a high level of resistance (*13*). Several groups have reported the emergence of resistant viruses in clade 1–infected influenza (H5N1) patients treated with oseltamivir and suggested that higher doses of oseltamivir may be needed (*1*,*8*). Because the clade 2 viruses studied here have a 15- to 30-fold decrease in sensitivity compared to the clade 1 viruses, this suggests the standard dosing of oseltamivir may be even less effective in treating clade 2 influenza (H5N1) A–infected patients.

Many laboratories are developing rapid PCR sequencing methods for detecting the known mutation (H274Y) that confers high-level resistance in influenza (H5N1). However, we have shown here the importance of phenotypic testing of isolates in an enzyme assay rather than just genotypic screening (*14*). Because the clade 2 virus is now spread through parts of Europe and Africa, continued global collaboration and phenotypic testing of drug sensitivity of circulating highly pathogenic avian isolates for NA inhibitor sensitivity are critical. This knowledge is essential for developing appropriate management strategies for pandemic planning. No altered sensitivity to zanamivir occurred, which further supports the hypothesis of minimalist drug design (*15*) and of maintaining the inhibitor as close as possible to the natural substrate to minimize the emergence of resistance. Our results suggest that zanamivir may also play an important role in pandemic stockpiles.

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Table 2. Mean IC_{50}s of isolates from each group against alternative source of oseltamivir*†

Virus	Oseltamivir, IC_{50}, nmol/L
A/Mississippi/3/2001 wt	3.2 (2.1)
A/Chicken/Vietnam/008/2004	0.7 (0.4)
A/Chicken/Cambodia/Kandal/23/2004	0.4 (0.2)
A/Goose/Cambodia/Kandal/2005	4.2 (0.5)
A/Chicken/Cambodia/Kandal/3/2005	4.8 (0.5)
A/Chicken/Indonesia/Wates/77/2005	25.6 (2.5)
A/Chicken/Indonesia/Wates/126/2005	14.7 (2.3)

*IC_{50}, drug concentration for 50% inhibition of enzyme activity; wt, wild type.
†Isolates were tested in duplicate in 2 separate assays. IC_{50}s and standard errors (in parentheses) were calculated by using the nonlinear curve-fitting function in Sigmaplot (Systat Software Inc., London, UK).

Dr McKimm-Breschkin is a chief research scientist in the CSIRO Division of Molecular and Health Technologies in Australia. Her institute was involved in the development of zanamivir. Her research interests focus on understanding mechanisms of influenza resistance to the neuraminidase inhibitors.

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New Clinico-epidemiologic Profile of Cutaneous Leishmaniasis, Morocco

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During the past 20 years, cutaneous leishmaniasis has emerged as a major public health threat in Morocco. We describe distribution of *Leishmania major* and *L. tropica* in Morocco and a new focus of cutaneous leishmaniasis due to *L. infantum.* We recommend using molecular techniques to diagnose suspected leishmaniasis cases.

Leishmaniasis, a vectorborne parasitic disease, affects 1.5–2 million people annually. In >100 countries whose populations are at risk for the disease, the disease inflicts a high economic cost (*1*,*2*). Additionally, large-scale emergence and reemergence have been recently reported in many Mediterranean countries, including Morocco (*1*,*3*).

Cutaneous leishmaniasis (CL) caused by *Leishmania major* has been reported in Morocco since 1914 (*4*); until recently, however, it was largely confined to arid Saharan regions (*4,5*). In 2001, the Moroccan Ministry of Health (MMH) reported 2,028 CL cases caused by *L. major* and *L. tropica* (*6*). Of the 3 clinically important *Leishmania* species (*L. major, L. tropica, L. infantum*), *L. tropica* has the largest geographic distribution and is considered a public health threat by the MMH. *L. tropica* CL has been reported in Azilal, Essaouira, Taza, Fes, the province of Chichaoua, and central Morocco (*5*,*7–10)*.

Accurate diagnosis and treatment of CL requires positive identification of the causative species of parasite (*11*). Often, however, traditional diagnostic methods such as analysis of clinical symptoms, microscopic identification, and parasite culture are performed in place of molecular diagnostic techniques, such as PCR. Problematically, all *Leishmania* species have similar morphology, and several species capable of causing both CL and visceral leishmaniasis (VL) may exist in the same locales.

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We update the current epidemiologic profile of *Leishmania* spp. in Morocco by using archived clinical samples tested by PCR. We provide economic and epidemiologic rationales for our recommendation that species-specific identification be performed for all cases of suspected leishmaniasis.

The Study

Tissue samples were taken from 27 patients with suspected CL who had consulted the health centers from March 2005 to March 2006. Local reference laboratories evaluated all stained slides by light microscopy and positively identified *Leishmania* amastigotes. Patients had no history of travel and were assumed to been infected in Morocco; all received free intralesional injections of meglumine antimoniate (Glucantime; Sanofi-Aventis, Bridgewater, NJ, USA) until total recovery, according to the protocol in the MMH leishmaniasis control manual. Samples were collected in areas of Morocco known for high CL incidence: north (Sidi Kacem), center (Beni Mellal and Boulemane), southeast (Errachidia), and southwest (Taroudant and Ouarzazate) (Figure 1).

DNA extraction and PCR analysis by amplification of the ribosomal internal transcribed spacer 1 (ITS1), using stained slides, was performed as described by Schonian et al. (*12*). We used 0.6-nM primers and PCR-Ready Supreme mix (Syntezza Bioscience, Jerusalem, Israel) in 25 µL of total reaction. *Leishmania* DNA (10 ng/reaction) from reference strains *L. tropica* (MHOM/AZ/1974/SAF-K27), *L. major* (MHOM/TM/1973/5ASKH), and *L. infantum* (MHOM/TN/1980/IPT1) were used as positive controls. Negative controls for extracted DNA and PCR analysis were included. After amplification, the PCR product was digested with 1.5 µL *Bsu*RI endonuclease (MBI Fermentas, Burlington, Ontario, Canada), and all digested products were analyzed by agarose gel electrophoresis (*12*).

All patients had classic symptoms of CL, from small erythematous papules to nodules and ulcerative lesions. Patients' ages varied from 1.25 to 70 years. The sample comprised 44% male and 56% female patients (Table). Papular lesions, nodular lesions, or both were present in 30% of the CL patients; ulcerative lesions, in 52%. Neither the papular/nodular nor the ulcerative forms correlated with a particular *Leishmania* species. The erythematous clinical form was present in 18% of total case-patients and in 63% of case-patients from the Sidi Kacem region.

Undigested ITS1 amplicons from the 27 slides produced a band of 300–350 bp (data not shown), which confirmed the presence of *Leishmania* DNA. Band patterns from the digested samples were compared with digested standards for each reference strain and identified the parasite species (Figure 2) as follows: *L. major,* 3 samples each

[1]These authors contributed equally to this article.

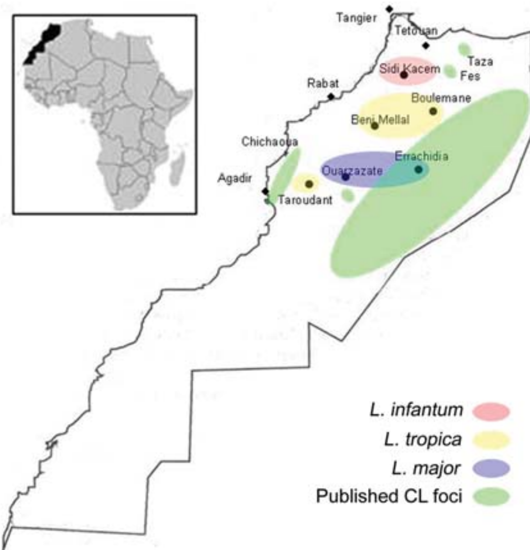

Figure 1. Molecular epidemiology of cutaneous leishmaniasis (CL) in Morocco. *L., Leishmania.*

from Ouarzazate and Errachidia; *L. tropica,* 2 samples from Taroudant, 4 from Beni Mellal, and 7 from Boulemane; *L. infantum,* 8 samples from Sidi Kacem.

Conclusions

CL caused by *L. major* or *L. tropica* and VL caused by *L. infantum* have been reported in Morocco (*5–9*). PCR on archived tissue samples enabled us to investigate the epidemiology of CL in disease-endemic regions of Morocco and identify those species responsible for this disease in several new foci (Table). Our results, together with those of previous studies (*5–9*; unpub. data from MMH, 2001), indicate that CL caused by *L. tropica* is found throughout the center of the country in a band stretching from the Atlantic Ocean along the length of the Atlas Mountains almost to the Mediterranean Sea. CL caused by *L. major* is present in the desert region south of the Atlas Mountains in a strip bordering the Sahara Desert (Figure 1).

We report on a focus of CL in Morocco caused by *L. infantum*, 8 samples from Sidi Kacem. In Morocco, the only previous human CL case caused by *L. infantum* was reported in 1996, within an active focus of VL (*13*). The northern coastal regions of Morocco are endemic for human and canine VL. As in other VL-endemic regions surrounding the Mediterranean Sea, this disease is caused by *L. infantum* (*3*). Although it is unusual for this parasite to cause CL, our finding is similar to a recent report from Tunisia, where *L. infantum* was shown to cause sporadic CL in regions endemic for VL. It appeared to have emerged in a new region of the country and was suggested to be more prevalent than originally indicated (*14*).

CL and VL overlap in many provinces of central Morocco; anthroponotic foci of *L. tropica* CL are found in Fes and Taza (*7–9*) (Figure 1), not far from existing VL foci including Sidi Kacem. Furthermore, several cases of canine VL caused by *L. tropica* have been reported in regions where canine VL is caused by *L. infantum*.

The nodular form of CL was caused by all 3 species; ulcerative lesions were seen only with CL caused by *L. tropica* and *L. major*. Of the 8 patients in Sidi Kacem with *L. infantum* infection, 5 showed the atypical erythematous papular form. These findings agree with results of studies in northern Morocco (*7*). The overlapping distribution of parasite species, causing diseases with similar clinical pictures, demonstrates the need for additional epidemiologic and ecologic studies of CL in conjunction with species identification. This is especially important as traditional methods of determining infection from patient history and microscopic examination prove increasingly unreliable. PCR can be performed rapidly on fresh or archived samples and does not require culturing of large amounts of parasites. In addition, PCR costs have come down considerably, and costs can be further reduced by sending samples by regular mail to a central facility.

Recent studies document the emergence of new *Leishmania* foci and the coexistence of multiple *Leishmania* species in the same geographic locale, including much of northern Africa (*14*). We recommend that treatment protocols, particularly in areas of coexistence, be predicated on diagnosis of not only the clinical form—CL versus VL—but additionally the disease-causing species.

In Morocco, local physicians and healthcare administrators often do not realize that different species of *Leish-*

Table. Distribution of cutaneous leishmaniasis, 27 patients, Morocco, 2005–2006

Geographic origin	No. cases	Age range, y	Sex	Clinical lesions	*Leishmania* species
Taroudant	2	5–20	1M, 1F	Nodular (*2*)	*L. tropica*
Beni Mellal	4	3–11	3M, 1F	Ulcerative (*4*)	*L. tropica*
Boulemane	7	1.25–60	4M, 3F	Ulcerative (*5*) Papulonodular (*2*)	*L. tropica*
Ouarzazate	3	0.25–52	1M, 2F	Ulcerative (*2*) Nodular (*1*)	*L. major*
Errachidia	3	3–39	1M, 2F	Ulcerative (*3*)	*L. major*
Sidi Kacem	8	2–70	2M, 6F	Erythematous (*5*) Papulonodular (*2*) Nodular (*1*)	*L. infantum*

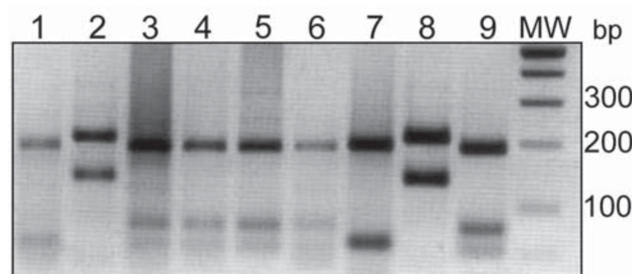

Figure 2. Comparison between endonuclease *BsuR*I digestion patterns of internal transcribed spacer 1–PCR products from clinical samples and reference *Leishmania* strains. Clinical samples, lanes 1–6: lane 1, Boulmane; lane 2, Ouarzazate; lanes 3–6, Sidi Kacem. Reference strains, lanes 7–9: lane 7, *L. tropica* (MHOM/SU/1974/SAF-K27); lane 8, *L. major* (MHOM/TM/1973/5ASKH); lane 9, *L. infantum* (MHOM/TN/1980/IPT1). MW, DNA molecular weight marker in base pairs (bp).

mania require differential treatments, which can result in a failure to diagnose more serious disease. Risk for metastatic lesions with *L. major* is almost zero. However, recurrent failure of local treatments (paromomycin and intralesional sodium stibogluconate) against *L. tropica* was evident (*11*). Because *L. tropica*, and now *L. infantum,* cause both VL and CL, a physician treating a cutaneous lesion may overlook visceral disease, and a host of costlier health problems may ensue. A simple, sensitive PCR test could easily reduce such risk. Further surveillance of cases and suspected cases from these foci should confirm the results of this limited study.

Recent implementation of PCR-based diagnosis in an outbreak in northern Algeria increased the positive diagnosis of CL by 69% over cases diagnosed by using microscopy alone (*15*). Furthermore, anthroponotic CL caused by *L. tropica* is limited to parts of southern Europe, Asia, and Africa; diagnosis and treatment of the disease at its earliest stage is of paramount importance for reduction of the human reservoir. Failure to promptly diagnose and treat all cases will result in continued dissemination of the parasite.

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Tuberculosis in Children and Adolescents, Taiwan, 1996–2003

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Analysis of data from Taiwan's National Tuberculosis (TB) Registry showed that incidence of TB in persons <20 years of age was 9.61/100,000 person-years, biphasic, and age-relevant, with a major peak in persons slightly >12 years. Aboriginal children were 8.1–17.4× more likely to have TB than non-Aboriginal children.

Because epidemiologic data on childhood tuberculosis (TB) are limited, we conducted a study in Taiwan to estimate the incidence of TB in children and adolescents and to characterize epidemiologic, geographic, and ethnic differences. To do this, we analyzed nationwide data obtained from Taiwan's National TB Registration, Center for Disease Control.

The Study

Taiwan's computer-based system for reporting cases of TB disease was established in 1996. In this system, even suspected cases of TB must be reported and registered. A diagnosis or confirmation of TB is made on the basis of clinical or laboratory findings (*1*). If no TB is confirmed or another diagnosis is made later, the TB registration is cancelled. To ensure compliance with the TB registration system, Taiwan's National Health Insurance Bureau, a universal healthcare system that has insured 96% of the population since 1996, introduced 2 policies in 1997. The first was the no-notification–no-reimbursement policy, which requires that no claim would be reimbursed for the treatment of a case of TB unless it is reported. The second was the notification-fee policy, which provides an extra cash award to physicians for reporting a new case of TB (*2*).

Population data for Taiwan, including those regarding Aboriginal and non-Aboriginal populations, were obtained from official publications of the Ministry of the Interior (*3*). Age- and gender-specific notification rates (per 100,000) were then calculated based on Taiwan's National TB Registry data and population data from 1996 through 2003.

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Differences in incidences between groups were measured by the χ^2 test. All reported p values were 2-tailed; $p<0.05$ was considered statistically significant. The strength of the associations between 2 variables was calculated by using Spearman rank order correlation. All analyses were performed with Epi Info 6.0 (available from www.cdc.gov/epiinfo/epi6/ei6dnjp.htm).

Between 1996 and 2003, a total of 5,062 cases were reported, and the overall incidence of TB in patients <20 years of age was 9.6/100,000 person-years, with no significant difference among the years studied (8.2–11.6/100,000 person-years, p = 0.55, with χ^2 for goodness of fit). Analyzed by age group, incidence of TB for newborns to those 3 years of age was slightly higher than that for those 4–11 years (Figure 1), and incidence increased sharply in children ≥12 years ($p<0.001$, χ^2 for goodness of fit). Analyzed by gender, the male-to-female ratio was 1.32, and boys in the 15- to 19-year-old group were 1.42× more likely than girls to have TB (95% confidence interval [CI] 1.33–1.52, $p<0.0001$).

We also analyzed the incidence of extrapulmonary TB. Extrapulmonary TB without lung involvement peaked in 1- to 2-year-olds (3.29/100,000 person-years). The distribution was monophasic (Figure 1). The risk of developing extrapulmonary TB was 2.88× higher for children <2 years of age than for children >2 years (95% CI 1.23–6.98, p = 0.012). From 1996 to 2003, the proportion of cases of extrapulmonary TB without lung involvement relative to total TB cases declined with age, from 60% during early childhood to 5% after adolescence. We also subdivided the incidence of extrapulmonary TB by site of involvement and the 4 age groups (Table). Bones and joints were the most frequent site of extrapulmonary TB for children <5 years, whereas lymph nodes were the most frequent site for those ≥5 years.

The indigenous Aboriginal people of Taiwan represent 1.9% of Taiwan's population of 22.1 million people (*3*), a proportion similar to that of the aborigines of Australia and Canada. The Aboriginal population in our study had an overall childhood TB incidence of 81.5/100,000 person-

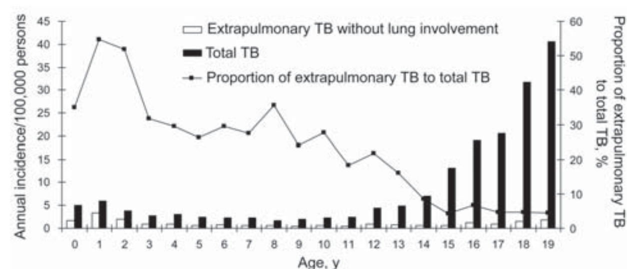

Figure 1. Annual incidence of tuberculosis (TB) and extrapulmonary TB without lung involvement in Taiwanese children, 1996–2003. The line indicates the proportion of extrapulmonary TB without lung involvement to total TB.

Table. Distribution of different sites of extrapulmonary tuberculosis among 4 age groups, Taiwan, 1996–2003

Site	Age group			
	≤4 y (n = 242), no. (%)	5–9 y (n = 96), no. (%)	10–14 y (n = 105), no. (%)	15–19 y (n = 242), no. (%)
Meninges	30 (12)	6 (6)	6 (6)	25 (10)
Lymph nodes	56 (24)	51 (54)	44 (41)	127 (53)
Bone and joint	92 (39)	11(11)	12 (11)	10 (4)
Genitourinary tract	1 (<1)	1 (1)	1 (1)	9 (4)
Skin and eye	15 (6)	3 (3)	6 (6)	10 (4)
Gastrointestinal tract	5 (2)	3 (3)	8 (8)	11 (5)
Others	42 (17)	21 (22)	28 (27)	49 (20)

years, which was 9.63× (95% CI 3.71–25.04) greater than the incidence for non-Aboriginal children (p<0.0001, χ^2 test). The ratios of the TB incidence of Aboriginal children to that of non-Aboriginal children were 11.3 in those ≤4 years old, 13.8 for the 5- to 9-year-old group, 17.4 for the 10- to 14-year-old group, and 8.1 for the 15- to 19-year-old group. Two peaks of extrapulmonary TB occurred in the Aboriginal population (Figure 2), the first in those ≤4 years of age and the second in those 10–14 years of age.

Geographically, the highest incidence of TB was found in Hualian County, located in eastern Taiwan. This county has a higher proportion of Aboriginal people (28.6%) than any other county in Taiwan. The incidences of TB by geographic area were significantly positively correlated with the percentages of Aboriginal populations for the 4 age groups (r = 0.44, p = 0.03 for those ≤4 years; r = 0.74, p = 0.00003 for those 5–9 years; r = 0.62, p = 0.0009 for those10–14 years; r = 0.56, p = 0.036 for those 15–19 years, with Spearman rank order correlation).

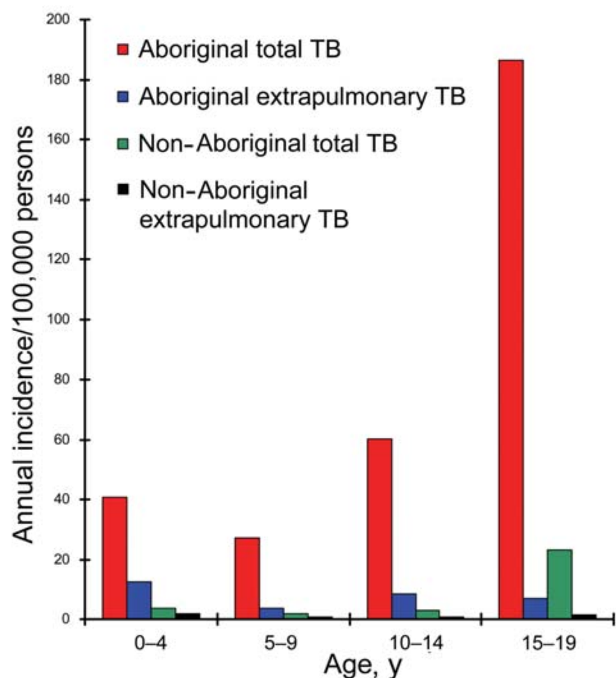

Figure 2. Annual incidence of total tuberculosis (TB) and extrapulmonary TB in aboriginal and non-Aboriginal children, Taiwan, 2000–2003.

Conclusions

In conclusion, the overall incidence of childhood TB is 9.61/100,000 person-years in Taiwan; the incidence is also biphasic and age-relevant, with a major peak found in those just above 12 years of age. The incidence of TB in children is higher in Taiwan than in Western countries (*4*,*5*). In Western countries that do not require bacillus Calmette-Guérin (BCG) vaccination, the highest incidence of childhood TB has been reported in children <5 years of age (*4*,*5*). One possible reason for this difference may be because almost all neonates in Taiwan receive BCG vaccinations (2001, 98%) (*6*), which may protect children <5 years of age from TB.

Previously, higher incidences of TB cases and TB-related deaths have been reported in Aboriginal areas than in non-Aboriginal areas in Taiwan (*1*,*7*). In our study, depending on the age group, the incidence of TB among Aboriginal children was 8.1–17.4× higher than that in non-Aboriginal children. The higher incidence in this population has been attributed to their lower socioeconomic status and an inherited susceptibility (*8*). Aboriginal children may also be exposed to more TB in adults than other groups are or have less access to medical resources than their counterparts (*1*). Although BCG vaccination coverage is high in Taiwan, a lower coverage rate may still play some role in a higher incidence of TB there. Checking the coverage rates of BCG in 2 counties from 2003 through 2005, we found that although 98.5% of all children in Taiwan received BCG vaccinations, only 92.2% of the children in Aboriginal areas did. The difference was significant (p = 0.03, with 2-tailed, 2-proportional *t* test). Therefore, childhood TB in Aboriginal areas might be reduced if the following measures were adopted: implementing directly observed therapy for infected persons, increasing BCG vaccination coverage, and providing more accessible treatment for latent TB infection for the indigenous people in these areas.

We found another peak in incidence of extrapulmonary TB in 10- to 14-year-old Aboriginal children. Although HIV-positive persons were found to have a significantly higher risk for extrapulmonary TB in Arkansas, USA (*9*),

we found that none of the Aboriginal people with extrapulmonary TB had reported HIV infection until late 2006. Thus, the increase in extrapulmonary TB in Aboriginal populations is likely related to causes other than HIV and should be investigated further.

In summary, the overall childhood TB incidence was 9.61/100,000 person-years and age-relevant in Taiwan. TB incidence among Aboriginal children was much higher than incidence among non-Aboriginal children. Therefore, efforts to reduce the incidence of childhood TB should be focused on areas with a larger proportion of the Aboriginal population.

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Risk Factors for Hantavirus Infection in Germany, 2005

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In 2005, a marked increase in hantavirus infections was observed in Germany. Large cities and areas where hantaviruses were not known to be endemic were affected. A case–control study identified the following independent risk factors for infection: occupational exposure for construction workers, living <100 m from forested areas, and exposure to mice.

Hantaviruses (family *Bunyaviridae*) are rodentborne pathogens found worldwide. They have caused recurrent epidemics in several countries (*1–3*). Hantaviruses circulating in North and South America can cause a fatal cardiopulmonary syndrome; hantavirus infections in Europe and Asia can result in a hemorrhagic fever with renal syndrome (HFRS) of varying severity. In Germany, the predominant serotype is Puumala; its main reservoir is bank voles (*Myodes glareolus*). A mild form of HFRS (nephropathia epidemica) usually develops in patients, but a substantial number require hospitalization and hemodialysis (*1*).

In 2001, hantavirus infection became a mandatory reportable disease in Germany. From January through May 2005, the number of reported case-patients (n = 158) almost tripled when compared with the number of patients seen in the same period in previous years. Unexpectedly, infections were also observed in larger cities and in rural regions where they were not known to have occurred previously. Thus far, risk factors for hantavirus infections have been assessed in rural settings (*4*,*5*). The unusual geographic pattern in 2005 in Germany prompted us to conduct a case–control study to investigate potentially new risk factors for human hantavirus infections.

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The Study

In Germany, all laboratory-confirmed hantavirus infections are reported to the local public health authorities and forwarded through the federal states to the Robert Koch Institute. Laboratory diagnosis is based on detection of nucleic acid, a marked rise of immunoglobulin (Ig) G antibodies in a paired sample, or detection of IgM or IgA antibodies confirmed by IgG antibodies. Local health departments identified eligible case-patients for the case–control study according to the following criteria: laboratory-confirmed hantavirus infection with clinical symptoms acquired in Germany and a reporting date between May and August 2005. Controls were selected from the population by sequential digital telephone dialing and matched individually by sex and residential area. An exclusion criterion for controls was having had a diagnosis of hantavirus infection or a disease with fever (>38.5°C) for at least 3 days, accompanied by back pain, abdominal pain, or headache in the 4 weeks preceding the interview. All participants were ≥18 years of age and provided informed consent.

Interviews were conducted by trained public health professionals who used a standardized questionnaire. The questionnaire covered demographic, clinical, and exposure data, e.g., type of residential area, residence distance from forested areas, handling of wood, outdoor activities, occupational exposures, contact with rodents and rodents' droppings, and travel history. The relevant period of exposure was 4 weeks preceding disease onset for case-patients and 4 weeks preceding the interview for controls. Controls were interviewed on average within 2 weeks after case-patients.

Matched odds ratios were calculated and variables with $p<0.2$ were considered for the conditional logistic regression model by using EpiInfo 3.3.2 (Centers for Disease Control and Prevention, Atlanta, GA, USA) and SAS statistical package version 8 (SAS Institute, Cary, NC, USA). A forward stepwise procedure was chosen. Variables with $p<0.05$ remained in the model. For each step the more complex model was compared with the previous model on the basis of likelihood ratio statistics. Selected variables were examined for collinearity, and interaction was tested in the conditional logistic regression model.

In the total year 2005, 448 hantavirus case-patients were reported. Notifications increased steeply in May and persisted at a high level until August. The annual incidence (0.54/100,000 inhabitants) almost doubled compared with that in 2001–2004 (Figure 1). Particularly high incidences were observed in some cities (Osnabrück 8.5, Aachen 8.1, and Cologne 4.2) (Figure 2).

In the case–control study (May–August), 154 (71.6%) of 215 eligible case-patients participated, and 150 matched

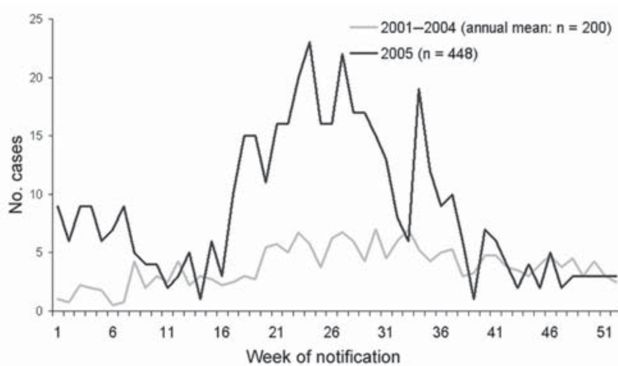

Figure 1. Reported hantavirus infections in 2005 compared with the annual average in 2001–2004, by week of report, Germany.

case–control pairs were analyzed. The male:female ratio was 2.1:1. The median age of case-patients was 42 years (range 19–75) and of controls, 46 years (range 20–87) (p<0.01). Of all case-patients, 40.7% lived in rural areas and 32.7% in cities with >100,000 inhabitants. Most case-patients had fever (87.7%); other major symptoms included back pain (74.8%), headache (73.9%), myalgia (73.7%), nausea (68.0%), vomiting (50.7%), blurred vision (45.3%), and abdominal pain (41.6%). Median duration of symptoms was 12 days. Of the case-patients, 73.4% were hospitalized (median duration 8 days), and 6.6% required hemodialysis. No hemorrhagic or fatal course was reported during the outbreak. Of the employed patients, 92.2% reported absence from work because of hantavirus infection (median duration 19 days).

Table 1 shows the associations of different exposure variables with the outcome in univariate analysis. Of note, 12.2% of the case-patients were forestry workers, and 11.5% were construction workers (27.5% of the male case-patients). Risk factors did not differ significantly between case-patients from urban and rural areas. Occupational exposure as a construction worker, noticing mice in the neighborhood, and living in a building <100 m from forested areas remained independent risk factors (adjusted for age) in the multivariate model (Table 2). No significant interaction was found.

Conclusions

The 2005 hantavirus epidemic in Germany caused substantial disease and showed remarkable epidemiologic characteristics. Compared with data from previous years, a relatively early and steep increase in patient numbers was observed in May, and the high disease activity extended over several months. A substantial number of patients acquired their infection in areas where the disease was previously not known to be endemic, most notably in urban settings near forests and wooded municipal parks. The main reason for the epidemic was a strong rise in the reservoir population, which has its habitat in forested areas. In fact, in some places an upsurge in the bank vole population had already occurred in 2004 because of the intense beech mast (F. Krüger, pers. commun.). Most likely, hantavirus-infected bank voles were also increasingly present in forested parts of inner city areas. In Cologne, environmental investigations detected Puumala virus in 66% of trapped bank voles (*6*). It is unclear, however, whether the virus has been newly introduced in these areas or had been present previously but only at very low levels or in small ecologic niches constituting only negligible risks for humans. A systematic monitoring system for rodents (population density, hantavirus prevalence) could be used to predict an increased human risk and facilitate recommendations for persons in at-risk areas.

Living close to forested areas was a major risk factor independent of a residence in more rural or urban areas. A substantial number of case-patients probably acquired infection in an area close to where they lived. Leisure activities in forested areas did not significantly increase the risk for hantavirus infection, as has been reported in other studies (*4*). If areas close to human residences are increasingly contaminated with virus-containing rodent excreta, the inhabitants are more likely exposed by common activities

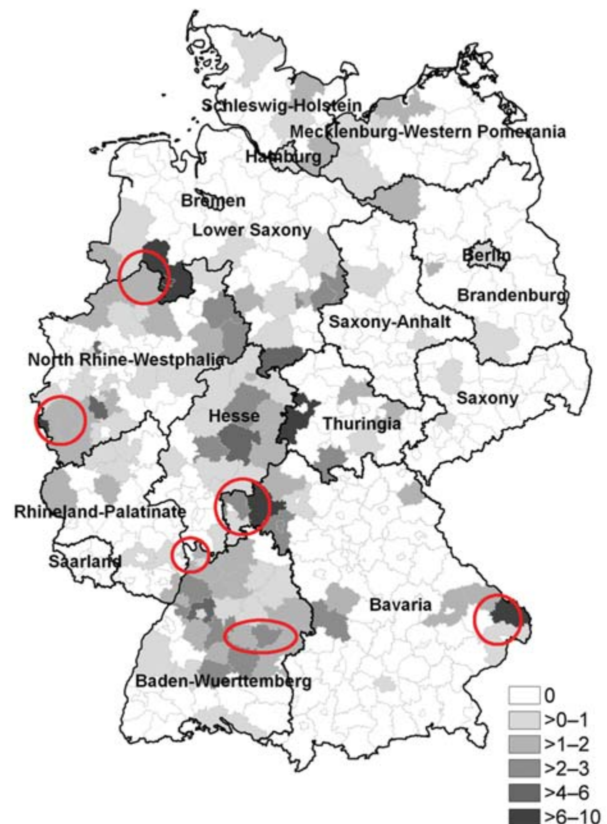

Figure 2. Incidences of reported hantavirus infections per 100,000 inhabitants by administrative district, Germany, 2005. Circles represent areas in which hantaviruses were known to be endemic.

Table 1. Univariate matched analysis for exposure variables for hantavirus infection, Germany, 2005*

Exposure	Case-patients, no. (%)	Controls, no. (%)	Matched OR	95% CI	p value
Noticing mice	75 (50.0)	48 (32.0)	2.5	1.4–4.5	<0.01
In forested areas	28 (18.7)	4 (2.7)	13.0	3.3–113.0	<0.01
Noticing mice droppings	43 (28.7)	21 (14.0)	2.5	1.3–5.0	<0.01
Living <100 m from forested areas	65 (43.3)	39 (26.0)	2.3	1.3–4.1	<0.01
Being a forestry worker	18 (12.2)	8 (5.4)	2.7	1.0–8.3	0.05
Being a construction worker	17 (11.5)	5 (3.4)	4.0	1.3–16.4	0.01
Entering empty rooms or buildings	26 (17.3)	10 (6.7)	2.8	1.3–6.8	0.01
Cutting or handling wood	35 (23.3)	21 (14.0)	2.0	1.0–4.2	0.05
Gardening	85 (56.7)	98 (65.8)	0.7	0.4–1.1	0.14

*OR, odds ratio; CI, confidence interval.

such as cleaning up around houses or sheds. Persons living close to areas with hantavirus-infected rodent populations should be informed about the potential exposure risks and follow recommendations for prevention and control (*7*). In residential areas, rodent control measures should be maintained at a high level.

Almost 30% of male case-patients were construction or forestry workers. Probably because of such occupational differences, but also because of recreational exposure patterns, men were predominantly affected in this epidemic, as has been described in other countries (*1*,*5*,*8*). Most case-patients who were construction workers mentioned having worked on restoring old buildings during the likely incubation period. Building sites near forested areas (and particularly older buildings in need of restoration) are likely infested by bank voles and pose considerable hazards to humans who work there. Several studies have shown that forestry workers, farmers, or soldiers in maneuvers are at increased risk (*4*,*5*,*9*,*10*).

In 2005, a similar marked increase of hantavirus infection was observed in Belgium and France, but case-patients living in more densely populated urban areas were reported only from Germany (*11*). To better understand the dynamics of the reservoir population as well as the epidemiologic characteristics and risk factors among humans, a concerted approach to monitoring of the reservoir and to surveillance and investigation of human cases are warranted in neighboring countries.

Acknowledgments

We thank all the participating local and regional public health offices for their contribution to the study.

Dr Abu Sin is a physician and was a fellow of the German Field Epidemiology Training Program at the Robert Koch Institute. Her major research interests include the epidemiology of zoonoses.

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Table 2. Risk factors for hantavirus infection, conditional logistic regression model, Germany, 2005

Exposure	Odds ratio*	95% Confidence interval	p value
Being a construction worker	4.8	1.4–17.1	0.01
Noticing mice	3.0	1.6–6.0	<0.01
Living <100 m from forested areas	2.5	1.3–4.7	<0.01

*Adjusted for age.

Coronavirus Antibodies in African Bat Species

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Asian bats have been identified as potential reservoir hosts of coronaviruses associated with severe acute respiratory syndrome (SARS-CoV). We detected antibody reactive with SARS-CoV antigen in 47 (6.7%) of 705 bat serum specimens comprising 26 species collected in Africa; thus, African bats may harbor agents related to putative group 4 CoV.

Severe acute respiratory syndrome (SARS) emerged as a newly recognized human disease in the People's Republic of China late in 2002 and spread globally, causing 8,422 infections with 916 (11%) deaths before it was brought under control in 2003 (*1*). The causative agent was identified as a coronavirus (SARS-CoV) (*2–4*), and related viruses found in palm civets (*Paguma larvata*), raccoon dogs (*Nycereutes procyonoides*) (*5*), and insectivorous bats in Asia cluster phylogenetically together with SARS-CoV in a putative group 4 (*6–10*). Farmed food animals such as civets may acquire SARS-like-CoV infection from bats, and adaptation of the viruses to these secondary hosts may occasionally give rise to strains capable of spreading and causing disease in humans (HCoV) (*10*).

The Study

Bat serum specimens (n = 705) collected from 1986 through 1999 in South Africa (SA) and the Democratic Republic of the Congo (DRC) were tested. The first 248 serum specimens were collected from 1986 through 1989 in the Mpumalanga and Limpopo Provinces of SA for studies on rabies-related viruses, with the approval of the provincial Directorates of Nature Conservation and the Animal Ethics Committee of the University of the Witwatersrand. The remaining 457 serum samples were collected in 1995–1999 in the Bandundu and Oriental Provinces of the DRC for studies on Ebola and Marburg viruses, under the auspices of the International Committees for the Control of Ebola hemorrhagic fever in Kikwit, and Marburg hemorrhagic fever in Durba-Watsa, coordinated by the World Health Organization on behalf of the government of DRC. Bats were caught in mist nets, anesthetized, and exsanguinated by cardiac puncture. Serum specimens were stored at –70°C until analyzed.

For screening of serum specimens we used the SARS-CoV ELISA kit (EUROIMMUN AG, Lübeck, Germany) with minor modifications. Bat serum samples were tested at a dilution of 1:50, and horseradish peroxidase–labeled goat antibat immunoglobulin (Ig) conjugate (Bethyl, Montgomery, AL, USA) was used as secondary antibody at a dilution of 1:2,000. Negative bat serum was obtained from a captive-bred *Rousettus aegyptiacus* at the National Institute for Communicable Diseases, Sandringham, SA. The cutoff was determined as 3× the mean optical density value at 450/605 nm observed in negative control samples. Positive serum samples were retested and their titers determined. To evaluate test specificity and to exclude possible cross-reactivity to other viruses, especially to HCoVs, which have a seroprevalence in humans >90% (*11*), 662 human serum specimens were screened (online Technical Appendix, available from www.cdc.gov/EID/content/13/9/1367-Techapp.htm), including those from 90 patients with other acute respiratory infections, 70 HCoV-229E–positive serum specimens and 4 HCoV-NL63–positive serum specimens (provided by L. van der Hoek).

A confirmatory Western blot (WB) was done by using protein lysates from Vero E6 cell cultures (American Type Culture Collection [Manassas, VA, USA] CRL 1586) infected with SARS-CoV Hong Kong isolate 6109 (GenBank accession no. AY278491) and from uninfected Vero E6 cultures. Bat serum specimens were applied at dilutions of 1:500 and 1:2,000. Secondary detection was performed with the SuperSignal West Dura Extended Substrate chemiluminescence detection assay (Pierce Biotechnology, Rockford, IL, USA). The signal intensity of the 150-kDa spike (S), 50-kDa nucleocapsid (N) proteins was evaluated independently by 2 operators. For a second confirmatory WB, recombinant SARS-CoV proteins were used. For prokaryotic expression of recombinant SARS-CoV N protein and a fragment of the S protein (amino acid positions 318–510), we followed the instructions of the Champion pET Directional TOPO Expression kit (Invitrogen, Karlsruhe, Germany) using plasmids pET101-N and pET102-Saa318–510. Purification and refolding of the protein on column were done as described previously (*12*). Purified recombinant protein (15 μg) was resolved by electrophoresis on a discontinuous 12% sodium dodecyl sulfate–polyacrylamide gel. After blotting, nitrocellulose strips were incubated with bat serum samples diluted 1:2,500 and 1:5,000. One positive bat serum specimen was used as a reference to exclude

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Table. Antibody to SARS-CoV in bat sera collected in 1986–1999 at 4 locations in central and southern Africa*

	ELISA: positive/tested (%)†					WB: positive/tested‡	IIFT: positive/tested‡
	Limpopo Province, SA	Mpumalanga Province, SA	Oriental Province, DRC	Bandundu Province, DRC	Total		
Fruit bats							
Casinycteris argynnis				0/3	0/3		
Eidolon helvum				0/6	0/6		
Epomophorus gambianus	0/4	0/6			0/10		
Epomophorus wahlbergi	0/2				0/2		
Epomops franqueti				0/5	0/5		
Hypsignathus monstrosus				1/11 (9.1)	1/11 (9.1)	1/1	0/1
Lyssonycteris angolensis			1/16 (6.3)	0/2	1/18 (5.6)	1/1	0/1
Myonycteris torquata				1/7 (14.3)	1/7 (14.3)		
Rousettus aegyptiacus	11/29 (37.9)		17/142 (12.0)		28/171 (16.4)	26/26	7/26
Insect bats							
Chaerephon pumila	0/35	0/18		0/1	0/54		
Hipposideros caffer	0/5		0/9		0/15		
Hipposideros commersoni			0/16		0/16		
Miniopterus inflatus			1/34 (2.9)		1/34 (2.9)		
Miniopterus schreibersi	0/1				0/1		
Mops condylurus	3/19 (15.8)	11/96 (11.5)			14/115 (12.2)	8/9	5/9
Mops midas	0/15				0/15		
Myotis bocagei	0/1				0/1		
Nycteris argae			0/1		0/1		
N. thebaica	0/6				0/6		
Pipistrellus capensis	0/1				0/1		
Rhinolophus darlingi	0/1				0/1		
Rhinolophus landeri	0/2				0/2		
Rhinolophus fumigatus			1/204 (0.5)		1/204 (0.5)		
Scotophilus borbonicus	0/1				0/1		
S. dinganii	0/5				0/5		
Taphozous mauritianus	0/1				0/1		
Totals	14/128 (10.9)	11/120 (9.2)	20/422 (4.7)	2/35 (5.7)	47/705 (6.7)	36/37	12/37

*SARS-CoV, severe acute respiratory syndrome–associated coronavirus; SA, South Africa; DRC, Democratic Republic of Congo; WB, Western blot; IIFT, indirect immunofluorescence test.
†Serum specimens were screened for antibody by modification of a commercially available ELISA kit. Titers ranged from 1:50 to 1:800.
‡Confirmatory tests were performed by 2 WB analyses and IIFT when sufficient sample was available.

variations in experimental procedures and signal intensities. Serum specimens that produced signals at a dilution 1:5,000 were considered positive as none of the control serum specimens showed reactivity at that dilution. To evaluate assay specificity, we tested 19 control serum specimens comprising 12 randomly selected bat serum specimens that were negative by ELISA, 2 SARS-CoV–positive human serum specimens, and 5 SARS-CoV–negative human serum specimens, including 4 HCoV-NL63–positive serum specimens.

A commercial indirect immunofluorescence test (SARS-CoV-IFTII kit, EUROIMMUN AG) was carried out as described by the manufacturer, except that bat serum samples were diluted 1:100, and slides were incubated at room temperature for 2 hours. Reactions were detected with goat-antibat immunogolublin (Ig) (Bethyl) at a dilution of 1:1,000 and fluorescein isothiocyanate–labeled donkey-antigoat Ig (Dianova, Hamburg, Germany) at a dilution of 1:100. Specificity of the indirect immunofluorescence test (IIFT) was determined by screening 572 human serum specimens. The sensitivity and correlation of IIFT versus ELISA were analyzed (online Technical Appendix). In addition, the 19 selected control serum samples were tested.

Virus neutralization tests were performed as described elsewhere (*13*) except for using Vero E6 cells cultured in Dulbecco's modified Eagle medium and SARS-CoV Hong Kong isolate 6109 (3.25×10^7 PFU/mL, diluted 1:5,000). Bat serum dilutions in quadruplicate ranged from 1:10 to

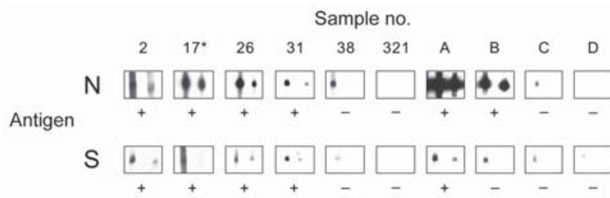

Figure 1. Results of Western blot analysis with recombinant severe acute respiratory syndrome–associated coronavirus (SARS-CoV) nucleocapsid (N) and spike (S) protein. Shown are examples for SARS-CoV ELISA–positive (2, 17, 26, 31) and –negative (38, 321) bat serum specimens tested using full-length recombinant SARS-CoV N and a fragment of the S protein (amino acids 318–510). Serum specimens were diluted 1:2,500 (left strips) and 1:5,000 (right strips). Secondary detection was performed by incubating the nitrocellulose strips with horseradish peroxidase (HRP)–labeled goat-antibat immunoglobulin (Ig) (Bethyl, Montgomery, AL, USA) (1:10,000). For chemiluminescence, SuperSignal Dura substrate (Pierce Biotechnology, Rockford, IL, USA) was added and films exposed for 1 min. Serum 17* was used as a reference for comparing blots. For evaluation purposes, strips were also incubated with human SARS-CoV–positive (A, B) and –negative serum specimens C and D (HCoV-NL63 positive) at the same dilutions, using goat-antihuman Ig HRP (1:20,000) for secondary detection. Serum specimens that produced signals at a dilution of 1:5,000 were recorded as positive (+).

1:320. After incubation at 37°C in 5% CO_2 for 3 days, the cells were fixed with 8% formaldehyde and results interpreted as described (*13*).

Viral RNA was extracted from serum by using a QIAamp viral RNA extraction kit (QIAGEN, Hilden, Germany), and reverse transcription–PCR (RT-PCR) was performed essentially as described elsewhere (*14*), with the exception that 140 µL was not available from every bat. In such cases, input volume was reduced and replaced with water. A minimum of 20 µL was usually tested.

Antibody activity to SARS-CoV antigen was detected by ELISA in 7 of 26 bat species tested at both collection sites with a seroprevalence of 6.7% (47/705). The highest prevalences were found in the fruit bat *Rousettus aegyptiacus* (Chiroptera: Pteropodidae) (16.4%) and the insectivorous bat *Mops condylurus* (Chiroptera: Molossidae) (12.2%) (Table). ELISA titers ranged from 50 (73% of the serum samples) to 800. Confirmatory WB analyses performed by 2 methods on ELISA-positive samples for which sufficient material remained available, were positive in 36 (97.3%) of 37 serum specimens, but IIFT was positive in only 12 (32.4%) of 37 samples (Table; Figures 1, 2; Figure in online Technical Appendix). None of the assays used detected antibodies to other human pathogenic coronaviruses (online Technical Appendix; Figures 1, 2). Neutralizing activity to SARS-CoV was not found in any of the ELISA-positive samples, and RT-PCR did not detect CoV nucleic acid in 262 serum specimens tested (data not shown).

Conclusions

The results of WB analyses support the specificity of the ELISA used in this study. The IF test is known to be less sensitive than ELISA but still provided confirmation in one third of the serum specimens tested. The negative results in the viral neutralization tests are not unexpected because this assay detects only antibodies that interfere with the specific entry mechanism of SARS-CoV, and putative group 4 CoVs from African bats may not use it. Moreover, deletions and mutations found in Asian bat SARS-like–CoV isolates lie in the S protein region essential for binding of SARS-CoV to the cellular receptor, angiotensin-converting enzyme 2, and thus are likely to affect cross-neutralization, as emphasized by conflicting results obtained in Asia (*6*,*7*,*15*). The negative findings obtained in RT-PCR can be explained by the unlikelihood of finding virus nucleic acid in serum. Studies in Asia used rectal swabs instead of serum samples, and the virus likely persists in the enteric tract but may not be found in serum at all.

Both bat species (*R. aegyptiacus* and *M. condylurus*) are widely distributed in Africa but vary in the degree of contact with humans. *R. aegyptiacus* roosts in caves but forages in orchards, whereas *M. condylurus* roosts in buildings. The results of this preliminary study suggest that some

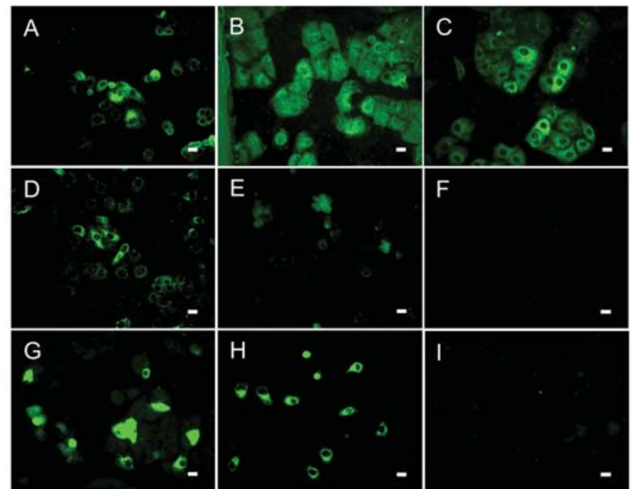

Figure 2. Results of indirect immunofluorescence test (IIFT) with Vero E6 cells infected with severe acute respiratory syndrome–associated coronavirus (SARS-CoV). The SARS-CoV diagnostic IIFT kit (EUROIMMUN AG, Lübeck, Germany) was used with minor modifications: bat and reference human serum specimens were diluted 1:100 (found to be the optimal dilution for bat sera) in sample buffer, and secondary detection was performed with goat-antibat immunoglobulin (Ig) (Bethyl, Montgomery, AL, USA) followed by fluorescein isothiocyanate (FITC)–labeled donkey-antigoat Ig (Dianova, Hamburg, Germany) (A–F) or FITC-labeled goat-antihuman Ig (G–I). Frames A–D, SARS-CoV ELISA–positive bat serum specimens 2, 17, 26, 31; E–F, ELISA-negative bat serum specimens 38 (showing unspecific signals) and 306; G–H, SARS-CoV–positive human control serum specimens A and B; I, negative human serum C. All photographs were taken at equivalent microscope settings. Scale bars represent 20 µm.

of the African bat species harbor agents related to putative group 4 CoV, and therefore further investigations should be undertaken to determine potential public health risks.

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Mr Müller is a scientist at the Center for Biological Safety of the Robert Koch-Institut, Berlin. He has been involved in establishing diagnostic tools for SARS-CoV detection, assisted international quality assurance studies on SARS diagnostics, and worked on SARS-CoV susceptibility studies. Currently he is finishing his PhD thesis on expression analysis of coronavirus structural proteins.

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Mokola Virus in Domestic Mammals, South Africa

Claude T. Sabeta,*† Wanda Markotter,† Debrah K. Mohale,* Wonderful Shumba,* Alexander I. Wandeler,‡ and Louis H. Nel†

We recently identified 2 Mokola viruses from domestic mammals (a dog and a cat) in South Africa. These cases occurred 8 years after the last reported case of infection with this virus. Our findings emphasize the endemicity of rabies-related lyssaviruses in South Africa and the need to better understand the epidemiology of Mokola viruses.

Mokola virus (MOKV) is classified as genotype (gt) 3 of the genus *Lyssavirus* in the family *Rhabdoviridae* (order Mononegavirales). Apart from MOKV, the genus *Lyssavirus* consists of 6 gts: classic rabies virus (gt1), Lagos bat virus (gt2), Duvenhage virus (gt4), European bat lyssavirus type 1 (gt5) and type 2 (gt6), and Australian bat lyssavirus (gt7). Some novel lyssaviruses identified in bat species in the former Soviet Union are considered putative gts within this genus (*1*).

Although gt1 viruses have a global distribution, gt5 and gt6 viruses are restricted to Europe and gt7 viruses are limited to Australia. Natural infections with gt2, gt3, and gt4 viruses have been found only in Africa. With the exception of MOKV, all lyssavirus gts and putative gts have been isolated exclusively or most frequently from chiropteran species. MOKV has never been isolated from these species, but only from terrestrial mammals. The first MOKV was isolated from shrews (*Crocidura* sp.) in Nigeria in 1968. Since then, ≥20 isolates of this lyssavirus have been found throughout Africa (Cameroon, Central African Republic, Ethiopia, South Africa, and Zimbabwe) (*2*–*12*) (Table 1).

We report the identification and characterization of 2 cases of infection with MOKV in South Africa. The first was in a domestic dog and is, to our knowledge, the first such case in South Africa. The second was in a domestic cat, the host species in which all previous isolates were found. The cat MOKV isolate belonged to 1 of 2 previously identified South African MOKV phylogenetic lineages, but the dog MOKV isolate appeared to have a different lineage not previously encountered in South Africa or elsewhere in Africa.

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The Study

In October 2004, a 3-month-old kitten (*Felis domesticus*) was adopted from the Society of the Prevention of Cruelty to Animals (East London, Eastern Cape Province, South Africa) and lived with its owner on a farm 23 km outside the city. It had been neutered and had been vaccinated at 10 months of age with an adjuvanted inactivated vaccine against rabies (Rabisin; Merial, Lyon, France), but no subsequent vaccinations were given. The cat spent most of the day indoors, but went out at night and returned in the morning. Unusual behavior was noticed in March 2006. It appeared dull and physically unbalanced and its pupils were dilated but it was not aggressive. The cat was humanely killed, and its brain was sent to the Onderstepoort Veterinary Institute for rabies testing.

On June 17, 2005, a 6-month-old puppy (*Canis familiaris*) was brought by its owner to a veterinarian in the rural town of Nkomazi (Mpumalanga Province, South Africa). The dog had a temperature of 39.8°C and no appetite. After symptoms were treated, the dog was discharged, but it was brought back 11 days later because it was paralyzed, dehydrated, and had a fixed stare. This animal had never been aggressive to other pets or humans. The dog was humanely killed, and its brain was sent to the Onderstepoort Veterinary Institute for rabies testing.

Direct immunofluorescent antibody test with an anti-rabies conjugate cross-reactive with African lyssaviruses showed numerous and strongly stained inclusion bodies in every field of impression smears of both brain samples. Isolation of virus was attempted by suckling mouse brain passage and cell culture (neuroblastoma cells; Diagnostic Hybrids, Athens, OH, USA); both methods were successful for the cat sample. However, neither method yielded an isolate from the dog sample, despite a lyssavirus-specific reaction in the original brain sample by direct immunofluorescent antibody test.

Subsequently, antigenic characterization was performed with a panel of 16 monoclonal antibodies to the nucleocapsid protein of rabies virus (Canadian Food Inspection Agency, Nepean, Ontario, Canada). Both samples showed reactivity patterns associated with MOKV (Table 2).

Final confirmation of MOKV in both case samples was obtained by reverse transcription–PCR, nucleotide sequencing, and phylogenetic analysis as described (*12*). Phylogenetic analysis (Figure) showed that the virus isolated from the cat sample (designated MOKV173/06) belonged to the same lineage of MOKV isolates that were recovered from cats in the same region of South Africa (*12*). However, the virus detected in the dog sample (designated MOKV404/05) appeared to represent a different South African MOKV lineage that was phylogenetically positioned between known South African and Zimbabwean lineages. This MOKV had nucleotide similarities of 88.1%–90.4%

Table 1. Moloka virus isolates identified in Africa

Location	Year of isolation	Species of origin	Reference
Ibadan, Nigeria	1968	Shrew (*Crocidura* sp.) (3 isolates)	(*2*)
Ibadan, Nigeria	1968	Human	(*3,4*)
Ibadan, Nigeria	1969	Shrew (*Crocidura* sp.)	(*3*)
Umhlanga Rocks, Kwazulu Natal Province, South Africa	1970 (identified in the 1980s)	Cat	(*12*)
Ibadan, Nigeria	1971	Human	(*3*)
Yaounde, Cameroon	1974	Shrew (*Crocidura* sp.)	(*5*)
Bangui, Central African Republic	1981	Rodent (*Lophuromys sikapusi*)	(*6*)
Bulawayo, Zimbabwe	1981	Dog (vaccinated) and cat (4 isolates)	(*7*)
Bulawayo, Zimbabwe	1982	Cat (2 isolates)	(*7*)
Addis Adaba, Ethiopia	1989–1990	Cat	(*8*)
Selous, Zimbabwe	1993	Cat	(*11*)
Mdantsane, Eastern Cape Province, South Africa	1995	Cat	(*9*)
East London, Eastern Cape Province, South Africa	1996	Cat	(*10*)
Yellow Sands, Eastern Cape Province, South Africa	1996	Cat (vaccinated)	(*10*)
Pinetown, Kwazulu Natal Province, South Africa	1997	Cat (vaccinated) (2 isolates)	(*10,12*)
Pietermaritzburg, Kwazulu Natal Province, South Africa	1998	Cat (vaccinated)	(*10,12*)
Nkomazi, Mpumalanga Province, South Africa	2005	Dog	This study
East London, Eastern Cape Province, South Africa	2006	Cat (vaccinated)	This study

and 85.3%–88.5% with viruses from Zimbabwe and South Africa, respectively.

Conclusions

Infections with MOKV are rare; only 23 isolates are known. During the past 2 decades, all MOKV isolates have been found in South Africa. Because these viruses are not exclusive to South Africa (*2–12*), lack of isolates from other regions of Africa indicates a lack of active surveillance and limited diagnostic capabilities in many African laboratories. To our knowledge, the 2 cases of infection with MOKV we report are the first in 8 years from South Africa. These cases suggest that other cases may not have been recognized. Clinical signs in the dog and cat, including general neurologic manifestations with a lack of aggression, are often signs that warrant submitting samples for rabies testing.

We have identified regional variations in the antigenic composition of MOKV. Whether these variations are caused by neutral genetic drift or reflect different epidemiologic features is not known. Phylogenetically, divergence of these viruses into different lineages indicates active

Table 2. Reactivity of virus isolates with 16 monoclonal antibodies to the nucleocapsid protein of rabies and rabies-related viruses*

Monoclonal antibody	Dog (gt1)	Mongoose (gt1)	Lagos bat (gt2)	Mokola (gt3)	Duvenhage (gt4)	MOKV404/05	MOKV173/06
26AB7	+	Var	–	–	–	–	–
26BE2	+	Var	–	–	–	–	–
38HF2 (positive control)	+	+	+	+	+	+	+
66–1C5 (negative control)	–	–	–	–	–	–	–
M1001	–	–	–	+	–	+	+
M1336	+	–	–	Var	–	–	–
M1349	Var	Var	–	Var	–	–	–
M1386	–	+	–	–	–	–	+
M1412	+	Var	–	–	–	–	–
M1494	Var	Var	–	–	+	–	–
M612	–	–	+	–	–	–	–
M837	–	–	–	–	+	–	–
M853	+	–	–	–	+	–	–
M856	+	–	–	–	+	–	–
M857	+	–	–	–	+	–	–
M879	+	–	–	Var	+	–	–

*gt, genotype; +, positive reactivity; Var, reactivity with some regional variants; –, negative reactivity.

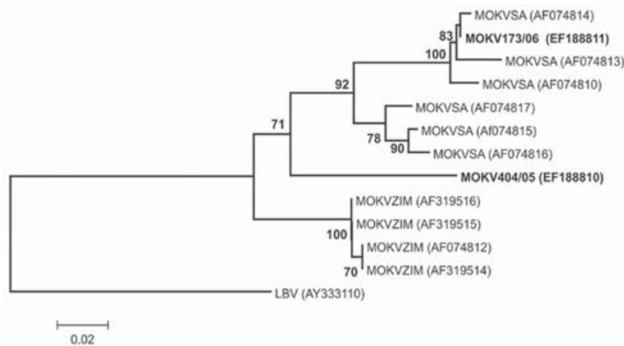

Figure. Phylogenetic tree based on 267 nt of partial nucleoprotein gene sequences of Moloka virus (MOKV) identified with the N1-N2 primer set as described (*12*). The tree shows phylogenetic positions of 2 recently identified cases of MOKV infection from South Africa (MOKV173/06 from a cat and MOKV404/05 from a dog) (in **boldface**) relative to previously characterized MOKV isolates from South Africa (SA) and Zimbabwe (ZIM) and Lagos bat virus (LBV) as the outgroup. GenBank accession nos. are shown in parenthesis. Bootstrap support values >70% are considered significant and indicated. Scale bar shows nucleotide substitutions per site.

cycles and evolutionary changes that occur independently, but in close proximity (a few hundred kilometers apart).

Although the epidemiology of MOKV is incomplete, the case for a reservoir host(s) among small terrestrial animals of limited range is supported by our findings. Together with recent isolations of rabies-related lyssaviruses in a human (*13*) and wild animals (*14*,*15*), these reports emphasize the endemicity of these lyssaviruses in South Africa. Public health implications of African rabies-related lyssaviruses should be recognized by laboratory workers, researchers, veterinarians, wildlife personnel, gamekeepers, and pet owners. A better understanding of the epidemiology of these viruses is vital and can only be achieved by improved surveillance and awareness.

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Dr Sabeta is a senior research scientist and head of the World Organisation for Animal Health Rabies Reference Laboratory in Onderstepoort, South Africa. His research interests include epidemiologic investigations of rabies and rabies-related viruses in southern Africa.

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Buruli Ulcer Surveillance, Benin, 2003–2005

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We reviewed Buruli ulcer (BU) surveillance in Benin, using the World Health Organization BU02 form. We report results of reliable routine data collected on 2,598 new and recurrent cases from 2003 through 2005.

Buruli ulcer (BU), a disease caused by *Mycobacterium ulcerans*, is one of the recently classified 13 neglected tropical diseases (*1*). It has been reported in >30 countries (*2*). The disease starts as a nonulcerative lesion-like nodule, plaque, or edema. Without treatment, these early lesions will progress to an ulcer. The disease affects the bone in an estimated 13% of patients (*3*). Treatment often requires multiple interventions, including specific antimicrobial agents, surgery, and physiotherapy. Recurrence is high in many countries (*4*,*5*), and the economic effects on affected households, communities, and the health system are considerable (*6*,*7*). The exact mode of transmission of the causative organism is not known. The only known risk factors are related to water, particularly the use of unsafe water (*8*), especially that obtained from swamps (*9*).

We describe Benin's surveillance system for BU from 2003 through 2005. The system is based on the use of the World Health Organization (WHO) BU02 form.

The Study

The study took place in Benin, West Africa. The BU control activities are organized by a National Control Programme. Five BU Detection and Treatment Centers (CDTUB) are distributed throughout the BU-endemic regions. The detection, referral, and follow-up of BU cases rely heavily on community-based surveillance teams composed of village volunteers and 1 or 2 teachers and supervised by health workers from the nearest health facility.

The BU02 form acts as a triple registry. A trained nurse registers each case on the form. Each quarter, the completed first sheet is sent to the national level. The second sheet is sent to the regional level, and the third is kept at the CDTUB for local analysis. A training workshop is performed annually for the surveillance team. At the national level, data are computerized for analysis and mapping, and feedback is provided annually at a review meeting with all BU management participants.

With the use of this system, from January 1, 2003, through December 31, 2005, a total of 2,598 new and recurrent cases were reported and treated in Benin (Tables 1 and 2). The rates of disease recurrence (6%) were much lower than the figures reported in other countries, e.g., 16% in Ghana (*4,5*). Euverte found a rate of 3% recurrence among 103 patients treated with streptomycin and rifampin in Oueme, Benin, in 2005 (*6*). During the same period, the total numbers of leprosy and tuberculosis cases were 1,163 and 8,556, respectively. Thus, BU has become the second most important mycobacterial disease after tuberculosis in some endemic countries, including Benin (*3*) and Ghana (*7*).

Consistent with other studies (*10*), our study found that 51% of the 2,598 cases were in children <15 years of age. Cases were equally distributed between male (49.7%) and female (50.3%) patients.

Of the total case-patients, 1,644 (63.3%) reported lesions on their lower limbs; 524 (20.2%), lesions on their upper limbs; 231 (8.9%), lesions on their head, neck, or trunk; 19 (0.7%), lesions in the perineal region; and 160 (6.2%), lesions in multiple areas. The location of a lesion was not noted on the BU02 form for 20 (0.8%) case-patients.

Many researchers believe that because legs and arms are the most exposed parts of the body they are more likely to be injured or to be bitten by an insect that may be associated with transmission of *M. tuberculosis*. However, why some lesions occur in the perineum, which is the least exposed area, remains unclear. In some villages, persons take baths in the swamps while carrying out domestic activities such as washing clothes or dishes. Lesions around the head, neck, and trunk were present in 9% of patients and in the perineum in almost 1%. Although these percentages are small, managing the technical and cosmetic aspects of lesions in the head, neck (*11*), and perineal regions (*12*) is difficult in Benin, where plastic surgeons are not available.

Nonulcerative early lesions (nodule, edema, and plaques) occurred in 27% of the total cases. Ulcers and mixed forms (an ulcer and some other form of the disease) occurred in 72% of the cases, and single ulcerative lesions occurred in 54%. The clinical form was not properly re-

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Table 1. Monthly trends for Buruli ulcer cases, Benin, 2003–2005

Year	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total (DR/10,000 inhabitants)*
2003	59	62	48	49	52	63	79	44	41	93	64	77	731 (1.56)
2004	86	60	87	57	75	73	77	56	44	77	60	70	822 (1.73)
2005	72	89	89	91	93	100	77	94	73	88	97	82	1,045 (2.13)
Total	217	211	224	197	220	236	233	194	158	258	221	229	2,598

*DR, detection rate.

corded for 2% of cases. Our figure of 72% is lower than the 94% rate reported elsewhere for Benin from 1989 through 1996 (*13*). The real challenge in Benin is how to further reduce the percentage of ulcers and sustain such surveillance efforts.

Regarding infection involving bone, Debacker et al. (*3*) reported a rate of 13% among 1,700 patients treated at CDTUB Zagnanado from 1997 through 2001. However, our results showed that bone involvement occurred in 6% of cases. Bone infection is a consequence of late disease (*14*). As progress is made in reducing late disease, bone lesions should be reduced.

Laboratory confirmation of BU is not frequently performed before treatment is begun. Although WHO strongly recommends laboratory confirmation of cases, in practice not all cases require it. Our study shows that 50% of cases are confirmed by at least 1 laboratory method under routine conditions.

The geographic distribution of cases shows that the BU-endemic areas are confined to the southern half of the country, Most BU-endemic villages occur along the Oueme and Couffo Rivers (online Appendix Figure 1, available from www.cdc.gov/EID/content/13/9/1374-appG1.htm, and Appendix Figure 2, available from www.cdc.gov/EID/content/13/9/1374-appG2.htm).

The Mono Region has the lowest incidence of BU in southern Benin. By contrast, the other BU-endemic regions are around rivers. This observation cannot be due to insufficiency of reporting because there is a CDTUB in the area and surveillance is good (online Appendix Figure 1). Unlike previous reports from Benin, our results suggest that the Oueme Region is now the most endemic for BU, not the Zou Region (online Appendix Figure 1). We believe that this finding may be due to the active community-level detection and antimicrobial drug treatment conducted by the new BU center established in the Ouémé/Plateau region in April 2004.

Conclusions

The data provided by Benin's BU surveillance system that used the BU02 form enabled the BU Program in Benin to reliably describe the epidemiologic situation, evaluate the results of actions, measure the results of the centers, and plan future interventions. The collected data are ≈98% complete. We conclude that the BU surveillance system is useful to the BU Program in Benin. Because the BU02 form has 3 parts, data can be submitted from the field without the difficulties of photocopying the pages of the register or entering the data in a computer, which may be problematic at a rural facility level. However, training and supervision of health workers are required.

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Table 2. Buruli ulcer cases reported in Benin by region, 2003–2005*

Region	2003, no. (%)	2004, no. (%)	2005, no. (%)	Total, no. (%)
Atlantique	171 (23)	171 (21)	263 (25)	605 (23)
Collines	2 (0)	0	0	2 (0)
Couffo	89 (12)	107 (13)	128 (12)	324 (12)
Littoral	8 (1)	18 (2)	31 (3)	57 (2)
Mono	14 (2)	13 (2)	20 (2)	47 (2)
Oueme	275 (38)	252 (31)	304 (29)	831 (32)
Plateau	26 (4)	43 (5)	79 (8)	148 (6)
Zou	124 (17)	201 (24)	198 (19)	523 (20)
Nigeria	4 (1)	3 (0)	6 (1)	13 (1)
Togo	2 (0)	2 (0)	1 (0)	5 (0)
Not specified	16 (2)	12 (1)	15 (1)	43 (2)
Total	731 (100)	822 (100)	1,045 (100)	2,598 (100)

*Benin surveillance captures data from the neighboring countries of Nigeria and Togo.

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Equine Rhinosporidiosis in United Kingdom

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We report 4 cases of equine rhinosporidiosis in the United Kingdom. These cases provide evidence of spread of infectious agents from rhinosporidiosis-endemic areas to nonendemic areas by increased international movement of livestock. Surveillance should continue for this infective agent of potential relevance for numerous species, including humans.

Rhinosporidiosis is caused by *Rhinosporidium seeberi*, an organism that was previously classified as a fungus but has been regrouped into the class Mesomycetozoa (family Rhinosporideacae). This class consists of several parasitic and saprophytic organisms, most of which infect fish and amphibians; only *R. seeberi* infects mammals (*1*,*2*). Rhinosporidiosis is endemic to India and Sri Lanka, although cases have been reported in Africa, the Americas, and Europe (*3*). Most affected patients have a history of temporary or permanent residence within rhinosporidiosis-endemic areas. Rhinosporidiosis is predominantly a human disease; however, it has been documented in many other species, including cats, dogs, cattle, and waterfowl (*4*). Equine cases are infrequent but have been reported from the southern United States (*5*), South America (*6*), and South Africa (*7*). The first equine case (1 of those detailed in this article) in the United Kingdom was recently reported (*8*).

The natural habitat of *R. seeberi* is thought to be stagnant or lacustrine water, although isolation of the organism from such environments has so far been unsuccessful (*9*). Nonetheless, epidemiologic evidence supports this hypothesis; the only report of an outbreak originating within Europe was associated with persons bathing in a lake in Serbia (*10*). Because the typical location of *R. seeberi*–associated lesions in all species is the nasal mucosa, drinking from contaminated water is likely the source of infection (*11*), possibly through superficial wounds in the mucosa. In addition, for ocular disease, dust particles are possible fomites for endospores (*4*). Rhinosporidiosis commonly causes single or multiple, sessile or pedunculated, papillomatous, polypoidal or compact masses within the nasal mucosa or, less frequently, the ocular mucosa. These masses are painless, slow-growing, and noninfiltrating. Surgical excision is the treatment of choice (*4*).

The Study

We describe 4 cases of rhinosporidiosis in polo ponies imported into the United Kingdom from Argentina and kept in different locations. Diagnoses were made over a 6-month period by routine histopathologic examination at 3 diagnostic centers. For confirmation of the causative agent, DNA was extracted from biopsy samples and skin of an unaffected horse (negative control) by using a commercially available kit (DNeasy Tissue Kit, QIAGEN Ltd., Crawley, West Sussex, UK) according to the manufacturer's protocol. *R. seeberi*–specific primers for the 18S rDNA sequence (*3*) were used in a PCR, and the *R. seeberi*–specific amplification product from 1 sample was sequenced by Lark Technologies (Takeley, Essex, UK), as described (*8*).

The ponies had clinical signs such as epistaxis, or they had been asymptomatic and a lesion was noticed during routine examination. One pony had a clinical history of epistaxis that first occurred 10 months after the animal had been imported. On gross examination, friable soft tissue masses, located unilaterally or bilaterally within the nasal mucosa, were observed. For all 4 ponies, histologic examination showed moderate multifocal hyperplasia and ulceration of the mucosa. Within the expanded mucosa, and particularly within the lamina propria mucosae, multiple spherical to polygonal organisms of variable appearance, consistent with *R. seeberi,* were seen (*6*). The smaller ($\leq$100 μm in diameter) structures had an eosinophilic and periodic acid Schiff–positive wall enclosing eosinophilic to basophilic fibrillar material (juvenile sporangia; Figure 1, panel B). The larger ($\leq$300 μm in diameter), spherical to polygonal structures had a thin eosinophilic wall with closely opposed basophilic stippled material and basophilic and eosinophilic ovoid structures (endospores) within the central lumen (mature sporangia; Figure 1, panel A). Surrounding these sporangia was a mild to moderate, multifocal, lymphoplasmacellular inflammatory infiltrate (Figure 1, panel B). Additionally, mild to marked multifocal pyogranulomatous infiltrates, most commonly associated with free endospores from ruptured mature sporangia, were noted. Mild hyperemia, mild multifocal hemorrhage, and mild multifocal hemosiderosis were also present.

PCR amplification using *R. seeberi*–specific primers provided bands of the expected size (377 bp [*3*]) in 3 of the 4 samples. PCR with primers for the housekeeping β-actin gene produced bands of the expected size in the same 3

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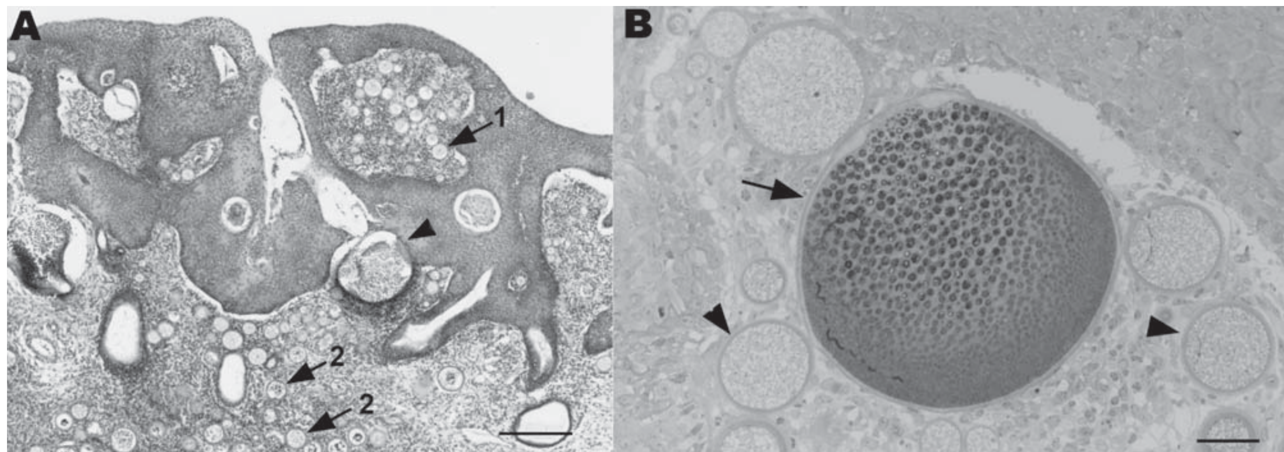

Figure 1. A) Section of nasal mucosa showing multifocal hyperplasia, juvenile sporangia (arrows) within the mucosal epithelium (1) and the lamina propria mucosae (2), and mature sporangia (arrowhead). A multifocal mixed inflammatory infiltrate can be seen within the mucosa. Stain, hematoxylin and eosin; magnification ×4; scale bar, 250 μm. B) Semithin section of nasal mucosa with juvenile sporangia (arrowheads) and a mature sporangium (arrow) with a lymphoplasmacellular inflammatory infiltrate within the lamina propria mucosae. Stain, toluidine blue; magnification ×10; scale bar, 40 μm.

samples and the noninfected control (Figure 2). Sequencing of 1 product was consistent with the published sequence for *R. seeberi* (*1*,*3*,*8*).

Conclusions

This report describes what we believe to be the first veterinary cases of rhinosporidiosis in the United Kingdom. The 4 affected polo ponies had all been imported from Argentina. Rhinosporidiosis is endemic throughout India and Sri Lanka, and disease-endemic foci are found in Uganda, the United States (Texas), Brazil, and Argentina (*4*). Within Argentina, rhinosporidiosis-endemic areas include the Rio Parana and the Rio de la Plata (*12*). Unfortunately, we could not obtain information about where in Argentina the affected polo ponies originated.

The morphologic features of the agent seen in all cases presented here are characteristic, and diagnostic, for *R. seeberi* (*13*). However, potential differential diagnoses that should be considered include polypoid or granulomatous rhinitis caused by fungal infection with *Coccidioides immitis* or *Chrysosporium parvum* (the causative agent of adiaspiromycosis) and neoplasia (*11*,*13*). The result of the *R. seeberi*–specific PCR and the sequencing of 1 of the amplification products provides definitive proof that *R. seeberi* was the infective organism in 3 of the 4 samples. Because the fourth sample did not yield a product with the *R. seeberi*–specific primers and also did not yield an amplification product in a PCR for the housekeeping β-actin gene, DNA extraction was likely not successful for this sample. Such extraction failure could result from prolonged formalin fixation and paraffin embedding because these techniques can have a profound effect on the molecular arrangement of DNA and may inhibit its amplification and extraction (*14*).

The treatment of choice for rhinosporidiosis is surgical excision of lesions. In humans, lesions have been found to recur after surgery in 11% of cases, possibly because of incomplete excision or intraoperative contamination of adjacent surfaces with endospores (*4*). To prevent recurrence, electrocauterization at the site of excision is recommended. Of the ponies in this study for which the outcome is known, excision was curative for 1, but recurrence of clinical signs has occurred in another, most likely due to incomplete excision. Pharmacologic treatment has not been successful, probably because of the impenetrability of the sporangial wall (*4*).

International movement of horses, particularly for competition, is now commonplace. Such travel increases risk for exposure to diseases and pathogens not usually encountered in the importing country. With regard to the United Kingdom, ≈1,000 polo ponies are imported from Argentina every year (A. Wardall, pers. comm.), and other ponies come from New Zealand, Australia, and the United States. Thus, a relatively large number of potentially exposed ponies are imported into the United Kingdom each year. For *R. seeberi*, the possibility of a prolonged incubation period before development of clinical signs (epistaxis in 1 pony reported here did not occur until 10 months after importation) could lead to introduction of this infective agent, because lesions may not be apparent at the time of importation. Direct transmission of *R. seeberi* between humans and animals has not been proven (*4*); furthermore, multiple host-specific strains may exist (*15*). However, because an outbreak was connected with a single body of water in Europe (*10*), infected animals imported into non–rhinosporidiosis-endemic areas may contaminate such water and lead to further, autochthonous outbreaks.

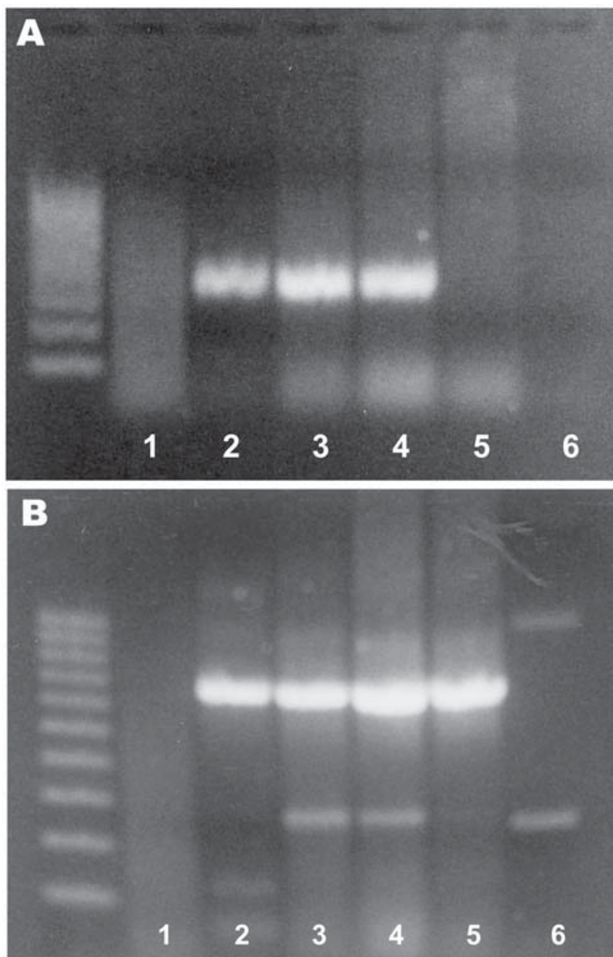

Figure 2. Agarose gel electrophoresis of PCR products from *Rhinosporidium seeberi*–specific primers (A) and β-actin primers (B). The left lane contains a 100-bp ladder. Samples 1–4, from horses with histologic diagnoses of rhinosporidiosis; sample 5, from the skin of a noninfected horse; sample 6, negative control (water).

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Poultry Drinking Water Used for Avian Influenza Surveillance

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Samples of drinking water from poultry cages, which can be collected conveniently and noninvasively, provide higher rates of influenza (H9N2) virus isolation than do samples of fecal droppings. Studies to confirm the usefulness of poultry drinking water for detecting influenza (H5N1) should be conducted in disease-endemic areas.

Pandemic influenza originates from influenza viruses of birds (*1*). Live poultry markets play a crucial role in maintenance, amplification, and dissemination of avian influenza viruses (*2–4*) and are a risk factor for zoonotic transmission of highly pathogenic avian influenza (H5N1) viruses to humans (*5,6*). Maintaining surveillance of live poultry markets for influenza viruses is therefore important. In routine surveillance of live poultry markets, handling birds for collecting tracheal or cloacal swabs is often unacceptable to the bird sellers. Because avian influenza viruses were believed to be transmitted primarily by the oral–fecal route (*7*), fecal droppings were therefore regarded as the noninvasive specimen of choice for surveillance purposes (*8*). However, emerging evidence from experimental studies indicates that H9N2 (*9*) and H5N1 (*10*) subtypes are shed in higher titers in the upper respiratory tract. We tested the hypothesis that sampling drinking water is a convenient, noninvasive, and sensitive method for conducting avian influenza surveillance in live poultry markets. Because vaccine-derived Newcastle disease virus (NDV) is also commonly isolated from poultry in Hong Kong, we used NDV isolation rates for comparison.

As part of our ongoing surveillance in live poultry markets in Hong Kong, 51–67 poultry stalls in 8 poultry markets were sampled monthly from August 2004 through July 2005. Typically, several poultry of the same type share a cage, and all birds in the same cage share a drinking water trough, which is intermittently filled from the municipal water supply. We collected paired samples: drinking water from the water trough supplying a cage and a fresh fecal dropping from the tray under that same cage. Because the numbers of minor poultry (poultry other than chickens) sampled during this period were smaller, we included additional data (413 paired specimens collected from August 2005 through November 2006) obtained from cages holding silkie chickens, guinea fowls, pigeons, chukars, and pheasants.

One fresh fecal swab and 0.5 mL of the drinking water were collected from each cage. The fecal dropping represents a sample from 1 bird, in contrast to the drinking water trough, which was shared by all the birds in the cage. A total of 2,503 specimen pairs were collected. The fecal sample swab and water sample were separately put into 1 mL of virus transport medium containing M199 (9.5 g/L), penicillin G (2×10^6 U/L), polymyxin B (10×10^6 U/L), gentamicin (2,500 mg/L), nystatin (0.5×10^6 U/L), ofloxacin HCl (100 mg/L), and sulfamethoxazole (1 g/L). A 200-µL aliquot of each sample was inoculated into a 9- to 11-day-old embryonic egg and incubated at 37°C for 3 days. Harvested allantoic fluid was tested for hemagglutination by using turkey erythrocytes. Hemagglutination-positive isolates were subtyped by using hemagglutination inhibition and neuraminidase inhibition tests with reference antiserum (*11*).

Of the 2,503 specimen pairs, influenza (H9N2) was isolated from 207 chickens (overall isolation rate 8.3%), 24 fecal samples alone (isolation rate 1.0%), 174 drinking water samples alone (7.0%), and 9 fecal and drinking water pairs (0.4%) (Table 1). The isolation rate for fecal samples was significantly lower than that for drinking water samples ($p<0.001$). The influenza (H9N2) isolation rates in drinking water and fecal droppings for silkie chickens were 5.8% and 0.6%, respectively ($p = 0.005$); for pigeons these rates were 3.8% and. 0%, respectively ($p = 0.01$). Isolation rates from pheasant gave a similar trend, although the results were not statistically significant ($p = 0.11$). The specimen numbers from guinea fowl and chukars were too small to be meaningfully analyzed.

In contrast, the isolation rate for NDV in chickens, silkie chickens, guinea fowl, and chukars did not show a comparable result (Table 2). In fact, for most of these species, the isolation rate from fecal samples was higher than that from drinking water samples, although these differences were not statistically significant.

To estimate survival of influenza (H9N2) in water troughs, we inoculated subtype H9N2 into a trough containing drinking water taken from a poultry cage (i.e., a trough containing some organic debris rather than chlorinated water directly taken from the municipal supply). The virus titer soon after inoculation was $10^{3.3}$ 50% egg infectious doses/mL of water, which is comparable to the titer of virus in subtype H9N2–infected water troughs in the retail

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Table 1. Avian influenza (H9N2) virus in chickens (August 2004–July 2005) and minor poultry (August 2004–November 2006)*

	No. pos/no. tested (% pos)			
Species	Overall isolation rate	Feces only	Drinking water only	Feces and drinking water
Chicken	207/2,503 (8.3)	24/2,503 (1.0)	174/2,503 (7.0)	9/2,503 (0.4)
Minor poultry				
Silkie chicken	10/171 (5.8)	0/171 (0)	9/171 (5.3)	1/171 (0.6)
Guinea fowl	1/13 (7.7)	1/13 (7.7)	0/13 (0)	0/13 (0)
Pigeon	6/158 (3.8)	0/158 (0)	6/158 (3.8)	0/158 (0)
Chukar	1/23 (4.3)	1/23 (4.3)	0/23 (0)	0/23 (0)
Pheasant	10/48 (20.8)	2/48 (4.2)	7/48 (14.6)	1/48 (2.1)

*Pos, positive for avian influenza (H9N2) virus.

market setting (unpub. data). Virus could be isolated from the water trough at 8, 12, 24, and 48 h postinoculation but not at 56 or 72 h postinoculation. When the experiment was repeated with fresh tap water or distilled water, virus remained viable for 8 and 12 h, respectively. This finding suggests that virus may survive in drinking water troughs for 8–48 h, perhaps depending on level of chlorination and organic content of the water.

During our study, only subtype H9N2 viruses were isolated from Hong Kong's poultry markets. The results from these field epidemiologic studies are compatible with data from experimental infection of poultry with subtype H9N2 viruses (*9*). Because titers of subtype H5N1 virus were higher in tracheal swabs than in cloacal swabs from ducks and other birds (*10,12*), subtype H5N1 virus isolation rates will likely be higher in drinking water than in fecal swabs, but this needs to be confirmed in studies conducted in regions where influenza (H5N1) is endemic. In contrast, NDV-infected chickens are reported to have virus detectable by reverse transcription–PCR for a longer period in the feces rather than the lungs (*13*), a finding consistent with our findings in live poultry markets.

The endemicity of highly pathogenic influenza (H5N1) in poultry in many countries across Asia and the continued detection of zoonotic transmission to humans, sometimes in regions where poultry outbreaks have not been reported, highlight the importance of systematic surveillance in live poultry markets. Systematic surveillance is especially important in regions where use of subtype H5 poultry vaccine is widespread. Whenever such studies have been conducted, previously unsuspected levels of virus activity have been found (*14*). Therefore, conducting such studies more widely, especially in areas known to be affected by subtype H5N1, is crucial. Such studies are the only way to determine the extent of virus transmission. They will also suggest potential interventions in the live poultry market systems that may effectively interrupt virus transmission in poultry; such interventions have been implemented in Hong Kong (*15*).

Our results provide evidence that taking samples from poultry drinking water troughs is an efficient way to conduct avian influenza surveillance. However, some caveats need to be noted. Drinking water potentially samples all the birds in a cage, whereas a fecal swab represents a single bird. Although the possibility for cage-to-cage transmission by infected water remains, NDV serves as a useful comparison in this regard. Different subtypes of avian influenza may have different shedding patterns from the respiratory tract compared with feces, and this strategy may not be applicable to all subtypes. Therefore, fecal droppings (or cloacal swabs) should also be collected. With these caveats accepted, sampling water from the drinking water troughs in poultry cages at live poultry markets and also at farms is likely to be a convenient, noninvasive, and practical strategy for implementing avian influenza surveillance for subtype H9N2 and perhaps also subtype H5N1; this approach should be evaluated in influenza-endemic regions.

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Table 2. Newcastle disease virus in chickens (August 2004–July 2005) and minor poultry (August 2004–November 2006)*

	No. pos/no. tested (% pos)			
Species	Overall isolation rate	Feces only	Drinking water only	Feces and drinking water
Chicken	95/2,503 (3.8)	53/2,503 (2.1)	33/2,503 (1.3)	9/2,503 (0.4)
Minor poultry				
Silkie chicken	8/171 (4.7)	6/171 (3.5)	2/171 (1.2)	0/171 (0)
Guinea fowl	2/13 (15.4)	2/13 (15.4)	0/13 (0)	0/13 (0)
Pigeon	4/158 (2.5)	2/158 (1.3)	2/158 (1.3)	0/158 (0)
Chukar	1/23 (4.3)	1/23 (4.3)	0/23 (0)	0/23 (0)
Pheasant	0/48 (0)	0/48 (0)	0/48 (0)	0/48 (0)

*Pos, positive for Newcastle disease virus.

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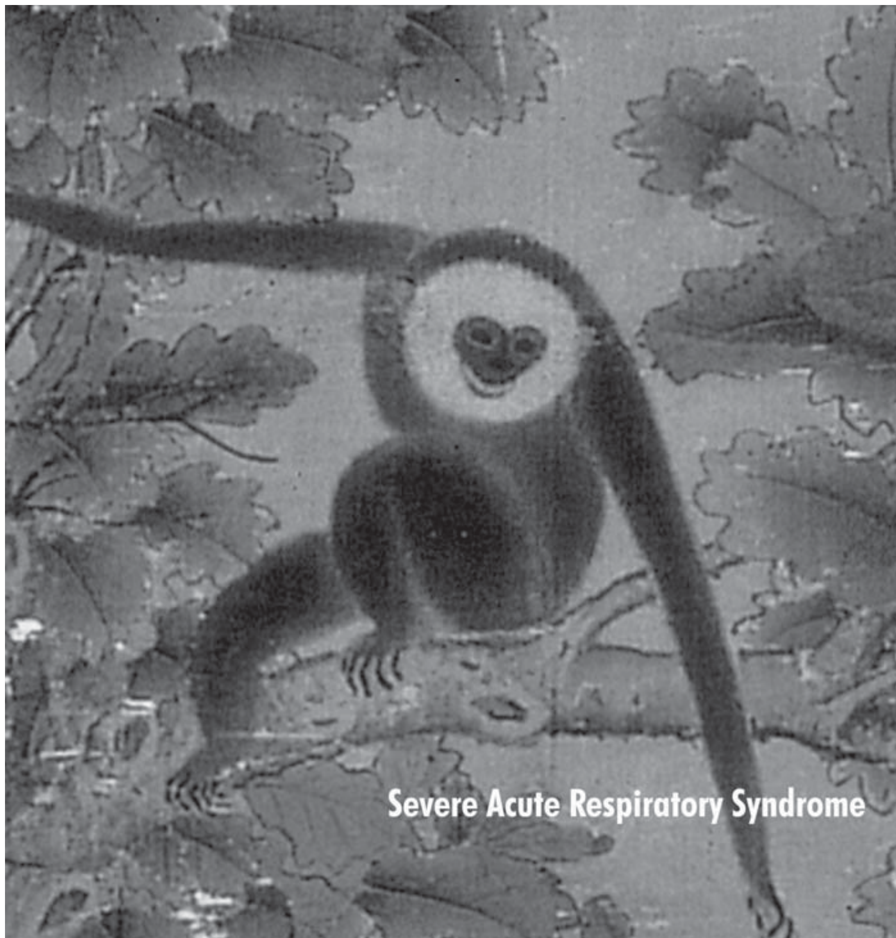

Endocarditis in Cattle Caused by *Bartonella bovis*

Renaud Maillard,* Elisabeth Petit,† Bruno Chomel,‡ Caroline Lacroux,§ François Schelcher,§ Muriel Vayssier-Taussat,† Nadia Haddad,* and Henri-Jean Boulouis*

This study aimed to determine the role of *Bartonella* as an endocarditis agent in cattle. *Bartonella bovis* was identified by PCR, gene sequences analysis, and specific internal transcribed spacer amplicon product size in 2 bovine endocarditis cases with high antibody titers, which demonstrates that *B. bovis* is a pathogen for cattle.

Bacteria-induced vegetative valvular endocarditis is one of the main cardiac disorders in adult cattle (*1*). The prevalence of endocarditis may reach 5.2 cases per 10,000 cows (*2*), but the disease is often misdiagnosed and only discovered during the slaughtering process or at necropsy. Bacterial endocarditis is often linked to a primary source of infection and the presence of other infectious lesions, such as mastitis, metritis, arthritis, or liver abscesses. The most frequent pathogens isolated from cardiac valves or the bloodstream of cows with endocarditis are *Arcanobacterium pyogenes* (up to 90% of the strains), *Streptococcus* sp., and numerous *Enterobacteriaceae* (*2*).

The development of molecular techniques (PCR) led to the identification of many noncultivable or poorly cultivable bacteria as agents of human endocarditis, such as *Coxiella burnetii,* different *Bartonella* species, or *Tropheryma whipplei* (*3*,*4*). In dogs, *Bartonella* species cause 8% of all bacterial endocarditis and up to 19% of noncultivable bacterial endocarditis (*5*). In cats, bacterial endocarditis is infrequent, but a fatal case caused by *B. henselae* was recently reported (*6*). Bartonellae can therefore be considered as potential agents of endocarditis even in their own reservoir species. Cattle host *B. bovis*, which has also been isolated from cats (*7*) and was recently suggested as the etiologic agent in a human case of bartonellosis (*8*). However, the role of *Bartonella* species in bovine endocarditis has never been explored. Therefore, our objective was to determine the putative role of *Bartonella* sp. as an agent of endocarditis in cattle.

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The Study

Twenty-two cases of bovine endocarditis were diagnosed in adult cows (ages 5–15 years, mean 7.4 years) at the School of Veterinary Medicine, Toulouse, France, from September 2004 through June 2006. Eighteen cows were hospitalized for poor condition, anorexia, weight loss, wasting syndrome, and abnormal cardiac auscultation. Endocarditis was diagnosed at physical examination. Lesions of the cardiac valves were confirmed at necropsy for all 18 animals. Four additional cases of endocarditis were identified at necropsy after an apparent sudden death. Most of the damaged valves of these 22 animals had large, cauliflower-like lesions.

For each cow, fragments of the vegetative valve and of 1 normal-appearing valve were collected. DNA extraction from each valve sample was performed by using Nucleospin Tissue extraction kit (Macherey-Nagel, Hoerdt, France) according to the supplier's recommendation.

PCR amplification was performed on all normal-appearing and vegetative valves for the hypervariable V3 zone of the eubacterial 16S rRNA detection, and the 3′ end of citrate synthase gene (*gltA*) *Bartonella* sp. DNA detection. Additional PCR amplification was performed on *gltA*-positive valves for the following *Bartonella*-specific genes or genomic region: *rpoB*, *ribC*, *groEL*, internal transcribed spacer (ITS) of 16S–23S rRNA (*9*,*10*). Amplification products, including those for the 16S rRNA, were subsequently sequenced.

Serology by indirect fluorescent antibody assay (IFA) was performed as reported elsewhere (*11*) on the supernatant extracted from a cardiac blood clot from each cow. Vero cells infected with the type strain of *B. bovis* (CIP 106692[T]), *B. chomelii* (CIP 107869[T]), and *B. schoenbuchensis* (NCTC 13165[T]), respectively, were used as antigens.

The 22 vegetative valves included 8 pulmonary valves, 7 tricuspid valves, 6 aortic valves, and 1 mitral valve. The only vegetative mitral valve and 1 of the 6 aortic vegetative valves showed positive results for *Bartonella*-specific *gltA* gene amplification. For both cows, the normal-appearing control valve was PCR negative for this gene. The PCR-positive cows (nos. 04–927 and 05–1406) were the 2 oldest cows (13 and 15 years old, respectively). ITS 16–23 rRNA amplification was obtained only for the damaged valve of cow 04–927; the size of the amplicon product was ≈190 bp, which was identical to the size of the product obtained with the *B. bovis* reference strain (Figure). Amplification of the 16S rRNA and all the other genes studied were PCR positive for the damaged valves. A 16S rRNA PCR amplicon has been obtained from the normal-appearing valve of cow 05–1406 but could not be sequenced. Amplification of all the other genes studied were PCR negative for normal-appearing control valves of both cows. The genes *rpoB*

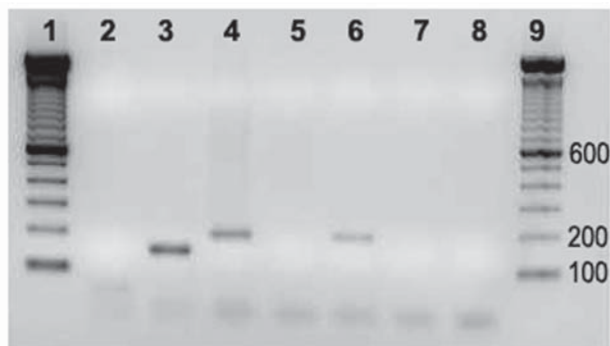

Figure. PCR amplification of internal transcribed spacer 16S–23S on vegative and normal-appearing valves of cows 04–927 and 05–1406. 1, Molecular weight marker; 2, negative control; 3, *Bartonella quintana*; 4, *B. bovis*; 5 and 6, normal appearing and vegetative valves (Cow 04–927); 7 and 8, normal appearing and vegetative valves (Cow 05–1406); 9, molecular weight marker.

(GenBank accession nos. EF432062, EF432061), *ribC* (accession nos. EF432060, EF432059), *gltA* (accession nos. EF432055, EF432056), *groEL* (accession nos. EF432058, EF432057) were partially sequenced. Sequence identities were respectively 100% with *B. bovis* (*gltA*) and 99% with *B. bovis* (*groEL*, *ribC*, *rpoB*). The sequence obtained with 16S rRNA (accession no. EF432054) had a 99% identity with *B. bovis*.

None of the cultures on rabbit blood agar (*12*) of fragments from the 22 vegetative valves yielded *Bartonella* isolates. The 2 PCR-positive cows had high IFA titers (5,120 and 640 for *B. bovis* antigen), whereas the 20 PCR-negative cows had low or negative titers (Table)

Conclusions

This is the first description, to our knowledge, of endocarditis associated with *Bartonella* in cattle. PCR amplification of the *gltA* gene, used for identification of *Bartonella* infection, gave an identity of 100% with the previously reported *B. bovis* gene sequence. The sequences of 4 additional genes (*groEL, ribC, rpoB,* and 16S rRNA) shared 99% identity with *B. bovis* genes. ITS amplification of 1 vegetative valve gave a fragment of ≈190 bp, which is the size expected for *B. bovis* (*10*). The lack of PCR amplification of the same genes from healthy-appearing valves indicated that the PCR amplification obtained with the vegetative valves was not the result of a *B. bovis* bacteremia. No definitive evidence exists that *B. bovis* had induced the primary lesion leading to the endocarditis. However, PCR amplification with universal primers for bacterial 16S rRNA allowed us to identify only *Bartonella* sequence in the damaged valves without apparent contamination with DNA from other bacteria.

Moreover, the high IFA antibody titer against *B. bovis* antigen and the low antibody titers of the PCR-negative endocarditis cases reinforced the likely role of *B. bovis* as the causative agent of these 2 bovine endocarditis cases. High antibody titers are commonly observed in human

Table. Serologic and PCR results for the vegetative heart valves from 22 cows*

Case no.	Age, y	Valve	*Bartonella* PCR (*gltA*)	Indirect fluorescent antibody titer		
				B. bovis	*B. chomelii*	*B. schoenbuchensis*
05–1406	13	Aortic	+	5,120	320	640
04–927	15	Mitral	+	640	160	160
05–379	8	Aortic	–	40	–	–
04–269	7	Aortic	–	–	–	–
4071	3.5	Pulmonary	–	40	40	80
3977	5.5	Aortic	–	–	–	–
556	8.5	Pulmonary	–	80	40	–
5988	6	Pulmonary	–	–	–	–
1507	6.5	Tricuspid	–	–	–	–
766	12	Tricuspid	–	–	–	–
4002	5	Tricuspid	–	–	–	–
4815	8	Pulmonary	–	–	–	–
4768	5	Pulmonary	–	–	–	–
4921	6	Tricuspid	–	–	–	–
4784	5.5	Aortic	–	–	–	–
239	10	Pulmonary	–	–	–	–
269	7	Aortic	–	–	–	–
304	12	Pulmonary	–	40	40	40
528	6	Tricuspid	–	–	–	–
975	6.5	Tricuspid	–	–	–	–
1289	10	Tricuspid	–	–	–	–
116	6	Pulmonary	–	–	–	–
379	8	Aortic	–	–	–	–

*$gltA$, citrate synthase; +, positive; –, negative.

and canine cases of *Bartonella* endocarditis. In fact, high antibody titers are considered a major diagnostic criterion for *Bartonella* endocarditis in humans (*3*). Finally, the 2 *B. bovis*–infected vegetative valves were aortic and mitral valves, which are the most frequently involved valves in human and canine *Bartonella* endocarditis cases (*5*,*13*,*14*).

Two (9.1%) of the 22 endocarditis cases were *Bartonella* DNA positive. This percentage is within the range of 3% reported for human cases of endocarditis (*14*) and 19% (*5*) to 28% (*13*) reported for dogs. The 2 cases occurred in the oldest animals (Table), which suggests that *B. bovis* endocarditis could develop in geriatric cows, following chronic bacteremia in an overtly healthy animal. Nearly 30% of cattle >7 years of age are reportedly *Bartonella* bacteremic (*12*).

Cattle are the main reservoir for *B. bovis,* and diseases attributed to infection with this *Bartonella* species in cows are scarce (*12*). Nevertheless, these 2 cases demonstrated that *B. bovis* is a potential bovine pathogen and that *B. bovis* can induce endocarditis in the animal reservoir host, as previously shown for *B. quintana, B. henselae,* and *B. vinsonii* subsp. *berkhoffii* in humans, cats, and dogs, respectively (*6*,*14*,*15*). This study confirms that *B. bovis* can cause endocarditis in cows like *B. henselae* and *B quintana* in their respective feline and human reservoirs.

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Parenteral Transmission of the Novel Human Parvovirus PARV4

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Transmission routes of PARV4, a newly discovered human parvovirus, were investigated by determining frequencies of persistent infections using autopsy samples from different risk groups. Predominantly parenteral routes of transmission were demonstrated by infection restricted to injection drug users and persons with hemophilia and absence of infection in homosexual men with AIDS and low-risk controls.

The novel human parvovirus PARV4, is a recently discovered member of the family *Parvoviridae* (*1*). PARV4 was originally cloned from a sample obtained from a person at risk for HIV infection through injection drug use or sexual contact who was enrolled in the San Francisco-based Option Project cohort. Since the original report, PARV4 infections have been detected in samples of pooled plasma from a variety of manufacturers (*2,3*), and at low frequency in pooled and individual plasma samples from US blood donors (≤2%) (*3*). A higher frequency of PARV4 viremia found among other persons enrolled in the Option Project cohort (6%) (*3*) and detection of PARV4 in autopsy blood from a person with a history of injection drug use (*4*) are indicators of a potential association between PARV4 infection and high-risk behavior for HIV infection.

Despite the acute, resolving nature of many parvovirus infections, it is now well established that many members of the family *Parvoviridae*, such as the human erythrovirus B19, can establish lifelong persistence with restricted replication and absence or rarity of detectable long-term viremia (*5–7*). Using a highly sensitive nested PCR, we recently investigated a range of autopsy tissues taken from high-risk persons (HIV-infected with histories of injection drug use or male homosexual contact) and low-risk persons (uninfected with HIV and hepatitis C virus [HCV]) who did not have a history of parenteral exposure or contact with multiple sexual partners. We checked for evidence of similar persistence of PARV4 (*8*). Remarkably, bone marrow, lymphoid tissue, or both from 17 of 24 study subjects in the high-risk group were positive for PARV4, but both sample types were uniformly negative in low-risk controls. These findings not only confirm the ability of PARV4 to establish persistent infections in humans, but also the existence of shared risk factors with HIV for transmission.

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The Study

We tested well-defined risk groups for HIV and parenterally transmitted infections to more precisely determine the transmission route of PARV4. Autopsy tissue samples used in this project were obtained from the Edinburgh Medical Research Council (MRC) HIV Brain and Tissue Bank at the Western General Hospital, Edinburgh. Consent for use of postmortem tissue was obtained from the Lothian Research Ethics Committee (LREC2002/4/36). Study subjects were divided into 4 groups of approximately equal size (n = 11–13, Table 1): 1) injection drug users (IDUs) without HIV infection (all positive for antibodies to HCV when testing was available); 2) Men who had sex with men (MSM) with AIDS without a history of parenteral exposure (all negative for antibodies to HCV when testing was available); 3) IDUs infected with HIV with AIDS-defining illnesses; and 4) IDUs infected with HIV who died of other causes while presymptomatic.

We also tested samples from 2 persons with hemophilia treated with nonvirally inactivated factor VIII concentrates from the late 1970s onward, both of whom became infected with HIV and HCV. Study subjects showed similar demographic characteristics, with similar age ranges and dates of death largely restricted to the 1990s (Table 1). The IDUs, MSM, and patients with hemophilia with AIDS showed similar mean CD4 counts before death, indicating profound immunosuppression. Samples of lymphoid tissue (lymph node or spleen) and bone marrow were assayed for parvovirus B19 and PARV4 DNA sequences by nested PCR as described (*8*). In all samples, ≥0.5 μg of genomic DNA was tested, which provided a test sensitivity of ≈6 copies of target sequence/10^6 cells. Detection of both parvovirus B19 and PARV4 sequences was highly reproducible between the 2 tissues analyzed (Table 2), which enabled generally unambiguous categorization of study subjects into infected and uninfected categories. Persons in whom B19 or PARV4 was detected in 1 of the 2 tissues were considered infected, although the same conclusions for risk group associations would have been reached if the 5 persons with discrepant results had been excluded from analysis or considered uninfected (data not shown).

Parvovirus B19 infection frequency increased with age of the patients and corresponded closely to frequencies of B19 seropositivity in the general population in the United Kingdom recorded previously for different age ranges (*9*). Infections were absent in 2 young patients with hemophilia (22 and 26 years of age at death), ranged from 46%

Table 1. Detection of parvovirus B19 and PARV4 in study groups*

Participant category (no.)	M/F	Age at death, y (range)†	Year of death (range)†	Mean CD4/µL (range)†	AIDS?	B19 positive, no. (%)	PARV4 positive, no. (%)
HIV+ IDU, AIDS‡ (13)	10/3	35 (2–48)	1995 (1991–1998)	44 (1–137)	Y	6 (46)	11 (85)
HIV+ IDU, pre-AIDS‡ (11)	7/4	33 (29–40)	1996 (1992–1998)	268 (167–496)	N	6 (55)	6 (55)
HIV+ MSM (13)	13/0	39 (28–49)	1993 (1990–1996)	25 (1–160)	Y	7 (54)	0
HIV– IDU (12)	10/2	35 (24–49)	1999 (1992–2005)	ND	NA	8 (67)	1 (8)
Hemophilia (2)	2/0	22, 26	1994, 1995	0	Y	0	1 (50)
Low-risk control§ (8)	3/5	54 (28–89)	All 2005	ND	NA	8 (100)	0

*IDU, injection drug user; M, male; F, female; Y, yes; N, no; MSM, men who had sex with men; ND, not done; NA, not applicable.
†Values are means (ranges) or individual values for those with hemophilia.
‡These study groups overlap with the previously analyzed HIV-positive group (*8*), restricted to those in whom parenteral risk factors for infection have been identified.
§Previously described in (*8*).

to 67% in IDUs and MSM (mean ages 33–39 years), and were found in all 8 low-risk controls (mean age 54 years). These findings provide further evidence for high frequencies of or potentially universal persistence of infection with B19 in those exposed (*5–7*). In contrast, infections with PARV4 were restricted to those with a history of parenteral exposure (IDUs and patients with hemophilia). Frequencies of infection ranged from 8.3% (1/12) of the HIV-negative IDUs to 55% and 85%, respectively, in HIV-infected IDUs before and after AIDS developed. Similarly, 1 of the 2 patients with hemophilia was positive for PARV4. No PARV4 infections were found in the MSM group, despite profound immunosuppression associated with AIDS and histories of frequent past exposure to sexually transmitted infections, such as HIV.

Conclusions

The absence of detectable PARV4 in the MSM group demonstrates that PARV4 infections are not specifically associated with HIV co-infection. Instead, its specific risk group association with injection drug use and hemophilia (and absence in MSM and low-risk controls) provides evidence for a predominantly or exclusively parenteral route of transmission. The higher frequency of PARV4 detection in HIV-positive persons may be an indirect reflection of the greater degree of parenteral exposure among IDUs who become infected with HIV. In Edinburgh, HIV infections are much less prevalent in the IDU population than are HCV infections, because the transmission of HIV is less efficient through the bloodborne route (*10*). The higher frequency of PARV4 infection in the IDU-AIDS group compared with the frequency in the pre-AIDS group may also have originated through differential parenteral exposure; AIDS is more likely to be diagnosed among those exposed early to HIV in their period of injection.

Table 2. Concordance of parvovirus B19 and PARV4 detection between bone marrow and lymphoid tissue*

Virus detected and risk group	Lymphoid tissue, Positive	Lymphoid tissue, Negative
B19 (all risk groups)		
Bone marrow		
Positive	27	1
Negative	3	29
PARV4 (IDUs and persons with hemophilia)		
Bone marrow		
Positive	14	1
Negative	2	21

*IDUs, injection drug users.

It could be argued that the higher frequency of PARV4 infection in HIV-infected IDUs and persons with hemophilia may be the result of greater ease of detection in immunosuppressed persons. PARV4 infections may be widespread like B19 infections but may only persist in detectable amounts in persons whose compromised immune system allows viral reactivation. Although this hypothesis was difficult to discount in our original study (*8*), our new observation of an absence of PARV4 infection in MSM with AIDS removes the proposed link between immunosuppression and PARV4 detection.

The findings of our study provide evidence that PARV4 is primarily or exclusively transmitted through parenteral routes, a marked contrast to predominantly respiratory routes of transmission of parvoviruses in other genera, including B19 and human bocavirus (*11*). Although no information was provided on the specific risk factors for persons at high risk who were enrolled in the Options Project cohort (i.e., whether an IDU or MSM), the finding of a higher prevalence of viremia in this group compared with blood donor controls (*3*) is also consistent with evidence obtained in the current study for a predominantly parenteral route of transmission.

Previous observations of the remarkable sequence homogeneity of PARV4 nucleotide sequences between variants detected in the United Kingdom and in the United States (*3,8*) indicate the recent origin and spread of this virus in this specific risk group. Although we currently understand little about its pathogenicity or the clinical outcome

of infection, PARV4 infection represents a potential newly emerging, additional bloodborne virus in IDUs. Given the resistance of parvoviruses to viral inactivation procedures, recipients of a wide range of plasma-derived therapeutics may also be at risk for PARV4 infection.

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Norovirus and Gastroenteritis in Hospitalized Children, Italy

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Noroviruses were detected in 48.4% of 192 children (<3 years of age) hospitalized for gastroenteritis in Palermo, Italy, during 2004; predominant genotypes were GGIIb/Hilversum and GGII.4 Hunter. Of children with viral enteritis, 19.6% had a mixed norovirus-rotavirus infection. The severity of infection was lower for norovirus than for rotavirus but increased in co-infection.

Noroviruses (NoVs) were the first viruses to be clearly associated with acute gastroenteritis, but for many years, knowledge of their role in infection and disease has been limited (*1*). The introduction of the reverse transcription–PCR (RT-PCR) method defined the relevant role of these agents in outbreaks and sporadic cases of gastroenteritis throughout the world and showed the broad heterogeneity and rapid evolution of NoV strains (*2–5*).

Italy has no surveillance system for nonbacterial gastroenteritis. Recently 2 outbreaks of confirmed NoV gastroenteritis were reported (*6,7*). With regard to sporadic enteritis in Italian children, the few studies performed have reported prevalence rates from 2.1% to 18.6 % (*8–11*).

The Study

This report aims to describe the clinical and epidemiologic features of NoV infection in children hospitalized for enteritis and to compare the severity score related to the different viral agents and NoV genotypes. From January to December 2004, 390 fecal specimens were obtained from 365 children with acute gastroenteritis within 24 hours of admission to the Department of Infectious Diseases at the G. Di Cristina Children's Hospital in Palermo, Italy. Gastroenteritis was defined as ≥3 stools that were looser than normal stools per day or 1 episode of vomiting. Demographic and clinical data were collected for most patients. A 14-point scoring system was used to summarize the clinical severity of the cases (Table 1). All the specimens were examined for the presence of *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., and *Yersinia* spp. One hundred ninety-nine samples negative for bacteria and from 192 children (100 boys and 92 girls; median age 11.75 months) were examined for NoVs, group A rotaviruses (HRVs), adenoviruses (AdVs), and astroviruses (HAstVs). NoV detection was carried out by single-step or nested RT-PCR (*12*). Positive and negative controls were included in all amplification reactions, and contamination of reactions by PCR products was avoided by strict separation of working areas and the use of filter-plugged pipette tips. The genotyping of NoV strains was obtained by sequence analysis performed on the RNA-dependent RNA polymerase gene and the sequences were aligned and compared with a selection of representative sequences from the various NoV genotypes available in online databases (*12*).

HRV, AdV, and HAstV were detected by enzyme immunoassays (EIAs) (IDEIA Rotavirus, IDEIA Adenovirus, and Amplified IDEIA Astrovirus; DakoCytomation, Angel Drove, UK). AdV-positive specimens were tested for enteric subgenus F serotypes 40 and 41 with the Premier Adenoclone-type 40–41 EIA (EIA Cambridge Bioscence, Worcester, MA, USA). HAstV-positive samples were confirmed by RT-PCR (*10*). Statistical analysis was carried out by using the χ^2 test, and a significance level of 5% was adopted.

NoVs were detected in 93 (48.4%) of 192 patients, and at least 1 of the gastroenteric viruses tested was found in 148 (77.1%) patients. Among 148 patients positive for enteric viruses, NoVs were the only viruses detected in 58 (39.2%), while 1 or 2 more viruses were present in 35 (23.6%) (Table 2). Of the 93 NoVs-positive patients, 74 (79.6%) were detected after the first PCR step, and 19 (20.4%) after the nested-PCR step. A total of 36 RT-PCR amplicons, 28/74 first step–positive and 8/19 nested-positive, were submitted for sequencing.

Table 1. Clinical parameters for evaluating gastroenteritis severity and related score

Clinical parameter/value	Score
Duration of diarrhea, d	
<2	1
2–4	2
>4	3
Maximum no. bowel movements/d	
3	
4–5	2
>5	3
Duration of vomiting, d	
No vomiting	0
1–2	1
>2	3
Fever	
No	0
Yes	2
Intravenous rehydration	
No	0
Yes	3

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Table 2. Severity score of 148 cases of infantile viral gastroenteritis related to their etiology*

Infection	No. cases (%)	Median severity score
NoV	58 (39.2)	8
HRV	50 (33.8)	10
AdV	4 (2.7)	8
NoV-HRV	27 (18.2)	10.5
NoV-AdV	3 (2)	10
NoV-HAstV	3 (2)	8
NoV-HRV-HAstV	2 (1.4)	14
HRV-AdV	1 (0.7)	ND

*NoV, norovirus; HRV, rotavirus; AdV, adenovirus; HAstV, astrovirus; ND, not determined.

HRVs were identified in 80 (41.6%) of 192 patients, AdVs in 8 (4.2%) patients, with 1 (0.5%) strain belonging to serotype 40/41, and HAstVs in 5 (2.6%) patients. Overall, single viral infections were found in 112 (58.3%) of 192 patients; double viral infections were detected in 34 (17.7%) patients, and 2 (1%) patients were infected with 3 viral agents.

All the NoV strains sequenced were characterized as GGII NoVs and could be attributed to a defined genotype. The 2 predominant strains were GGIIb/Hilversum (44.4%) and GGII.4 Hunter (52.8%); a single strain belonged to the GGII.4 Farmington Hills cluster. Both the GGIIb/Hilversum- and the GGII.4-positive patients were also infected with another virus in 37.5% and 35% of cases, respectively.

NoV infections were detected in almost every month of the year; the highest incidence was recorded from February through May. Most of the GGIIb/Hilversum strains were isolated from February through April; the GGII.4 strains were detected at a higher frequency from January through March and again from October through December.

Children infected by NoVs comprised 46 (49.5%) boys and 47 (50.5%) girls. The median age was 12 months. The median duration of diarrhea was 4 days (range 1–17 days) with a median number of bowel movements per day of 7.5 (range 1–21). Vomiting and fever were present in 46 (49.5%) and 48 (51.6%) children, respectively. Thirty nine (41.9%) children showed signs of dehydration. There was no clinical difference in the median age and in the severity of illness caused by each of the 2 prevalent NoV genotypes ($p>0.05$).

The severity score in all the groups of infected children is shown in Table 2. Though not statistically significant ($p>0.05$), HRVs were associated with the highest severity score among single infections. The severity score for NoV co-infections was higher than that for NoV single infections, except for double infections with HAstV. In the last few years many studies have confirmed the growing importance of NoVs as agents of sporadic enteritis. In Italy, the NoV detection rate in pediatric enteritis appears to be increasing. In 2002 in northern Italy, RT-PCR found NoVs to be the second most common viral agents of enteritis after HRVs, with a rate of 10.4% of single infections (*11*). In our study, NoVs emerged as the principal cause of viral enteritis ($p<0.05$) responsible for 39.2% of cases of diarrhea positive for at least 1 viral agent.

The 2 predominant NoV genotypes circulating in southern Italy in 2004 were GGII.4 Hunter and GGIIb/Hilversum. The first was identified in Australia during 2002–2004 and was then related to an increase in gastroenteritis outbreaks in the Netherlands; the second appeared in France in 2000 and soon became prevalent in Europe (*3,4,13*). GGIIb/Hilversum has been described as a strain highly prone to recombinational events, and it may play a peculiar role in children (*3,14*). Detection of both GGII.4 and GGIIb/Hilversum NoV genotypes in sporadic cases of gastroenteritis occurring throughout the year in our area demonstrates that 2 distinct NoV strains can be introduced in a local population and be maintained over a long period. Emergence of new genetic variants may be the cause of increasing NoV infections (*5*).

Conclusions

Our results highlight the need to apply molecular diagnostic tools widely to determine the actual etiology of acute childhood enteritis when the causative agent is not known. This procedure enabled us to define the real prevalence of NoV infection and its frequent occurrence in association with another etiologic agent (18.2%), as reported (*2,11*). The protracted duration of virus shedding reported both in HRV and NoV infections makes it difficult to attribute the main clinical role to one or the other as either may well represent asymptomatic shedding after an early infection (*15*). The lower severity score observed in NoV single infections with regard to both NoV-HRV mixed infections and HRV single infections, suggests that the clinical picture might be dominated by HRVs. However, both viruses could be acting in synergy, and this option might also be contemplated for NoV-AdV co-infections because their score was higher than that of single infections with each of the 2 viruses. To our knowledge, this is the first report relating the clinical picture to NoV genotypes. This study did not show any statistically significant difference in the clinical parameters evaluated in patients infected with GGII.4 or GGIIb/Hilversum types ($p>0.05$).

In conclusion, NoVs emerged in our area as the main cause of sporadic viral gastroenteritis in hospitalized children during 2004, reaching epidemiologic effects of HRV. Analysis of the genetic variability of NoV permitted confirmation of the changing epidemiologic features of these emerging pathogens.

Dr Colomba is a medical researcher at the Infectious Diseases Department, University of Palermo. Her primary research interests include enteric pathogens and zoonotic diseases such as leishmaniasis and rickettsioses.

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Anaplasma platys in Dogs, Chile

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We conducted a 16S rRNA nested PCR for the genus *Ehrlichia* and *Ehrlichia spp.* with blood samples from 30 ill dogs in Chile. Phylogenetic analysis was performed by using *groESL* gene amplification. We identified *Anaplasma platys* as 1 of the etiologic agents of canine ehrlichiosis.

Ehrlichioses are recognized as important emerging tick-borne diseases in humans and wild and domestic animals. The brown dog tick, *Rhipicephalus sanguineus*, is the main tick that infests dogs in Chile (*1*). This tick species is a vector of *Ehrlichia canis* and has been implicated, but not confirmed, as a vector of *Anaplasma platys* (*2*). Serologic and clinical evidence of canine ehrlichiosis and serologic evidence of human ehrlichiosis have been reported in Chile (*3*,*4*). The purpose of this study was to identify the etiologic agent of canine ehrlichiosis in Chile.

The Study

Blood samples were obtained from 30 pet dogs seen in a private veterinary clinic in Santiago, Chile, with tick infestation and clinical signs compatible with ehrlichiosis (hemorrhagic manifestations and thrombocytopenia). We performed a nested PCR to amplify a portion of the 16S rRNA gene by using specific primers for the genus *Ehrlichia* and for *Ehrlichia* spp. DNA was extracted from 300 μL of whole blood by using the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). For *Ehrlichia* genus–specific PCR, 2.5 μL of DNA was amplified by using outer primers EHR-OUT1 and EHR-OUT2 and inner primers GE2F and EHRL3-IP2 in 1 reaction with a final volume of 25 μL (*5*) (Table 1).

The first-round amplification included 20 cycles of denaturation at 94°C for 45 s, annealing at 72°C for 1.5 min, and chain extension at 72°C for 1.5 min. The second-round amplification included 50 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min, and chain extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. Amplification products were analyzed by agarose gel electrophoresis. The expected size of the amplification product was 120 bp. *A. phagocytophilum* DNA was used as a positive control (provided by Didier Raoult). For *Ehrlichia* spp.–specific amplification, we used the same set of outer primers for *Anaplasmataceae* and specific inner primers for *A. phagocytophilum* (*6*), *E. chaffeensis*, *E. ewingii*, and *E. canis* (*5*) (Table 1). For *A. platys* amplification, we used inner primers developed by Kordick et al. (EHRL3-IP2–*E. platys*) (*7*) (Table 1). Expected sizes of amplification products were 546, 395, 395, 389, and 151 bp, respectively.

The *Ehrlichia* genus PCR resulted in the expected DNA band in 6 of 30 dogs (dogs 7, 12, 17, 19, 23, and 25). These 6 samples were positive only for *A. platys*, showing the expected 151-bp product, and negative for other species tested (Figure 1, panel A). *A. platys* PCR was also conducted on the remaining 24 *Ehrlichia*-negative samples; none were positive.

DNA obtained from 3 16S rRNA PCR products (dogs 7, 17, and 25) was purified by using a commercial kit (Rapid Gel Extraction System; Marligen Biosciences, Ljamsville, Germany) and sequenced twice with an ABI 3100 genetic analyzer (Model 3100; Applied Biosystems, Foster City, CA, USA). The 16S rRNA sequences obtained were compared by using BLAST (www.ncbi.nlm.nih.gov/blast) with sequences available at GenBank. Sequences obtained were similar to that of *A. platys* strain Okinawa 1 (GenBank accession no. AF536828), with similarities of 98%, 95%, and 98%, respectively. GenBank accession nos. for 16S rRNA sequences of *A. platys* strains obtained in this study are DQ125260 and DQ125261, which correspond to strains from dogs 7 and 17, respectively.

For phylogenetic analysis, the *groESL* gene of *A. platys* was amplified from samples positive for *A. platys* 16S rRNA that had sufficient amounts of DNA (dogs 17, 23, and 25) and from 1 negative sample (dog 13). Reactions contained 2 μL of purified DNA as template in a total volume of 25 μL. Amplifications contained 1.25 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 3 mmol/L $MgCl_2$, 2.5 mmol/L deoxynucleotide triphosphates (Invitrogen), and 0.2 pmol/L of primers EEgro1F and EEgro2R (*8*) (Table 1). DNA was denatured by heating at 95°C for 10 min. PCR amplification included 40 cycles of denaturation at 95°C for 1.5 min, annealing at 52°C for 2 min, and extension at 72°C for 1.5 min, followed by a final extension at 72°C for 10 min. For nested amplifications, 1 μL of primary PCR products was used as the template in a total volume of 25 μL. Reaction conditions were the same as for primary amplifications. The primers used were SQ3F, SQ5F, SQ4R, and SQ6R (*9*) (Table 1). PCR products were analyzed by 1.5% agarose gel electrophoresis.

We amplified 3 overlapping fragments (790, 1,170, and 360 bp) in 3 16S rRNA–positive samples (Figure 1, panel B). These DNAs were purified by using a commercial kit (Rapid Gel Extraction System; Marligen), sequenced, and analyzed for phylogenetic relationships. Multiple alignment

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Table 1. *Ehrlichia/Anaplasma* spp. PCR primers used in this study

Ehrlichia/Anaplasma spp. (primer type)	Primer	Primer sequence (5′→3′)	Region	Reference
Ehrlichia spp., *A. phagocytophilum*,	EHR-OUT1	CTGGCGGCAAGCCTAACACATGCCAACAT	16S rRNA	(*5*)
E. canis, *E. chaffeensis*, *E. ewingii, A. platys* (outer)	EHR-OUT2	GCTCGTTGCGGGACTTAACCCAACATCTCACGAC	16S rRNA	(*5*)
Ehrlichia spp. (inner)	GE2F	GTTAGTGGCATACGGGTGAAT	16S rRNA	(*5*)
	EHRL3-IP2	TCATCTAATAGCGATAAATC	16S rRNA	(*5*)
A. phagocytophilum (inner)	ge9f	AACGGATTATTCTTTATAGCTTGCT	16S rRNA	(*6*)
	ge2	GGCAGTATTAAAAGCAGCTCCAGG	16S rRNA	(*6*)
E. canis, *E. chaffeensis*, *E. ewingii* (inner)	HE3-R	CTTCTATAGGTACCGTCATTATCTTCCCTAT	16S rRNA	(*5*)
E. canis (inner)	*E. canis*	CAATTATTTATAGCCTCTGGCTATAGGAA	16S rRNA	(*5*)
E. chaffeensis (inner)	*E. chaffeensis*	CAATTGCTTATAACCTTTTGGTTATAAATA	16S rRNA	(*5*)
E. ewingii (inner)	*E. ewingii*	CAATTCCTAAATAGTCTCTGACTATT	16S rRNA	(*5*)
E. equi (inner)	*E. equi-3-IP2*	GTCGAACGGATTATTCTTTATAGCTTG	16S rRNA	(*5*)
E. platys (inner)	*EHRL3-IP2*	TCATCTAATAGCGATAAATC	16S rRNA	(*5,7*)
	E. platys	GATTTTTGTCGTAGCTTGCTA	16S rRNA	(*7*)
E. platys (outer)	EEgro1F	GAGTTCGACGGTAAGAAGTTCA	*groESL*	(*8*)
	EEgro2R	CAGCGTCGTTCTTACTAGGAAC	*groESL*	(*8*)
A. platys (inner)	SQ3F	ATTAGCAAGCCTTATGGGTC	*groESL*	(*9*)
	SQ5F	TCAGTGTGTGAAGGAAGTTG	*groESL*	(*9*)
	SQ4R	CTTTAGGCTATCAAGAGATG	*groESL*	(*9*)
	SQ6R	TGCTTCCTATGTTCTTATCG	*groESL*	(*9*)

analysis was performed with the ClustalW program (www.ebi.ac.uk/clustalw). Calculation of distance matrices and construction of a phylogenetic tree were made with MEGA 3.1 software (www.megasoftware.net). A phylogenetic tree was constructed by the neighbor-joining method and distance matrices for the aligned sequences were calculated by using the Kimura 2-parameter method. Stability of the tree was estimated by bootstrap analysis of 1,000 replications. A final sequence of 686 bp obtained from the overlapping fragments was used for comparison and showed 100% identity between the 3 Chilean sequences and 99.8% similarity with sequences of the *A. platys groESL* gene deposited in GenBank (Table 2). Phylogenetic relationships of Chilean *A. platys* strains with other *Anaplasmataceae* species are shown in Figure 2. GenBank accession no. for the *groESL* gene sequence of *A. platys* is EF201806 (corresponding to dogs 17, 23, and 25).

Conclusions

We identified *A. platys* DNA in the blood of 6 dogs with clinical signs indicative of ehrlichiosis. These findings support the conclusion that *A. platys* is an etiologic agent of canine ehrlichiosis in Chile.

Since its first report in the United States in 1978 (*10*), *A. platys* has been described in several countries as the etiologic agent of cyclic thrombocytopenia in dogs. A tick vector of *A. platys* has not been determined, although *R. sanguineus* is the most suspected species (*2*). Because *R. sanguineus* is the only tick species that infests dogs in Santiago (*1*), our results support the conclusion that this species is the vector of *A. platys* in Chile.

A wide range of clinical manifestations of canine cyclic thrombocytopenia has been described. Cases from the United States have been described as mild or asymptomatic (*10*), and cases from Spain have more severe symptoms (*11*), which also seems to be the case in Chile. This variability in clinical symptoms of infection has not been clearly associated with strain variations (*11–13*).

Low diversity was observed when *groESL* gene sequences of Chilean strains were compared with other *A. platys* strains available in GenBank. This finding has also been observed in strains from different geographic origins (*13*).

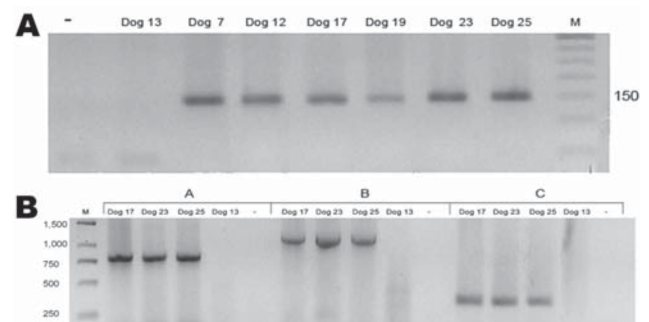

Figure 1. A) *Anaplasma platys* nested PCR products of 30 blood samples from dogs in Chile. Positive samples from dogs 7, 12, 17, 19, 23, and 25 are indicated by a 150-bp band. –, PCR-negative control; dog 13, negative control; M, 50-bp DNA ladder. Value on the right is in basepairs. B) Second-round *A. platys groESL* nested PCR products of dog DNA samples with 3 sets of primers. Group A, SQ5F/SQ4R (790 bp); group B, SQ3F/SQ4R (1,170 bp); group C, SQ3F/SQ6R (360 bp). M, GeneRuler 1-kb DNA ladder (Fermentas, Hanover, MD, USA); Dog 13, negative control; –, PCR-negative control. Values on the left are in basepairs.

Table 2. Nucleotide sequence differences among *groESL* genes from different strains of *Anaplasma platys*

Strain	Similarity,* %	Nucleotide position†		
		591	1259	1271
A. platys Sommieres	100	G	A	C
A. platys Lara	100	–	–	–
A. platys RDC	100	–	–	–
A. platys Okinawa	100	–	–	–
A. platys Louisiana	99.7	–	G	T
Dog 17	99.8	T	–	–
Dog 23	99.8	T	–	–
Dog 25	99.8	T	–	–

*Percentages of nucleotide sequence identities for 686-bp region determined from pairwise alignment.
†Nucleotide positions of *A. platys* Sommieres strain, GenBank accession no. AY0441621. –, same base as the type strain.

Recent studies have shown more genetic variability when sequences of the *gltA* gene were used (*11*,*12*).

Evidence of the zoonotic potential of *A. platys* is scarce. In Venezuela, a few symptomatic human cases have been diagnosed since 1992 by the presence of platelet morulae in blood smears (*14*). Monocytic and platelet morulae were reported in a 17-month-old girl with fever and rash (*15*). However, none of these cases have been confirmed by molecular assays. Further studies that investigate the pathogenic and zoonotic role of *A. platys* should be conducted.

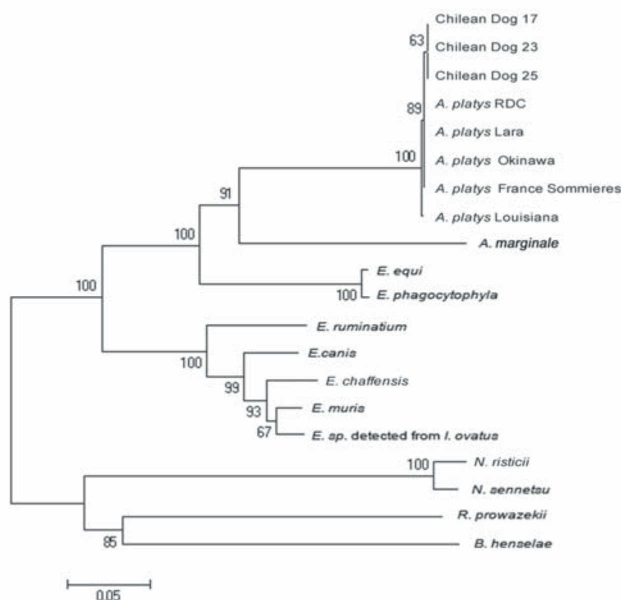

Figure 2. Phylogenetic relationship between 3 Chilean *Anaplasma platys* strains and other strains of the families *Rickettsiaceae* and *Anaplasmataceae* based on the *groESL* gene nucleotide sequences. GenBank accession nos. of *groESL* sequences used to construct the phylogenetic tree were the following: *A. platys* France Sommieres AY044161*; A. platys* Lara Venezuelan dog AF399916; *A. platys* from *Rhipicephalus sanguineus* ticks in the Democratic Republic of Congo AF478129; *A. platys* from a dog in Okinawa, Japan AY077621; *A. platys* from a dog in Louisiana, USA AY008300; *A. marginale* AF165812; *Ehrlichia equi* AF172162; *E. phagocytophyla* U96729*; E. chaffeensis* L10917; *E. canis* U96731; *E. muris* AF210459; *Ehrlichia* sp. from *Ixodes ovatus* AB032711; *E. ruminantium* U13638; *Neorickettsia risticii* U96732; *N. sennetsu* U88092; *Rickettsia prowazekii* Y15783; and *Bartonella henselae* U96734. Scale bar at the lower left indicates 0.05 substitutions per nucleotide.

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Human Metapneumovirus in Children, Singapore

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Four hundred specimens were collected from pediatric patients hospitalized in Singapore; 21 of these specimens tested positive for human metapneumovirus (HMPV), with the A2 genotype predominating. A 5% infection rate was estimated, suggesting that HMPV is a significant cause of morbidity among the pediatric population of Singapore.

Human metapneumovirus (HMPV) is a new member of the family *Paramyxoviridae*. It was first identified in children with respiratory diseases in the Netherlands and is now recognized as a substantial cause of acute respiratory infection in pediatric patients (*1*). The clinical symptoms in children are similar to those observed during respiratory syncytial virus (RSV) infections and vary from upper respiratory tract infection (URTI) to bronchiolitis and pneumonia. HMPV infections have been detected in young children 5 years of age (*2*) as well as in adults of all age groups (*3*). Sequence analysis of HMPV isolates has identified 2 main lineages, A and B; each group is further subdivided into 2 more lineages, A1 and A2, and B1 and B2 (*4,5*). Both virus genotypes were reported in various countries in the Americas, Europe, and Asia. This study aims to assess the importance of HMPV infection among hospitalized pediatric patients in Singapore.

The Study

Kandang Kerbau Women's and Children's hospital is one of the major centers in Singapore for the admission of sick children, including those showing respiratory illness. After obtaining prior approval from the Hospital's ethics committee (approval number EC/043/2004), we collected nasopharyngeal swabs from 400 pediatric patients between October 2005 and January 2007. When admitted to the hospital, these patients exhibited symptoms of acute lower respiratory tract infections (LRTI) (bronchiolitis, bronchitis, pneumonia, asthma, and wheezing) and URTI (pharnygitis). Specimens were sent to the hospital's microbiologic laboratory for routine testing for influenza A and B viruses, RSV, adenovirus, and parainfluenza virus (serotypes 1–3) by immunofluorescence assay (LIGHTDIAGNOSTICS, Chemicon, Tamacula, CA, USA). The clinical specimens were stored at –80°C until further analysis for HMPV was performed (not longer than a week after collection). Viral RNA (vRNA) was extracted from each of the thawed nasopharyngeal swabs with the QIAamp viral RNA minikit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturer's instructions. Of the total RNA extracted from the clinical specimens, 5 μL was subjected to real-time reverse transcription–PCR (RT-PCR) testing by using the N gene specific primer set NL–N (*6*). This was performed with the OneStep RT–PCR kit (QIAGEN) on a Corbett Research Rotorgene 3000 (Corbett Life Science, Sydney, NSW, Australia). The PCR cycling conditions were 50°C for 30 min, 95°C for 15 min, and 45 cycles (95°C for 20 s and 60°C for 60 s). Specimens that tested positive by real-time RT-PCR analysis were confirmed by conventional RT–PCR by using the NL–N primer set. The amplified products (163 bp) were detected by using agarose gel electrophoresis, and their identity was confirmed by DNA sequencing.

Of the 400 samples collected, 21 tested positive for HMPV infection, which suggests an incidence rate of ≈5.3%, compared with an 11.5% incidence rate for RSV (Table 1). Previous reports have suggested that in some cases severe symptoms exhibited by RSV-infected patients are associated with dual infections involving HMPV (*7*). Although we detected the presence of HMPV and RSV in the patients screened, no evidence for co-infections was observed, which suggests a low occurrence for these viruses in Singapore. In a recent study in Australia, only 8 of 10,000 screened hospitalized patients showed evidence of co-infection with HMPV and RSV (*8*). In contrast, several recent studies suggest that co-infections may account for a substantial number of instances in which HMPV has been detected. For example, a recent study in Brazil, which used a lower sample size than in our study, reported an 8% incidence rate for pediatric patients who had RSV and HMPV co-infections (*9*). Therefore, environmental factors may be a key feature in the development of co-infections.

Table 1. Positive test results for respiratory viruses from clinical specimens (n = 400)

Virus	No. positive (%)
Respiratory syncytial virus	46 (11.5)
Influenza A virus	3 (0.8)
Influenza B virus	1 (0.3)
Parainfluenza 1 virus	4 (1.0)
Parainfluenza 2 virus	0 (0)
Parainfluenza 3 virus	8 (2.0)
Adenovirus	1 (0.3)
Human metapneumovirus	21 (5.3)
Total	84 (21.0)

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The entire P gene sequences were amplified directly from the specimens by RT–PCR using the primers hmptPF 5′-ATGTCGTTCCCTGAAGGAAAAGATATTC-3′ and hmptPR 5′-TTAAACTACATAATTAAGTGGTAAAT-3′. Amplicons 884 bp in size were generated and corresponded to 1209 nt–2093 nt of the HMPV genome (strain JPS03-240, AY530095). PCR cycling was performed on a conventional thermal cycler by using a "touch-down" procedure; conditions were 94°C for 5 min followed by 30 cycles of 94°C for 15 s, 62°C (reducing by 0.5°C/cycle) for 30 s, 72°C for 1 min, and a final extension step of 72°C for 7 min. The sizes of the respective PCR-amplified products were examined by using agarose gel electrophoresis, gel-purified, and confirmed by DNA sequencing. The sequences were submitted to GenBank under accession nos. EF409351–EF409371. The genetic relationship between the Singapore HMPV isolates and those HMPV isolates described previously was analyzed by comparing the P gene sequences (*10*). Alignments of nucleic acid sequences were created by using ClustalX version 1.83 (bips.u-strasbg.fr/fr/documentation/clustalx). Phylogenetic trees were constructed by using the neighbor-joining method (1,000 bootstrap replicates) and edited with MEGA 3.1 (*11*). Comparisons were made with representatives of the 4 genetic lineages (Figure). This analysis shows that although isolates representing both A and B genotypes were detected, the Singapore isolates clustered more predominantly with representative HMPV strains in lineage A, in particular the sublineage A2. In this study HMPV was detected throughout the year, which suggests that in Singapore, HPMV is present in the pediatric community throughout the year. We also noted a slight increase in the incidence of B genotypes (B1 and B2) during the last quarter of 2006, but the implications of this finding are unclear.

The age and clinical characteristics of the HMPV patients were next compared with the different HMPV lineages (Table 2). Children with HMPV infection were 1 month to 12 years in age; 67% were ≤1 year of age compared with 63% of RSV-infected children. Of the HMPV-infected patients, 52% exhibited LRTI; of these, 82% were infected with the HMPV sublineage A2. In contrast, ≈43% of the patients exhibited URTI caused by the sublineages A2 and B2. In comparison, 61% and 20% of the RSV patients had a clinical diagnosis of LRTI or URTI, respectively. Our data suggested an increased association of sublineage A2 with LRTI in the HMPV-infected patients. The implications of this are unclear, but several reports note a correlation between severity of infection and the presence of the A genotype (*12,13*). Unfortunately, we were not able to make a strict comparison of our data with data from recent studies in Southeast Asia (*14,15*); these studies used significantly smaller sample sizes and a different selection criterion for

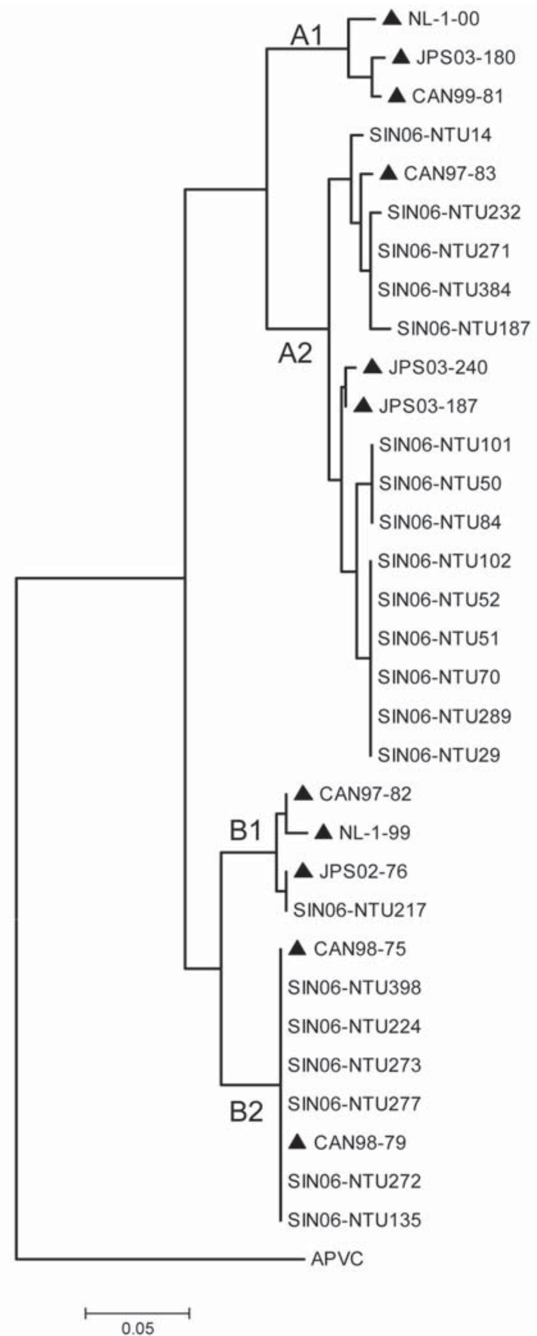

Figure. Phylogenetic analyses of nucleotide sequences of HMPV phosphoprotein showing comparisons with Singapore-Nanyang Technological University (SIN06-NTU*) sequences. The specimen number acquired during the course of the investigation (e.g., SIN06-NTU14) was made with known strains (highlighted ▲) from Canada [CAN99-81 (AY145294, AY145249), CAN97-83 (AY297749), CAN97-82 (AY145295, AY145250), CAN98-75 (AY297748), CAN98-79 (AY145293, AY145248)], Japan [JPS03-180 (AY530092), JPS03-240 (AY530095), JPS03-187 (AY530093), JPS02-76 (AY530089)], and the Netherlands [NL-1-00 (AF371337), NL-17-00 (AY304360), NL-1-99 (AY525843), NL-1-94 (AY304362)]. Avian pneumovirus type C (APVC AY590688) was used as the outgroup.

Table 2. Characteristics of pediatric patients with human metapneumovirus (HMPV) infection

Patient no.	Age	Clinical diagnosis*	Lineage of HMPV
14	2 y	L	A2
29	1 y	O	A2
50	6 mo	U	A2
51	1 y	L	A2
52	1 y	U	A2
70	11 mo	U	A2
84	6 mo	U	A2
101	1 y	L	A2
102	2 y	L	A2
135	1 y	U	B2
187	3 mo	L	A2
217	1 mo	L	B1
224	1 y	U	B2
232	1 y	L	A2
271	12 y	L	A2
272	4 y	U	B2
273	1 y	U	B2
277	5 y	U	B2
289	1 y	L	A2
384	2 y	L	A2
398	7 y	L	B2

*L, lower respiratory infections including bronchiolitis, bronchitis, pneumonia, asthma, wheezing or chest infection; U, upper respiratory infections including infantile pyrexia and pharyngitis; O, febrile fit.

the patients screened (i.e., LRTI [*14*] and wheezing and asthma [*15*]).

Conclusions

Our study is the first, to our knowledge, that has attempted to assess the importance of HMPV among the pediatric population in Singapore. We analyzed 400 samples that were collected from pediatric patients who were admitted to a hospital over a 16-month period. An infection rate of 5.3% was observed, which is consistent with the reported infection rates of several other industrialized countries. We also noted that of the viruses detected, ≈67% were of the A subtype and 33% were of the B subtype, which suggests that the former was the predominant HMPV subtype causing illness in these patients. Furthermore, a significant proportion of the HMPV-infected patients had LRTI. Our findings suggest that HMPV is a substantial cause of illness among the pediatric population of Singapore.

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Fecal Viral Concentration and Diarrhea in Norovirus Gastroenteritis

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Fecal viral concentrations of 40 patients infected with norovirus genogroup GII.4 correlated with diarrhea duration and frequency of vomiting. Higher viral concentration and older age were independently associated with prolonged diarrhea (≥4 days). These findings provide information on the pathogenesis and transmission of norovirus infections.

Norovirus is a major cause of viral gastroenteritis worldwide, accounting for at least 28% of all foodborne outbreaks (*1*). However, its pathogenesis is poorly understood (*2*). Although the disease is usually perceived as mild and self-limiting (symptoms generally subside within 2–3 days in otherwise healthy persons) (*1*,*2*), protracted diarrhea and serious complications may develop in elderly or immunocompromised patients (*2*–*4*).

We have previously shown that patients infected with norovirus genogroup GII have at least 100-fold higher fecal viral concentrations than those infected with genogroup GI (*5*), which may help explain the former's global predominance (*6*,*7*). However, whether fecal viral concentration has any association with disease manifestation is unknown. In this study, we postulated that a higher viral concentration is associated with more severe symptoms. We studied potential associations in patients infected with norovirus GII.4, the predominant norovirus genotype circulating in Hong Kong during the study period (*6*,*7*).

The Study

During a 2-year period (November 2004–November 2006), 44 adult (≥16 years of age) patients at 2 regional hospitals in Hong Kong Special Administrative Region with acute gastroenteritis were shown to be infected with norovirus genogroup GII.4. Clinical records were reviewed and baseline characteristics, clinical features, and output charts were studied. Cases were included for analysis if stool samples were collected ≤96 hours from symptom onset. Diarrhea was defined as having ≥3 loose stools per day. Duration of diarrhea was defined as the number of days (inclusive) between the first and final dates of symptoms (*3*).

Stool samples were collected from all patients when initially observed and processed immediately for RNA extraction. Diagnosis of norovirus infection and its quantitation were based on real-time reverse transcription–PCR assay of stool samples as described (*5*). The lower detection limit of the assay was 2×10^4 copies of cDNA/g stool. Phylogenetic studies were also performed as described (*5*).

Associations between clinical parameters and fecal viral cDNA concentrations were determined. Univariate associations between fecal viral concentration ($\log_{10}$ copies cDNA/g fecal specimen), baseline characteristics, and clinical variables were examined by using the Mann-Whitney test or χ^2 test as appropriate. Variables with a p value <0.1 in univariate analyses were entered into multivariate models as covariates. Stepwise backward logistic regression was performed to identify independent variables associated with prolonged diarrhea, defined as ≥4 days of diarrhea. This cutoff was based on the results of many observational studies (*1*–*4*) and was also above the median duration of diarrhea in this cohort. Spearman rank correlation coefficient (ρ) was used to assess correlations between viral cDNA concentration and other continuous variables. A p value <0.05 was considered statistically significant. All probabilities were 2-tailed. Statistical analysis was performed with SPSS software version 13.0 (SPSS Inc., Chicago, IL, USA).

Stool samples from 40 patients were analyzed. Mean ± SD age of patients was 60.4 ± 24.3 years and 15 (37.5%) were males. Seventeen patients (42.5%) had pre-existing medical conditions, and 21 (52.5%) were hospitalized. Diarrhea was observed in 100%, whereas vomiting and fever were observed in 64.9% and 36.8%, respectively. Median duration of diarrhea was 3 days (range 2–6 days). Median fecal cDNA concentration was 8.93 $\log_{10}$ copies/g stool (interquartile range 8.22–10.24 $\log_{10}$ copies/g stool).

Fecal viral cDNA concentration was examined in relation to baseline characteristics and clinical symptoms (Table). Higher viral concentrations were associated with older age (p = 0.064). Higher fecal viral concentration was significantly associated with prolonged diarrhea ≥4 days (2.11 $\log_{10}$ copies/g stool; p = 0.001, by Mann-Whitney test) than with limited diarrhea (Figure 1). Viral concentration was positively correlated with total duration of diarrhea (Spearman ρ 0.47, p = 0.004) and total frequency of vomiting (Spearman ρ 0.34, p = 0.043) during the course of illness (online Appendix Figure, available from www.cdc.gov/EID/content/13/9/1399-appG.htm). Fever developed more frequently in patients with prolonged diarrhea (64.3% vs. 21.7%; p = 0.010, by χ^2 test). Mean total frequency of diarrhea and vomiting was 14.9 and 3.1, respectively, in in-

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Table. Fecal viral concentrations of 40 patients infected with norovirus*

Comparison groups (%)	Median fecal viral concentration, log_{10} copies cDNA/g stool (IQR)	p value
Age, y		
<65 (47.5)	8.48 (7.79–10.11)	0.064
≥65 (52.5)	8.97 (8.54–10.70)	
Sex		
Male (37.5)	8.97 (7.79–10.72)	0.706
Female (62.5)	8.88 (8.24–10.22)	
Pre-existing medical conditions†		
No (57.5)	8.95 (7.86–10.26)	0.520
Yes (42.5)	8.91 (8.31–10.14)	
Diarrhea duration‡		
Limited (62.2)	8.38 (7.89–9.45)	0.001
Prolonged (37.8)	10.49 (8.84–10.94)	
Vomiting		
No (35.1)	8.71 (7.71–9.91)	0.215
Yes (64.9)	9.10 (8.25–10.40)	
Fever§		
No (62.2)	8.77 (8.15–10.17)	0.380
Yes (36.8)	9.13 (8.23–10.75)	

*IQR, interquartile range.
†Includes diabetes mellitus, chronic cardiovascular/pulmonary/hepatic diseases, and underlying malignancies. No patient had conditions associated with profound immunosuppression in this cohort.
‡Limited (62.2%) is defined as a total duration of diarrhea of 1–3 d, including both hospitalized and nonhospitalized patients (followed up by the same hospital's emergency departments as outpatients).Prolonged (37.8%) is defined as a total duration of diarrhea ≥4 d; all but 1 were hospitalized patients.
§Temperature >37.5°C on ≥1 occasion.

patients with prolonged diarrhea and 11.8 and 1.2, respectively, in those with limited diarrhea. We did not observe an association between mean daily output and fecal viral concentrations in this cohort.

To rule out possible confounding by variations in sample collection time, fecal viral cDNA concentration was also examined by sample collection day (Figure 2). In general, samples collected from patients with prolonged diarrhea had higher viral concentrations on all collection days. The mean day of sample collection was slightly later in patients with prolonged diarrhea than in those with limited symptoms (2.4 ± 1.3 days vs. 1.5 ± 1.1 days; p = 0.045, by *t* test).

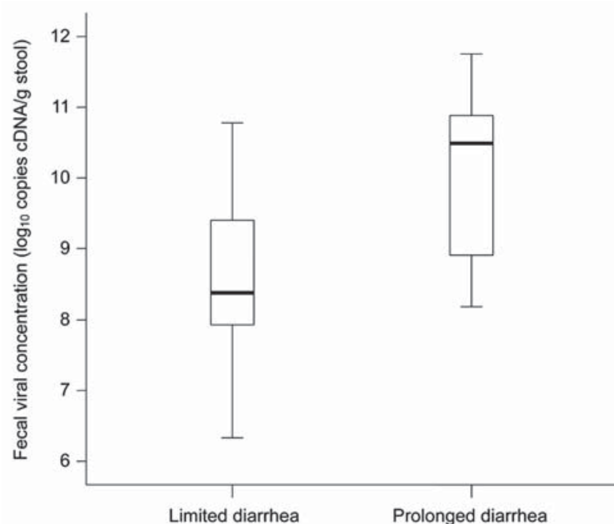

Figure 1. Box plot of median (black horizontal bars) and interquartile range (error bars) of fecal norovirus cDNA concentrations in patients with limited and prolonged diarrhea. Limited diarrhea is defined as a total duration of diarrhea of 1–3 days, and prolonged diarrhea is defined as a total duration of diarrhea ≥4 days.

Prolonged diarrhea ≥4 days was associated with older age and pre-existing medical conditions by univariate analyses ($p<0.05$ for both variables, by χ^2 test) and with fecal viral concentration. Stepwise backward logistic regression analysis showed that fecal viral concentration (odds ratio [OR] 9.56, 95% confidence interval [CI] 1.18–77.57 per log_{10} copies; p = 0.035) and age (OR 1.15, 95% CI 1.03–1.28) per year; p = 0.013) were 2 independent factors associated with prolonged diarrhea caused by norovirus genotype GII.4.

Conclusions

To our knowledge, this is the first clinical study to demonstrate that fecal viral concentration correlates with duration of illness in norovirus gastroenteritis. It has been reported that severe protracted diarrhea caused by norovirus infection can develop in hospitalized, elderly, and immunocompromised patients (*3*,*4*,*8*). Such patients often shed virus for prolonged periods, which probably indicates active viral replication and slow viral clearance (*4*,*8*). In an animal model, norovirus was shown to infect and possibly replicate in enterocytes, resulting in disease (*9*). Results of our study thus provide preliminary evidence that active viral replication determines clinical disease in norovirus gastroenteritis, as in most other viral infections (*10*). These findings also suggest that more stringent infection control measures need

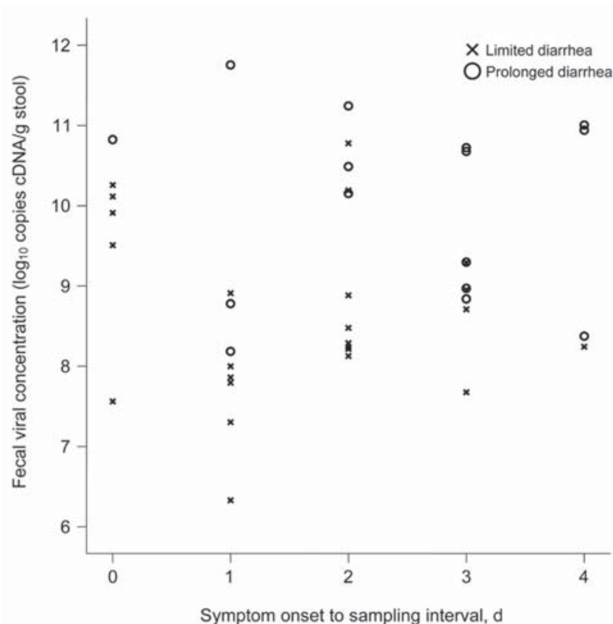

Figure 2. Scatter plot showing fecal norovirus cDNA concentrations in patients with limited and prolonged diarrhea, according to sampling day. Limited diarrhea is defined as a total duration of diarrhea of 1–3 days, and prolonged diarrhea is defined as a total duration of diarrhea ≥4 days.

to be implemented in patients with severe diarrhea because of high fecal viral concentrations (*1–4*,*8*).

This study was limited by a small sample size and fecal viral concentration, which was studied only at 1 time point for each patient. Further research on changes in fecal viral concentrations and their relationships with disease severity are warranted. Because no previous clinical studies describe temporal changes of norovirus concentration in relation to symptoms, we analyzed viral concentration data only in patients with acute diarrhea (days 0–4). Although our definition of prolonged diarrhea (≥4 days, which was above the median in our cohort) seemed arbitrary, it is supported by the results of many observational studies, which show that in most patients (even elderly or hospitalized patients), acute symptoms subside within 2–3 days (*1–4*,*8*). Inclusion of only norovirus GII.4 infections in the analysis removed the possible confounder of strain variation on viral concentration (*5*). Whether similar correlations can be observed with other norovirus strains remains uncertain. Given that genogroup GII.4 is the predominant circulating strain in most countries with major outbreaks (*6*), these results have implications with regard to pathogenesis and infection control of norovirus infections.

In conclusion, these results provide preliminary evidence that a high fecal viral concentration is independently associated with prolonged norovirus gastroenteritis. Further studies are needed to confirm the role of enhanced viral replication on pathogenesis and transmission of this disease. In addition, the approach of quantifying norovirus by real-time PCR can be used for future evaluation of antiviral treatment (*11*) and to study factors associated with delayed viral clearance (*3*).

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Malaria Reemergence in Northern Afghanistan

Michael K. Faulde,* Ralf Hoffmann,* Khair M. Fazilat,† and Achim Hoerauf‡

Field investigations were conducted in Kundoz Province, an Afghan high-risk area, to determine factors responsible for the rapid reemergence of malaria in that country, where 3 million cases were estimated to have occurred during 2002. Results indicate the presence of nonrice-field–dependent *Plasmodium falciparum* and rice-field–associated *P. vivax* malaria.

In 2002, the total malaria incidence in Afghanistan was estimated to be 3 million cases per year, most of them in Kundoz Province. Field investigations from 2001 through 2005 showed a rapid reemergence of malaria caused by *Plasmodium falciparum* and *P. vivax*, with annual incidence rates from 0.0088 to 4.39 and from 3.58 to 13.37 episodes per 1,000 person-years, respectively. Both diseases peaked during 2002 and then declined independently. Although control campaigns against falciparum malaria, transmitted by the freshwater breeder *Anopheles superpictus*, have been successful, *P. vivax* malaria remains highly endemic and is associated with rice-growing areas, where it is transmitted by the endophilic and exophilic rice-field breeders, *A. pulcherrimus* and *A. hyrcanus*. *P. vivax* polymorph VK 247 prevailed in 90% of infected mosquito pools. Field data showed anthropogenically induced increases in rice-field vivax malaria in northern Afghanistan and the need for further control strategies, including large-scale larval mosquito eradication, in rice-growing areas.

Malaria is endemic to large areas of Afghanistan that are <2,000 meters above sea level, but high-altitude epidemic *P. falciparum* malaria may occur in areas ≥2,400 meters above sea level (*1*). From the 1950s through 1979, malaria control in Afghanistan was implemented by the government (*2,3*). During the 1970s, the number of recorded cases of malaria varied from 40,000 to 80,000 annually (*4*). At that time, vivax malaria chiefly occurred in the irrigated zones of northeastern Afghanistan (*3*). Rice fields were located ≥5 km away from villages to exceed the flight range of vector-competent, widely DDT-resistant anopheline mosquitoes, and larvivorous *Gambusia affinis* fish were continuously reared and widely introduced (*5,6*). After 1980, chronic political instability resulted in the progressive breakdown of malaria control activities (*2*).

Although existing malaria control efforts have focused mainly on the Kabul area, little is known about the situation in the irrigated rice-growing high-risk areas of northeastern Afghanistan (*7*). During 1996–2001, from 202,767 to 395,581 malaria cases were reported annually, sharply increasing in 2002 and 2003 with 590,176 and 591,441 cases confirmed, respectively (*7*), and 3 million cases estimated annually (*8*). Takhar and Kundoz Provinces were most affected (*7*). In late 2003, *P. falciparum* incidence ranged from 0.002% in Wardak to 31% in Takhar Province. The other malaria cases were attributable to *P. vivax* (*7*). Our aim was to analyze the current status, risk factors, and epidemiology of malaria in Kundoz Province, a previously underreported risk area.

The Study

Newly contracted (excluding all follow-up patients with *P. vivax* relapses) malaria cases were confirmed by light microscopy, using standard Giemsa staining according to the World Health Organization (WHO) national malaria treatment and diagnosis guidelines (7–9), and were detected passively in febrile patients seeking treatment in the Provincial Malaria Center, Kundoz City, from January 2001 through December 2005. Annual cases of *P. vivax* and *P. falciparum* malaria reported from Kundoz Province from January 2001 through December 2005 are depicted in the Figure. A marked increase in case numbers of both vivax and falciparum malaria occurred from 2001 through 2002, showing an 8.9-fold increase for *P. falciparum.* After 2002, the height of the epidemic, malaria case numbers steadily declined, with *P. vivax* cases falling to 10,946 and *P. falciparum* cases falling to only 27 during 2005. With an estimated population of 3,058,100, the annual incidence rates for *P. falciparum* malaria in Kundoz Province were from 0.0088 (in 2005) to 4.39 (in 2002) per 1,000 person-years; for *P. vivax*, the rates were from 3.57 (in 2005) to 13.37 (in 2002) per 1,000 person-years.

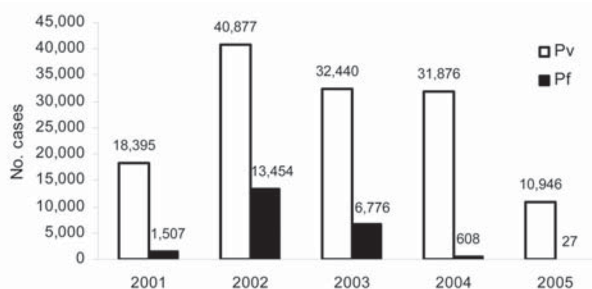

Figure. *Plasmodium vivax* (Pv) and *P. falciparum* (Pf) malaria cases reported in Kundoz Province, northern Afghanistan, January 2001–December 2005.

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From January 2004 through December 2005, adult anopheline mosquitoes were collected outdoors by using New Standard Miniature Light Traps (No. 1012, John W. Hook Co., Gainesville, FL, USA) without an additional CO_2 generator and indoors by using an aspirator in the rice-growing areas of Kundoz City, Kanam, Khanabad, Angor Bag, Alchira, Malaghi, and Jan Guzar. Light Traps were set in housing areas within a 5-km radius of rice fields, which are located in or close to towns, villages, and housing areas. Anopheline larval monitoring was carried out using the WHO-recommended Frisbee disk method (*10*) once a month from May through October in rice fields associated with mosquito trapping sites. Results represent mean values obtained after 10 replicates.

Indoor trapping showed the following: of 299 anopheline mosquitoes trapped in 2004, 82.6% were *A. pulcherrimus,* 16.7% were *A. superpictus,* and 0.7% were *A. culicifacies*; of 403 anophelines trapped in 2005, 81.1% were *A. pulcherrimus* and 18.9% were *A. superpictus* (*11*). All specimens were female and blood-fed.

Outdoor entomologic surveys showed the following: of 439 anophelines collected in 2004, 60.1% were *A. pulcherrimus*, 30.0% were *A. hyrcanus*, and 9.9% were *A. superpictus;* of 456 anophelines collected in 2005, 47.4% were *A. hyrcanus*, 42.1% were *A. pulcherrimus*, and 10.5% were *A. superpictus*. Among all mosquitoes trapped, 80.8% were female, and 22.9% of these were blood-fed. The mean trap rate was 4.8 ± 3.9 anophelines per trap night (range 0–17 per trap night).

Anopheline adult outdoor abundance peaked in late August, with the following percentage monthly means: May (1.2%), June (9.5%), July (18.6%), August (35.2%), September (26.8%), and October (8.7%). Anopheline larval monitoring yielded 54.7% *A. hyrcanus* (0–68 larvae per dip; mean 12.3), and 45.3% *A. pulcherrimus* (0–49 larvae per dip; mean 9.8). No *A. superpictus* or *A. culicifacies* larvae could be detected in rice field samples. Anopheline larval abundance peaked in late July and early August with the following monthly means: May (0%), June (17.9%), July (32.3%), August (36.2%), September (12.8%), and October (0.8%).

The *P. falciparum* and *P. vivax* polymorphs VK 210 and VK 247 circumsporozoite protein (CSP) positivity rates in anopheline pools (5 females per species) trapped indoors and outdoors from 2004 through 2005 were detected by using the VecTest Malaria Panel Assay dipstick ELISA (Medical Analysis Systems, Inc., Camarillo, CA, USA) and are listed in the Table. The available data indicate that *A. superpictus* is the principal *P. falciparum* vector. Three *A. pulcherrimus* pools positive for *P. falciparum* CSP indicate that this species may be partly involved in *P. falciparum* malaria transmission. *Plasmodium* CSP positivity values were higher in indoor-trapped *A. superpictus* (2004: $\chi^2 = 4.9$; df = 1; p = 0.025). Of *P. vivax* CSP-positive pools, 90.6% were VK 247-reactive, and 9.4% were reactive against both VK 247 and VK 210, indicating a similar *P. vivax* genospecies distribution pattern as reported previously from eastern Afghanistan (*12*).

Table. Estimated annual malaria incidence rates, Kundoz Province, northern Afghanistan, 2001–2005*

Year	*Plasmodium falciparum* malaria incidence	*P. vivax* malaria incidence	Total malaria incidence
2001	0.49	6.01	6.50
2002	4.39	13.36	17.76
2003	2.21	10.60	12.82
2004	0.19	10.42	10.62
2005	000.88	3.57	3.58

*Cases per 1,000 population.

Conclusions

Our results show that malaria quickly reemerged in rice-growing Kundoz Province of northeastern Afghanistan. This may be due to various factors: 1) introduction of *P. falciparum* and *P. vivax* malaria by returning refugees (*13*); 2) environmental changes caused by intensified rice growing in close proximity to towns, villages, and housing areas and therefore within flight range of endemic anopheline vectors (*3,5*); 3) increased abundance and breeding of the local principal vectors of *P. vivax* malaria stemming from intensified rice growing and irrigation systems that serve as preferred breeding sites for *A. pulcherrimus* and *A. hyrcanus* (*3,5*); and 4) absence of widespread biological and chemical vector control measures, including effective larviciding in flooded rice fields (*8*).

Habitat and breeding site preferences of malaria vectors may play a major role in the differing epidemiologies of local *P. falciparum* malaria and rice-field–dependent, exophilic and endophilic *P. vivax* malaria. Possible reasons for the decline in annual malaria cases after 2002, especially in endophilic *P. falciparum* malaria not dependent on rice fields, may include the introduction of insecticide-treated bednets, increased indoor spraying, and improved treatment and health education (*7,8*), as well as inhibiting climatic conditions (e.g., the extraordinarily cold 2005 spring/summer season). Current *P. vivax* malaria incidence rates indicate that future control efforts should emphasize large-scale management of potential mosquito breeding sites in rice-growing areas, including biological or chemical larviciding or both. The effectiveness of personal protection from exophilic *P. vivax* malaria vectors such as *A. hyrcanus* may be enhanced by simultaneous use of skin repellents and insecticide-treated clothing (*14,15*).

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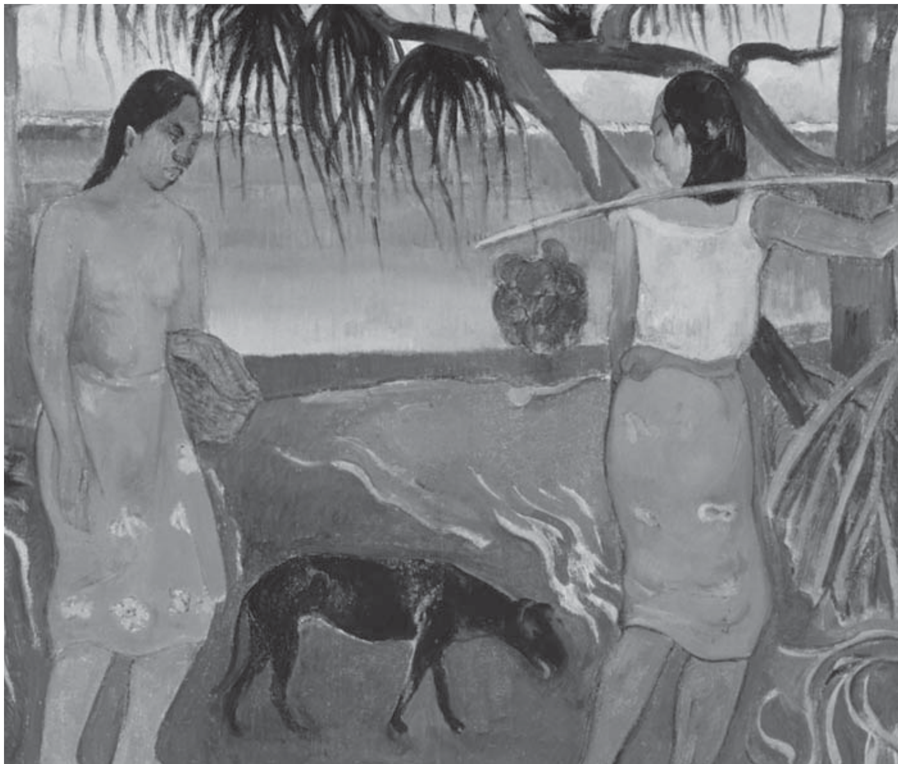

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Rickettsia monacensis and Human Disease, Spain

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We identified *Rickettsia monacensis* as a cause of acute tickborne rickettsiosis in 2 humans. Its pathogenic role was assessed by culture and detection of the organism in patients' blood samples. This finding increases the number of recognized human rickettsial pathogens and expands the known geographic distribution of Mediterranean spotted fever–like cases.

Tickborne rickettsioses are produced by spotted fever group (SFG) rickettsiae and cause an expanding spectrum of clinical signs. *Rickettsia conorii* is the etiologic agent of Mediterranean spotted fever (MSF) and is transmitted by *Rhipicephalus sanguineus*. *Rickettsia helvetica*, a widespread species, is carried by *Ixodes ricinus* (*1*). Recently, other SFG rickettsiae have been found in *I. ricinus* from Spain (*2*), Slovakia (*3*), and northeastern Italy (*4*), as well as in *I. nipponensis* from Japan (*5*). Subsequently, a new rickettsia species, *R. monacensis*, was isolated from *I. ricinus* from Germany (*6*) and detected in Hungary (*7*). The pathogenicity of this species is unknown. It constitutes a new rickettsial genotype and forms a separate cluster among the SFG rickettsiae (*3*), close to strain Cooleyi, which was isolated from *I. scapularis* in Texas (*8*). *I. ricinus* is well established in areas of northern Spain (*9*), where MSF-like cases are increasingly reported.

Our study aim was to identify the SFG rickettsial species involved in MSF-like rickettsioses in 2 patients in northern Spain. We report an association between *R. monacensis* and these rickettsioses.

The Study

Patient 1 was an 84-year-old man from La Rioja, who sought medical attention on June 19, 2003, 7 days after onset of fever (39.5ºC), general discomfort, headache, and joint pain. At the time of the physical examination, he had a nonpruritic, disseminated maculopapular rash, with no inoculation eschar, of the trunk and lower extremities, including palms and soles. Other than a slightly low platelet count (82,000/mm^3), examination findings were within normal limits. MSF was diagnosed, and serum and defibrinated blood samples were taken before a course of oral doxycycline (100 mg/12 h for 10 d) was initiated. Three days later, fever and rash were gone without sequelae. Additional serial serum samples were taken during weeks 4, 13, and 26 after onset and reserved for serologic analysis (Table).

Patient 2 was a 59-year-old woman from Basque Country, who sought medical attention on September 20, 2003, 4 days after onset of fever (38ºC), headache, and an erythematous rash, with no inoculation eschar, at the site of a tick bite. The patient reported a history of tick bites, most recently 1 week before symptom onset. Blood cell counts and other blood chemistry values were normal. MSF was diagnosed, and oral doxycycline (100 mg/12 h for 10 d) was prescribed. Serial serum samples were taken the day of the visit and weeks 4 and 6 after onset and were reserved for serologic analysis (Table). Defibrinated blood was also taken 2 days after treatment was initiated. The patient recovered without sequelae.

DNA was extracted with the QIAGEN Tissue kit (IZASA S.A., Barcelona, Spain), and an *ompA*-nested PCR was designed. The first set of primers (Rr190.70p and Rr190.602n) have been described (*10*). Those used for the nested amplification were designed in this study: NompA-F (5′-AGC GAT AAT GCT GAG TAG TAG-3′) and NompA-R (5′-TAT ATT TCC TAA ACC TGT ATA A-3′) nucleotide positions 150–170 and 576–555, respectively, were numbered according to Regnery et al. (*10*). Amplification conditions were as described, except annealing temperature was 40ºC for the second PCR and AmpliTaq Gold DNA Polymerase (Applied Biosystems, Branchburg, NJ, USA) was used. The specificity of the method was tested against DNA obtained from Vero cells and *Coxiella burnetii*, and fragments of the expected sizes (532 and 427 bp) were obtained from different rickettsia species (data not shown). The amplicons obtained from blood samples were run in 1% low-melt agarose gels (Pronadisa, Barcelona, Spain), and the bands of interest were excised, purified with QIAquick Gel Extraction kit (IZASA S.A), and sequenced as described (*9*).

A phylogenetically informative fragment of 446 bp of *gltA* was also sequenced from samples by nested PCR with primers designed for this study: GLTA1F (5′-GAC GGT GAT AAA GGA ATC TTG-3′) and GLTA1R (5′-CAT TTC TTT CCA TTG TGC CAT C-3′) for the first run, and GLTA2F (5′-CTA CGA ACT TAC CGC TAT TAG-3′) and GLTA2R (5′-GAC CAA AAC CCA TTA ACC TAA AC-3′) for the second; nucleotide positions 279–299,

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Table. Microimmunofluorescence titers obtained with different rickettsial antigens, 2 patients, northern Spain, 2003*

Patient	Week†	*Rickettsia conorii*	*R. monacensis*	*R. helvetica*	*R. akari*	*R. australis*
1	1	<1:40	<40	<40	<40	<40
	4	1,280	2,560	2,560	1,280	640
	13	1,280	1,280	1,280	1,280	1,280
	26	1,280	1,280	1,280	320	320
2	1	640	2,560	2,560	640	320
	4	320	1,280	1,280	320	160
	6	640	1,280	1,280	320	160

*A 1-fold decrease in titer is considered not significant.
†Week after symptom onset in which the samples were extracted.

1011–989, 566–586, and 1298–1277, respectively, were numbered according to Regnery et al. (*10*). PCR conditions included annealing temperatures of 65ºC and 50ºC for the first and second runs, respectively. The rest of the parameters were identical to those used above, and samples were subjected to 35 cycles of denaturing (20 s at 95ºC), annealing (30 s), and extension (2 min at 60ºC), with an initial denaturing cycle of 9 min at 95ºC.

Blood samples from each patient were cultured by using shell vial technique (*11*). Giménez stain and PCR, performed after 7 days of incubation, confirmed the growth of a *Rickettsia*-like organism (strain Rp-Sp1) from patient 1. The sequences of *ompA* and *gltA* of this isolate (GenBank accession nos. DQ157778 and DQ517498, respectively) were identical to those obtained from the blood samples of each patient and to that of *R. monacensis* (*6*) (GenBank accession nos. AF201329 and DQ100163). The sequences generated in this study were subjected to phylogenetic analyses as described (*9*) and belonged to the same clade as *R. monacensis* and other related strains that have been detected in *I. ricinus* (*3*,*4*,*12*) (Figure).

In-house microimmunofluorescence assay (IFA) ([*1*] and references therein) that used *R. monacensis, R. conorii, R. helvetica, R. akari,* and *R. australis* as antigens was performed in serial serum samples from each patient (Table). The isolate Rp-Sp1 from patient 1 could not be used as antigen because of poor adaptation of this isolate to culture in Vero cell monolayers; *R. monacensis* slides for IFA were obtained from the Department of Entomology, University of Minnesota, Minneapolis, MN, USA. Seroconversion against the 5 rickettsia species was observed from patient 1's second serum sample (day 30 after the onset). Patient 2's first serum sample also had high titers against the 5 antigens. Although the reactivity against the 5 rickettsial antigens was similar, the titers observed were slightly higher against *R. monacensis* and *R. helvetica*, which are phylogenetically closer to each other than to the other species tested. However, because the serologic results may only loosely implicate a given rickettsia species, isolation of *R. monacensis* from patient 1 and its detection by PCR for both patients confirm it as the etiologic agent.

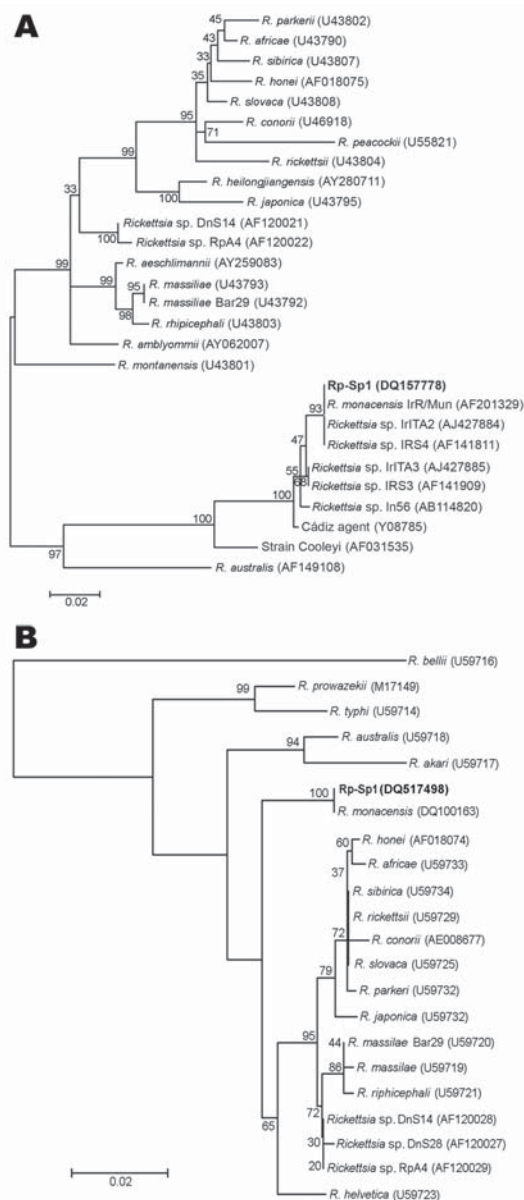

Figure. Neighbor-joining phylogenetic analysis based on *ompA* (panel A) and *gltA* (panel B). Mega 3 software (www.megasoftware.net) was used for the calculation of pairwise distances. Numbers near each node represent the bootstrap values. The isolate from patient 1 is shown in **boldface**. GenBank accession no. for each sequence is in parentheses.

Conclusions

We describe a new, to our knowledge, rickettsia species that caused human disease. *R. monacensis* was the etiologic agent of MSF-like illness in northern Spain. Strain Rp-Sp1 was obtained from 1 patient. Because the sequences of *ompA* and *gltA* were identical to this rickettsia species and also amplified from blood samples of each patient studied, we conclude that this rickettsia is responsible for the symptoms observed in these patients. Therefore, *R. monacensis* joins the list of autochthonous rickettsia species (*R. conorii* [*13*], *R. slovaca* [*14*], *R. typhi* [*15*]) confirmed as human pathogens in Spain.

We were not able to study the vectors involved; however, each patient contracted the disease in areas where *I. ricinus* is the most prevalent tick species (*9*), and strains close to *R. monacensis* have been recently detected in *I. ricinus* in Spain (*2*,*12*). Thus, *I. ricinus* may eventually be shown to be the vector. Studies of *R. monacensis* incidence in autochthonous *I. ricinus* specimens are in progress to evaluate the risk of its transmission to humans.

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Methicillin-Resistant *Staphylococcus aureus* in Pigs with Exudative Epidermitis

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Despite a strict control program for methicillin-resistant *Staphylococcus aureus* (MRSA) in human medicine in the Netherlands, MRSA was cultured from exudative epidermitis lesions of 4 piglets on a breeding farm, 20 pigs on a supplier farm, and 2 workers on these farms. The MRSA strains were indistinguishable, suggesting direct transmission.

Worldwide, methicillin-resistant *Staphylococcus aureus* (MRSA) causes hospital- and community-acquired infections in humans. In the Netherlands, the proportion of clinical human isolates that are methicillin resistant is still very low (2%) (*1*). A 2005 study in France identified pig farming as a risk factor for increased nasal colonization with *S. aureus* (*2*). The strains found in farmers were not found in nonfarmers but often caused swine infections, which suggests transmission between pigs and farmers. During 2004–2005, MRSA was cultured from 3 Dutch patients who had had contact with pigs (*3*). Investigators also found 6 carriers of MRSA among a group of 26 pig farmers. In the Netherlands, contact with pigs is now recognized as a risk factor for MRSA carriage.

The Study

In June 2006, a Dutch farmer contacted the Pig Health Unit of the Veterinary Faculty of Utrecht University about an outbreak of exudative epidermitis among his swine. On his breeding farm (farm A), which had 200 sows (22.5 piglets/sow/year), a high preweaning mortality rate (20%) was caused by exudative epidermitis. Exudative epidermitis is a skin disease normally caused by *S. hyicus* and is usually an acute infection in suckling and weanling piglets. On farm A, a litter of 3-week-old piglets housed in a crate had clinical signs of exudative epidermitis. Other pigs on this farm had been unsuccessfully treated with ceftiofur, cefquinome, tylosin, and trimethoprim/sulfonamides.

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A skin lesion sample from 1 piglet was sent to the Veterinary Microbiological Diagnostic Center of Utrecht University, where it was plated on sheep blood agar and MacConkey agar and incubated at 37°C for 24 h. No growth was seen on MacConkey agar, but large numbers of hemolytic white colonies were found on the sheep blood agar. These colonies were identified as *S. aureus* by colony morphology examination, Gram staining, catalase and coagulase testing, and API ID32 Staph (bioMérieux, Marcy-l'Etoile, France). No *S. hyicus* was found. Antimicrobial drug susceptibility was determined by using an agar diffusion method with IsoSensitest agar (CM471, Oxoid, Basingstoke, UK) and Neosensitab discs (Rosco, Taastrup, Denmark). The breakpoints used were those recommended by the Dutch Committee on Guidelines for Susceptibility Testing (*4*). The *S. aureus* was susceptible to enrofloxacin, trimethoprim/sulfamethoxazole, and fusidic acid and resistant to ampicillin, gentamicin, kanamycin, tetracycline, erythromycin, lincomycin, and tylosin. Because this *S. aureus* was resistant to multiple drugs, it was suspected of being MRSA, and presence of the *mec*A gene was confirmed by PCR (*5*).

Farm A was revisited 2 weeks later. Additional samples were taken from the skin lesions, nares, or both of 5 other 3-week-old piglets with exudative epidermitis from a different litter than the first piglet; from the nares of 1 healthy sow; and from the nares and throats of 2 veterinary students. The students had had contact with the pigs on the day the samples were taken and 1 week earlier. To investigate the source of the MRSA, samples were also taken from the nares of 12 healthy weanling pigs and 10 healthy gilts on a supplier farm (farm B) that had provided gilts for breeding to farm A. Nares samples were also taken from 2 farmers on farm B (Table). The samples were plated on sheep blood agar and incubated in tryptic soy broth, 4% saline, 1% mannitol, phenol red (16 µg/mL), ceftizoxime (5 µg/mL), and aztreonam (50 µg/mL). After incubation at 37°C for 48 h, broth cultures were plated on sheep blood agar and incubated at 37°C for 24 h. Suspected colonies were identified as MRSA, and antimicrobial drug susceptibility was determined as described above. MRSA was cultured from the nares of 1 student (farm A), 1 farmer (farm B), 1 sow and 4 piglets (farm A), 20 pigs (farm B), and from skin lesions of 3 piglets (farm A). Susceptibility testing showed that all MRSA isolates were susceptible to fusidic acid, trimethoprim/sulfamethoxazole, and enrofloxacin and resistant to ampicillin, tetracycline, gentamicin, and kanamycin. Of the 32 isolates, 18 were susceptible to lincomycin, tylosin, and erythromycin, and 14 were resistant to these antimicrobial drugs. Both resistance patterns were detected on both

Table. Methicillin-resistant *Staphylococcus aureus* (MRSA) from 2 pig farms, the Netherlands, 2006

Sample source	No. investigated	Sample site (no.)	Farm	No. MRSA-positive pigs or persons*	MRSA-positive sample site (no.)	Resistance pattern†
Piglets	6	Skin (4) Nares (5)	A	6	Skin (4) and/or nares (4)	TKG or TKGTyLE
Sow	1	Nares (1)	A	1‡	Nares	TKG or TKGTyLE
Students	2	Nares (2), throat (2)	A	1	Nares	TKG
Farmers	2	Nares (2)	B	1	Nares	TKG
Gilts	10	Nares (10)	B	8	Nares	TKG or TKGTyLE
Weanlings	12	Nares (12)	B	12	Nares	TKG or TKGTyLE

*For some, >1 positive sample or 2 types of MRSA were obtained from the same pig or person.
†T, tetracycline; K, kanamycin; G, gentamicin; Ty, tylosin; L, lincomycin; E, erythromycin; all isolates were also resistant to ampicillin.
‡2 isolates with different resistance patterns.

farms. The phenotypic resistance of the isolates to oxacillin was confirmed by Etest (AbBioDisk, Solna, Sweden) according to manufacturer's guidelines. MICs were >128 mg/L for all MRSA isolates.

MRSA isolates were genotyped by using pulsed-field gel electrophoresis (PFGE) with *Sma*I according to the Harmony protocol (*6*), *spa* typing (*7*), and multilocus sequence typing (MLST) (*8*). Typing of the staphylococcal cassette chromosome (SCC*mec*) was performed by using PCR (*9–11*).

All isolates were nontypeable by PFGE with the *Sma*I restriction enzyme. Nontypeable MRSA associated with pig farming possesses DNA methylase, which methylates the *Sma*I-recognition sequence and leads to uninterpretable results (*12*). Genotyping showed that all isolates had *spa* type t011 and MLST 398 and SCC*mec* type IV (*ccrA/B* gene type 2, *mec* complex non–class A). Although 2 distinct resistance profiles were observed, all isolates belonged to the MLST/*spa* genotype associated with pigs in the Netherlands.

Conclusions

Because farm B regularly sells gilts to farm A, farm B is probably the source of the MRSA isolated from farm A. Farm B is a closed farm that has not purchased pigs since 1996. Further research is necessary to identify the source of MRSA on farm B.

Since 2002, human MRSA isolates sent to the National Institute for Public Health and the Environment by the regional laboratories are typed by PFGE, and nontypeable MRSA in persons who are not in contact with pigs is rare. The MRSA-positive farmer and student had no other known risk factors for MRSA carriage. The differences in the resistance patterns may be caused either by erythromycin-lincomycin-tylosin resistance genes located on mobile elements such as plasmids or transposons (like Tn*554*) or by differences in the expression of the resistance genes.

Transmission of MRSA between pigs and pig farmers has been previously reported by Voss et al. (*3*). However, to our knowledge, ours is the first report of culturing MRSA from clinically diseased pigs. The infected piglets were only 3 weeks of age, which suggests that they might have been infected through contact with their mother. The isolation of MRSA from piglets with exudative epidermitis was unexpected. That large numbers of *S. aureus* but no *S. hyicus* were cultured from the skin lesions indicates clinical relevance.

The sale of pigs to many different breeding farms favors the spread of MRSA. It was recently reported that 209 (39%) of 540 finishing pigs at Dutch slaughterhouses were MRSA positive and that all MRSA had MLST 398 and were resistant to tetracycline (*13*). Antimicrobial drugs, especially β-lactams and tetracyclines, may select for this MRSA strain. Farm A used many different antimicrobial drugs, including third-generation and fourth-generation cephalosporins, for treatment of exudative epidermitis; farm B regularly used amoxicillin. The diseased piglets were treated with enrofloxacin and recovered. Healthy pigs carrying MRSA should not be treated with antimicrobial drugs. Currently, no precautions are taken before and during slaughter of MRSA-positive pigs.

In conclusion, MRSA was cultured from clinically diseased and asymptomatic pigs. Colonization with MRSA seems to be widespread in Dutch pigs; supplier farms that sell MRSA-colonized pigs to other farms play a role in spreading the organism. Because the Netherlands exports pigs to other countries, further research on the prevalence of MRSA in pigs in foreign countries is warranted.

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Molecular Evidence for *Anaplasma phagocytophilum* in Israel

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Sequences from the *Anaplasma phagocytophilum* 16S rRNA gene were detected in 5 ticks representing 3 species (*Hyalomma marginatum, Rhipicephalus turanicus,* and *Boophilus kohlsi*) collected from roe deer (*Capreolus capreolus*) in Mount Carmel, Israel. The sequences were all identical to those of Ap-variant 1 strain.

Anaplasma phagocytophilum is the causative agent of granulocytic anaplasmosis (ehrlichiosis) in humans, horses, sheep, cattle, dogs, and cats (*1*). Although serologic evidence for the presence of *A. phagocytophilum* in humans (*2*), jackals (*3*), and domestic dogs (*4*) has been available in Israel since 1999, no direct verification has been presented to confirm its occurrence. In this study, we present molecular evidence for the occurrence of *A. phagocytophilum* in ticks in Israel collected from roe deer.

Ticks were collected from 4 female roe deer (*Capreolus capreolus*) between 2004 and 2005. The deer were part of a reintroduction program initiated in Israel, with deer imported since 1991 from France, Italy, and Hungary and brought to the Hai-Bar Carmel breeding facility on Mount Carmel (*5*). The collected ticks were kept in a 70% ethanol solution for identification and DNA extraction.

Extraction of DNA was performed by using the QIAamp Minikit Catalogue no. 51304 (QIAGEN Inc., Valencia, CA, USA). The DNA extract from each tick was tested for *A. phagocytophilum* by using a nested PCR assay that amplified a 456-bp portion of the 5′ region of the 16S rRNA gene as previously described (*6*). Each positive PCR product was subjected to DNA sequencing with fluorescent-labeled dideoxynucleotide technology (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster City, CA, USA). Sequencing reaction products were separated, and data were collected by using an ABI 3100 Genetic Analyzer automated DNA sequencer (Applied Biosystems).

Seventy ticks were collected from roe deer. DNA extracted from 5 (7.1%) of the 70 ticks produced products when primers specific to the16S rRNA gene of *A. phagocytophilum* were used (Table). DNA sequences from 16S rRNA of *A. phagocytophilum* from the ticks showed a high degree of homology with those reported in the GenBank database. All sequences examined from the ticks were identical. They all differed by 2 bp from the sequence of the human agent (Ap-ha) (GenBank accession no. U02521) but were identical to the variant strain referred to as AP-variant 1 (GenBank accession no. AY193887) (*6*).

Rhipicephalus sanguineus and *R. turanicus* ticks are common in Israel and found on a large variety of domestic and wild animals (*7*). *Hyalomm marginatum* ticks have a worldwide distribution and have been documented on mountain gazelles and Nubian ibexes in Israel (*8*); *Boophilus kohlsi* has been documented on sheep and goats in Jordan (*9*). In a study conducted in Spain, *A. phagocytophilum* was found in *Dermacenter marginatus, Ixodes ricinus, R. bursa,* and *Hemophysalis punctata* (*10*). Santos-Silva et al. were not able to demonstrate the presence of *A. phagocytophilum* in *H. marginatum* or *R. turanicus* in Portugal (*11*). Existing evidence cannot determine whether these ticks could act as vectors of *A. phagocytophilum* or were merely infected during a blood meal from an infected roe deer.

The presence of *A. phagocytophilum* in roe deer has been demonstrated in Slovakia (*12*), Germany (*13*), the Czech Republic, and Austria (*14*). These data indicate that roe deer may act as reservoirs for *A. phagocytophilum* in Israel. The primary reservoir for the Ap-variant 1 strain in the United States has been reported to be white-tailed deer (*15*). Although this strain has never been associated with a human infection, additional studies are needed to define its host range and pathogen potential. Our study presents molecular evidence of the presence of *A. phagocytophilum* in ticks in Israel and could have important implications for both medical and veterinary healthcare providers.

Table. PCR positivity to *Anaplasma phagocytophilum* in ticks collected from roe deer (*Capreolus capreolus*), Mount Carmel, Israel

Tick species	No. ticks tested	Ticks with *A. phagocytophilum* DNA
Rhipicephalus turanicus		
Females	25	2
Males	16	1
R. sanguineus	1	0
Hyalomma marginatum		
Females	4	0
Males	10	1
Boophilus kohlsi		
Males	1	0
Nymphs	13	1

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Sympatric Occurrence of *Taenia solium*, *T. saginata*, and *T. asiatica*, Thailand

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We confirmed sympatric occurrence of *Taenia solium, T. saginata,* and *T. asiatica* in western Thailand. DNA analysis of morphologically identified *T. saginata,* in a dual infection with *T. solium*, indicated it was *T. asiatica.* To our knowledge, this report is the first of *T. asiatica* and a dual *Taenia* infection from Thailand.

Taeniid tapeworm infections in the human intestine are caused by *Taenia solium, T. saginata,* and *T. asiatica* in Asia and the Pacific (*1–3*). Taeniasis caused by *T. solium* is a serious public health problem worldwide because eggs and proglottids expelled in the stool can infect humans through contamination of the environment and cause fatal neurocysticercosis. Neurocysticercosis cases caused by *T. solium* have increased in non–taeniasis-endemic areas (*3–5*).

A related taeniid tapeworm, Asian *Taenia* (= *T. asiatica*), has been described in Taiwan and the Republic of Korea (*1–3*,*6–8*). Although *T. asiatica* is phylogenetically closely related and is considered to be a sister species of *T. saginata* (*1–3*,*6*,*7*), the important intermediate host for *T. asiatica* is the domestic pig and the metacestodes mainly develop in the pigs' liver (*6*). The morphologic characteristics of adult *T. asiatica* are very similar to those of *T. saginata*. Morphologic differentiation by either scolex or gravid proglottid of these 2 species is practically impossible (*1*,*3*). On the basis of molecular analysis of taeniid isolates from Asia and the Pacific, *T. asiatica* is distributed in Taiwan, the Republic of Korea, Malaysia, People's Republic of China, Philippines, Indonesia, and Vietnam (*1–3*,*6–11*). However, there has been no evidence of the distribution of *T. asiatica* in Thailand (*8*,*12*).

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The Study

The field investigation was conducted during 2002–2005 in communities in Thong Pha Phum District, 150 km northwest of Kanchanaburi Province, Thailand, close to the Myanmar border (Figure 1). The region is mountainous terrain that acts as a natural border between Thailand and Myanmar, and it mostly comprises natural parks and

Figure 1. A) Map of Thailand showing Kanchanaburi Province (shaded area). B) The study area in Thong Pha Phum District (arrow).

[1]These authors contributed equally to this article.

a water reservoir. The major studied population is Karen; Mon and Thai are minorities. In our study, molecular identification of these taeniid samples was conducted in Asahikawa, Japan in 2006. The study team, the Faculty of Tropical Medicine at Mahidol University, obtained ethical approval for a human stool survey for control of helminthic infections.

All taeniasis patients were informed of the study objectives and worm collection procedure through discussions, including about expulsion of proglottids. Tapeworms, either with scolices or without scolices, were expelled from 24 persons with taeniasis after the persons received 2 g of niclosamide or 40 mg/kg of praziquantel and a purgative. A total of 29 scolices were confirmed. Scolices with hooks expelled from 5 patients (nos. 2, 4, 7, 9, 11) were identified as *T. solium.* Scolices without hooks from 12 patients were identified as *T. saginata* because no molecular evidence on the distribution of *T. asiatica* in Thailand exists and all *Taenia* scolices without hooks were confirmed to be *T. saginata* in other areas in Thailand (*8*,*10*,*12*). Morphologic identification of the species was based on scolex only. Specimens without scolices were not identified morphologically. Most patients (12/17) harbored a single scolex. However, several patients harbored 2–6 scolices, including 1 dual infection with 2 *T. solium* and 1 *T. saginata* (patient 7) (Table). Patients were 7–60 years of age; 16 were male, and 8 were female.

Nineteen *Taenia* samples were fixed in 80% ethanol and kept at –20°C until use. Four samples were fixed in 10% formalin. All scolices were fixed with alcohol-formalin-acetic acid and stained with acetocarmine for morphologic comparative examination. One scolex with or without hooklets each from a patient 7 was further processed for molecular studies.

DNA samples were extracted from taeniid proglottids except for patient 7, for whom DNA was extracted from 2 scolices. DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) was used for the samples kept in ethanol. A DNA Isolator PS kit (Wako Pure Chemicals, Osaka, Japan) and DEXPAT (TaKaRa Shuzo, Shiga, Japan) were used for the formalin-fixed proglottids. DNA samples from 2 scolices expelled from patient 7 stained with acetocarmine were prepared by using DEXPAT and 0.05 N NaOH/1% sodium dodecyl sulfate containing proteinase K. Mitochondrial DNA diagnosis of ethanol-fixed samples was performed by multiplex PCR by using cytochrome *c* oxidase subunit 1 gene (*cox1*), except for the use of a forward primer (5′-TTATTTATTTACGTCAATCTTATTG-3′, positions

Table. Characteristics of 24 taeniasis cases, Thailand, 2002–2005*

Patient				No. scolex/proglottids expelled	Morphologic identification of scolex	Preservative used	Molecular identification
No.	Sex	Age, y	Year				
1	M	60	2003	1 scolex without hook	*Taenia saginata*	NA	NT
2	F	34	2005	3 scolices with hooks	*T. solium*	NA	NT
3	M	55	2002	Segment without scolex†	–	Formalin	*T. solium*
4	M	29	2002	6 scolices with hooks	*T. solium*	Formalin	*T. solium*
5	F	38	2003	Segment without scolex†	–	Formalin	*T. solium*
6	F	46	2002	Segment without scolex†	–	Formalin	*T. solium*
7	F	28	2002	2 scolices with hooks, 1 scolex without hook	*T. solium,* *T. saginata*	AFA AFA	*T. solium* ***T. asiatica***‡
8	M	NK	2002	Segment without scolex†	–	Ethanol	*T. solium*
9	M	47	2004	1 scolex with hooks	*T. solium*	Ethanol	*T. solium*
10	M	7	2004	Segment without scolex†	–	Ethanol	*T. solium*
11	M	43	2004	3 scolices with hooks	*T. solium*	Ethanol	*T. solium*
12	M	10	2004	Segment without scolex†	–	Ethanol	*T. solium*
13	M	40	2003	Segment without scolex†	–	Ethanol	*T. saginata*
14	F	NK	2002	Segment without scolex†	–	Ethanol	*T. saginata*
15	M	40	2003	1 scolex without hook	*T. saginata*	Ethanol	*T. saginata*
16	M	55	2004	1 scolex without hook	*T. saginata*	Ethanol	*T. saginata*
17	F	30	2004	1 scolex without hook	*T. saginata*	Ethanol	*T. saginata*
18	M	13	2004	1 scolex without hook	*T. saginata*	Ethanol	*T. saginata*
19	M	45	2005	1 scolex without hook	*T. saginata*	Ethanol	*T. saginata*
20	F	42	2003	3 scolices without hook	*T. saginata*	Ethanol	***T. asiatica***‡§
21	F	28	2005	1 scolex without hook	*T. saginata*	Ethanol	***T. asiatica***‡
22	M	60	2005	1 scolex without hook	*T. saginata*	Ethanol	***T. asiatica***‡
23	M	32	2005	1 scolex without hook	*T. saginata*	Ethanol	***T. asiatica***‡
24	M	37	2005	1 scolex without hook	*T. saginata*	Ethanol	***T. asiatica***‡

*Year refers to year when specimen was collected; NA, not available; NT, not tested; Formalin, 10% formalin; AFA, alcohol-formalin-acetic acid; NK, not known; ethanol, 80% ethanol.
†These segments without scolices were not examined for morphologic identification.
‡These cases (**boldface**) were identified as *T. saginata* morphologically but were confirmed to be *T. asiatica* by mitochondrial DNA analysis.
§Three worms fixed separately.

561–585) for *T. asiatica* (*10*). The formalin-fixed and acetocarmine-stained specimens were identified by base excision sequence scanning thymine-base (BESS T-base) analysis that used either *cox1* or cytochrome *b* gene (*cob*) (*13*). For BESS T-base analysis, the following primers were used: F3 (5′-TATTTGATCGTAAATTTAGTTCT-3′, corresponding to nucleotide (nt) positions 629–651) and R7 (5′-ATTAACACATAAACCTCGGGA-3′, nt positions 740–720) for *cox1* of *T. solium,* F1 (5′-GTCAAAA-GATTCTTTTTTACTTGGT-3′, nt positions 180–205) and R2 (5′-CCCTTCTTTCTATAACTTGAATAAT-3′, nt positions 305–281) for *cob* for *T. solium*. DNA sequencing of the products amplified by multiplex PCR was performed for confirmation. DNA samples for sequencing were prepared with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). DNA sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), and the nucleotide sequence data were analyzed by using DNASTAR version 3.75 (DNASTAR Inc., Madison, WI, USA).

Multiplex PCR applied on 19 proglottids from 17 patients with *cox1* (*10*) showed that 5, 7, and 7 proglottids were *T. solium* (Asian genotype) (*10*,*13*), *T. saginata,* and *T. asiatica*, respectively (data not shown). These results were supported by DNA sequencing of the amplicons (data not shown). By contrast, small sizes of 112-bp *cox1* products were successfully amplified from samples taken from patients 3–6. These samples had been preserved in 10% formalin for years and BESS T-base analysis indicated that they were *T. solium* (Asian genotype) (Figure 2A) (*14*). BESS T-base analysis showed that scolices with and without hooklets from a dual infection (patient 7) were *T. solium* (Asian genotype) and *T. asiatica*, respectively (Figure 2B and C). To our knowledge, this is the first report demonstrating a dual infection with *T. solium* and *T. asiatica* in which 3 human taeniid cestodes are sympatrically distributed (*1*).

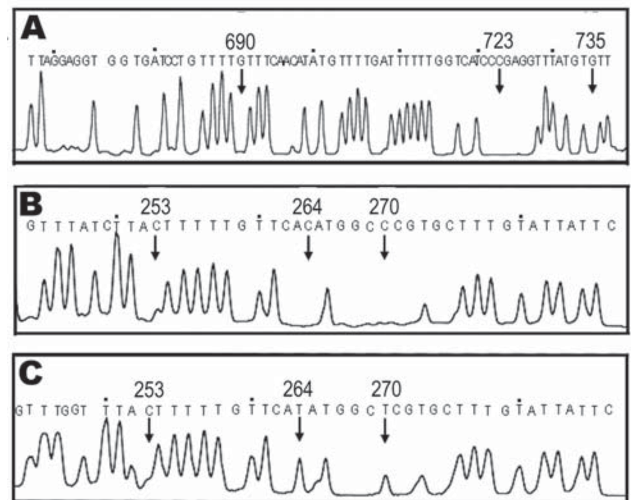

Figure 2. Molecular identification of formalin-fixed and acetocarmine-stained samples by base excision sequence scanning thymine-base (BESS) T-base analysis. BESS T-base profiles are shown in panels A, B, and C. A) Thymine-base profile using the 112-bp *cox1* products from patient samples 3–6. B) and C) BESS T-base analysis data that used 136-bp *cob* products from scolices with and without hooklets, respectively. Arrows indicate diagnostic positions. Nucleotide sequences indicated above the peaks are from GenBank databases (AB066485 for *cox1*, AB066570 and AB066580 for *cob*).

Conclusions

We documented sympatric distribution of *T. solium*, *T. saginata,* and *T. asiatica* in western Thailand on the basis of mitochondrial DNA analysis. Our study indicated that 53.3% (8/15) of taeniid specimens expected to be *T. saginata* were *T. asiatica* and that both *T. asiatica* and *T. saginata* are codistributed in Kanchanaburi Province. Although *T. solium* taeniasis has seldom been reported in the literature in Thailand (*15*), our study has shown infection with *T. solium* (Asian genotype) in 11 (45.8%) of 24 *Taenia*-infected patients. The number of *T. solium* organisms expelled from taeniasis patients varied from 1 to 6, and $\geq$2 tapeworms were found in 36.4% (4/11) of *T. solium* taeniasis patients. In addition, we confirmed a dual infection with *T. solium* and *T. asiatica* (in patient 7). This experience indicates that molecular analysis is preferable and necessary for precise re-identification of so-called *T. saginata* in Asia and the Pacific (*1*).

Although *T. solium* cysticercosis in humans has not been reported in this study area, these populations appear to pose a risk for environmental contamination and person-to-person spread of *T. solium* leading to cysticercosis in humans and swine. Raw or inadequately cooked beef, pork, or pig viscera, and fresh blood are commonly consumed by local people in the study areas, and consequently they are at high risk of acquiring taeniasis. Therefore, to improve sanitation and quality of life, sustainable health education should be introduced and stressed to the population in the community.

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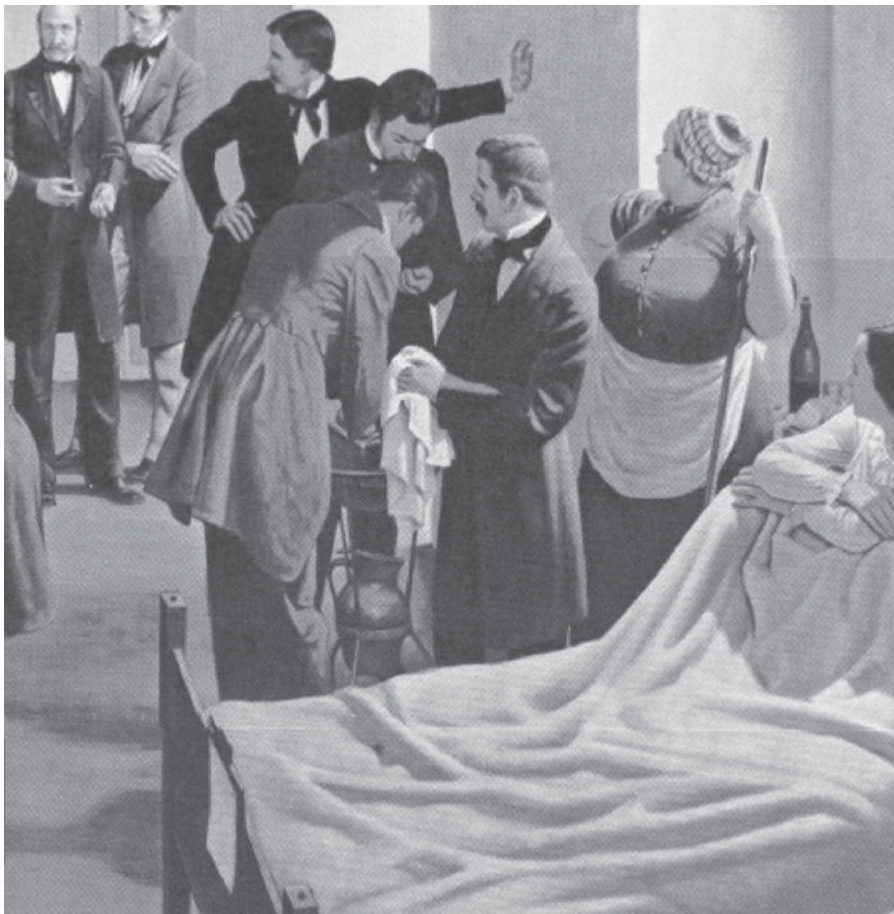

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Increase in *Clostridium difficile*–related Mortality Rates, United States, 1999–2004

Matthew D. Redelings,* Frank Sorvillo,*† and Laurene Mascola*

Reported mortality rates from *Clostridium difficile* disease in the United States increased from 5.7 per million population in 1999 to 23.7 per million in 2004. Increased rates may be due to emergence of a highly virulent strain of *C. difficile*. Rates were higher for whites than for other racial/ethnic groups.

Clostridium difficile is an anaerobic, gram-positive bacillus that can cause considerable disease, including diarrhea, colitis, and septicemia, resulting in death (*1*). *C. difficile*–associated disease (CDAD) primarily affects persons ≥65 years. Risk factors include residence in hospitals and long-term care facilities and the use of antimicrobial medications (*1–3*). Incidence of CDAD has been increasing, and severe cases are becoming more common (*4*,*5*). These changes in the incidence and severity of CDAD may be associated with the emergence of a more virulent strain of *C. difficile* bacteria (*5*,*6*). Death rates associated with *C. difficile* were reported to be increasing from 1999 to 2002 in the United States and from 2001 to 2005 in England and Wales (*7*,*8*). However, no trend analysis was conducted to evaluate the rate of increase. We incorporated mortality data for the United States through the year 2004 to conduct trend analyses of CDAD-related deaths and to examine demographic characteristics and coexisting conditions reported in deaths from *C. difficile* infection.

The Study

CDAD-related deaths were identified by using multiple cause-of-death data from national mortality records for 1999–2004. CDAD-related deaths were defined as all deaths for which the underlying cause of death or any of the contributing causes of death included the International Classification of Diseases, 10th revision (ICD-10) code A04.7 (enterocolitis due to *C. difficile*). Information about the size and demographic breakdown of the US population for each year during 1999–2004 was obtained from censal and intercensal year estimates with bridged race data (*9*,*10*). Age-adjusted mortality rates were calculated by using the age distribution of the 2000 US population as a standard (*11*). The US population was divided into 5 racial/ethnic categories: white, Hispanic, Asian/Pacific Islander, black, and American Indian/Alaska Native.

During 1999–2004, CDAD was reported as a cause of death for 20,642 persons. CDAD was reported as the underlying cause for 12,264 (59%) of these deaths. A total of 3,256 deaths were reported related to all other intestinal infectious diseases combined (ICD-10 codes A00 to A09, excluding A047) over the same period. The median age of death for CDAD patients was 82 years. Age-adjusted mortality rates from CDAD were slightly higher for men than for women (Table) and were higher for whites than for any other racial/ethnic group. Most CDAD-related deaths occurred in hospitals (n = 16,557, 80%); 1,634 (8%) occurred in long-term care facilities, and 2,027 (10%) occurred at home.

Common coexisting conditions for CDAD-related deaths included septicemia (n = 7,654, 37%), renal failure (n = 4,786, 23%), pneumonia (n = 3,430, 17%), urinary tract infection (n = 1,496, 7%), and anemia (n = 785, 4%). HIV was reported for 81 CDAD-related deaths (<1%). However, among the 697 deaths reported in persons 25–54 years of age, HIV was reported for 72 (10%).

The overall rate of *C. difficile*–related deaths during the study period was 12.2 deaths per million population. Mortality rates related to CDAD increased during the study period (Figure), rising from 5.7 deaths per million population in 1999 to 23.7 deaths per million population in 2004. Poisson regression estimates showed mortality rates increased by 35% per year (coefficient = 0.30, standard error = 0.004, 95% confidence interval = 0.29–0.31) during the study period.

Conclusions

Due to the inclusion of CDAD-related deaths when CDAD was not reported as the underlying cause of death, reported death rates in this study were higher than those published in an earlier analysis of CDAD-related deaths in the United States (*7*). *C. difficile* is a cause of a substantial and increasing proportion of deaths in the United States and may be underrecognized as a cause of death. Little attention has been paid to *C. difficile* prevention; media and public health awareness efforts have focused largely on the prevention of disease from other intestinal pathogens such as *Escherichia coli* or *Salmonella* spp. However, the incidence of deaths from *C. difficile* is greater than the extent of deaths from all other intestinal infectious diseases combined. *C. difficile*–related mortality rates were higher

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Table. Demographic characteristics of patients with *Clostridium difficile*–related deaths, United States, 1999–2004

Demographic group	*C. difficile*–related deaths, no. (%)	Age-adjusted mortality rate/million population
Sex		
Female	12,468 (60)	11.8
Male	8,174 (40)	12.7
Race/ethnicity		
White	18,534 (90)	12.9
Hispanic	602 (3)	7.2
Black	1.304 (6)	9.3
Asian/Pacific Islander	139 (1)	3.5
American Indian/Alaska Native	63 (<1)	7.9
Age group, y		
<1	17 (<1)	0.7*
1–4	11 (<1)	0.1*
5–14	12 (<1)	0.1*
15–24	24 (<1)	0.1*
25–34	62 (<1)	0.3*
35–44	171 (1)	0.6*
45–54	464 (2)	2.0*
55–64	1,159 (6)	7.6*
65–74	3,238 (16)	29.3*
75–84	7,859 (38)	104.0*
≥85	7,623 (37)	287.1*
Total	20,642	12.2

*This statistic is not age-adjusted because it only pertains to 1 age group.

for whites than for other racial/ethnic groups. Racial/ethnic differences in insurance status and access to care (*12*) may render elderly whites more likely to receive treatment with antimicrobial drugs that put them at risk for *C. difficile* infection. However, genetic or other factors may also be involved, and further research is needed to determine the causes of racial/ethnic differences in *C. difficile*–related deaths.

Previous research showed increases in CDAD-related mortality rates in the United States until 2002 (*7*,*8*). This analysis estimates the rate of increase at 35% per year, and shows that mortality rates continued to increase at least until 2004. Increases in incidence and deaths from CDAD may be associated with the emergence of a new and more virulent strain of *C. difficile* (*5*). The emergence of virulent strains of *C. difficile* makes continued assessment of mortality statistics important.

Infection with *C. difficile* is associated with recent use of antimicrobial medications and with residence in hospitals. Most CDAD cases are acquired in healthcare settings (*1*), and as many as 90% of cases may be associated with antimicrobial drug use (*2*,*3*). High *C. difficile* death rates call attention to the importance of proper infection control practices in hospitals and long-term care facilities and the judicious use of antimicrobial medications. Further research is needed to explore current questions concerning which antimicrobial medications, if any, will lead to CDAD (*13*,*14*).

Infections such as septicemia, pneumonia, and urinary tract infections were commonly reported in conjunction with *C. difficile*–related deaths. For some of these patients, the administration of antimicrobial medications to treat infections from other pathogens may have paved the way for infection with *C. difficile*. However, other risk factors are known, so that in many cases the careful use of antimicrobial agents may not be enough to prevent *C. difficile* infection. HIV infection was only reported in a small fraction of CDAD–related deaths. However, immunosuppression and the use of prophylactic antimicrobial drugs in persons with AIDS may increase the risk for CDAD (*15*), and the effects of HIV should not be overlooked. In persons 25–54 years

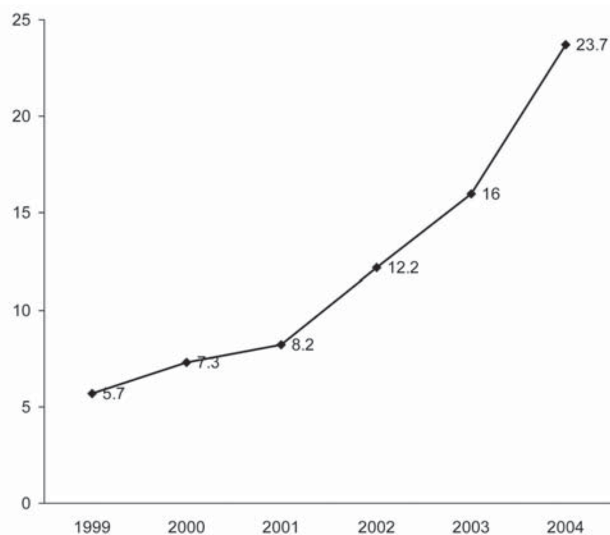

Figure. Yearly *Clostridium difficile*–related mortality rates per million population, United States, 1999–2004.

of age, in whom HIV infection is most common, HIV infection was reported in approximately one tenth of CDAD-related deaths. Thus, HIV can considerably increase *C. difficile* death rates for demographic groups in which HIV prevalence is high.

Death certificate data may underrepresent the extent of CDAD-related deaths. This analysis was limited to deaths in which ICD-10 code A047 (enterocolitis due to *C. difficile*) was mentioned and may have failed to capture CDAD-related deaths in which colitis was not present. In addition, death certificate data may be affected by reporting error. Supplemental information such as decedents' medical histories was unavailable. No data were available regarding which strains of *C. difficile* were responsible for reported CDAD–related deaths.

C. difficile is an underrecognized cause of severe illness and death. This analysis underscores the importance of CDAD as a public health problem and the increasing incidence of CDAD-related deaths in the United States.

Mr Redelings is an epidemiology analyst with the Los Angeles County Department of Public Health. He previously served as a humanitarian worker in Sudan. His current research interests include the epidemiology of gastrointestinal infections and the prevention of disease in refugee situations.

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Hantavirus in Northern Short-tailed Shrew, United States

Satoru Arai,* Jin-Won Song,† Laarni Sumibcay,* Shannon N. Bennett,* Vivek R. Nerurkar,* Cheryl Parmenter,‡ Joseph A. Cook,‡ Terry L. Yates,‡ and Richard Yanagihara*

Phylogenetic analyses, based on partial medium- and large-segment sequences, support an ancient evolutionary origin of a genetically distinct hantavirus detected by reverse transcription–PCR in tissues of northern short-tailed shrews (*Blarina brevicauda*) captured in Minnesota in August 1998. To our knowledge, this is the first evidence of hantaviruses harbored by shrews in the Americas.

Rodents and their ectoparasites serve as reservoirs and vectors of myriad viruses and other pathogenic microbes. In contrast, the role of insectivores (or soricomorphs) in the transmission and ecology of zoonoses is largely unknown. Because some soricomorphs share habitats with rodents, shrews might also be involved in the maintenance of the enzootic cycle and contribute to the evolutionary history and genetic diversity of hantaviruses.

Hantavirus antigens have been detected in the Eurasian common shrew (*Sorex araneus*), alpine shrew (*Sorex alpinus*), Eurasian water shrew (*Neomys fodiens*), and common mole (*Talpa europea*) in Russia and the former Yugoslavia (*1–3*). More than 20 years ago, when Prospect Hill virus was discovered in meadow voles (*Microtus pennsylvanicus*) captured in Frederick, Maryland, USA, serologic evidence suggestive of hantavirus infection was found in the northern short-tailed shrew (*Blarina brevicauda*) (*4*). However, virus isolation attempts were unsuccessful, and molecular tools such as PCR were unavailable. Empowered by robust gene-amplification techniques and the complete genome of Thottapalayam virus (TPMV) isolated from the Asian house shrew (*Suncus murinus*) (*5,6*), we have identified a genetically distinct hantavirus in the northern short-tailed shrew.

The Study

After obtaining approval from the University of Hawaii Institutional Animal Care and Use Committee, we retrieved lung and liver tissues of 30 northern short-tailed shrews captured from several regions within the United States during 1994–1999 (Table 1) from deep-freeze storage at the University of New Mexico Museum of Southwestern Biology. Total RNA was extracted from shrew tissues by using the PureLink Micro-to-Midi Total RNA Purification Kit (Invitrogen, San Diego, CA, USA). cDNA was then prepared by using the SuperScript III First-Strand Synthesis System (Invitrogen) for reverse transcription–PCR (RT-PCR) with oligonucleotide primers designed from TPMV and other hantaviruses: medium (M) (outer: 5′-GGACCAGGTGCADCTTGTGAAGC-3′, 5′-GAACCCCADGCCCCNTCYAT-3′; inner: 5′-TAAVTTCAMCAACATGTCT-3′, 5′-CATGAYATCTCCAGGGTCHCC-3′) and large (L) (outer: 5′-CAGTCWACARTTGGTGCAAGTGG-3′, 5′-TCCATKATWGACATBGMRCCA-3′; inner: 5′-YTMATGTATGTTAGTGCAGATGC-3′, 5′-GRTTAAACATACTCTTCCACATCTC-3′). For confirmation, RNA extraction and RT-PCR were performed independently in a laboratory in which hantaviruses had never been handled. Amplicons were sequenced directly by using an ABI Prism 377XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Of the 30 northern short-tailed shrews tested, hantavirus M-segment sequences were amplified from lung tissues of 3 of 12 animals captured in Camp Ripley (46.185°N, 94.4337°W), a 53,000-acre, state-owned military and civilian training center near Little Falls, in Morrison County, Minnesota, USA, in August 1998 (Table 1). Pairwise alignment and comparison of the 1,390-nt region (463 aa) spanning the Gn and Gc glycoprotein–encoding M segment indicated differences of 33.6%–41.9% and 32.7%–47.4% at the nucleotide and amino acid levels, respectively, from representative hantaviruses harbored by *Murinae*, *Arvicolinae*, *Neotominae*, and *Sigmodontinae* rodents (Table 2). No insertions or deletions were found in the regions sequenced compared with sequences of other hantaviruses. The new hantavirus, designated Camp Ripley virus (RPLV), showed sequence similarity of 98.1%–98.5% among the 3 strains.

Analysis of a 490-nt (163-aa) region of the L genomic segment amplified from 2 of the 3 shrews indicat-

Table 1. Reverse transcription–PCR detection of hantavirus sequences in tissues of *Blarina brevicauda*, United States

State	County	Trapping date	No. tested	No. positive
Indiana	Porter	Jul 1994	2	0
	Westchester	Jul 1994	1	0
Iowa	Allamakee	Aug 1994	5	0
Maryland	Charles	Sep 1997	3	0
Michigan	Benzie	Jul 1994	1	0
	Crawford	Jul 1999	2	0
Minnesota	Morrison	Aug 1998	12	3
Ohio	Summit	Jul 1994	2	0
Virginia	Appomatox	Jul 1994	1	0
	Page	Mar 1995	1	0

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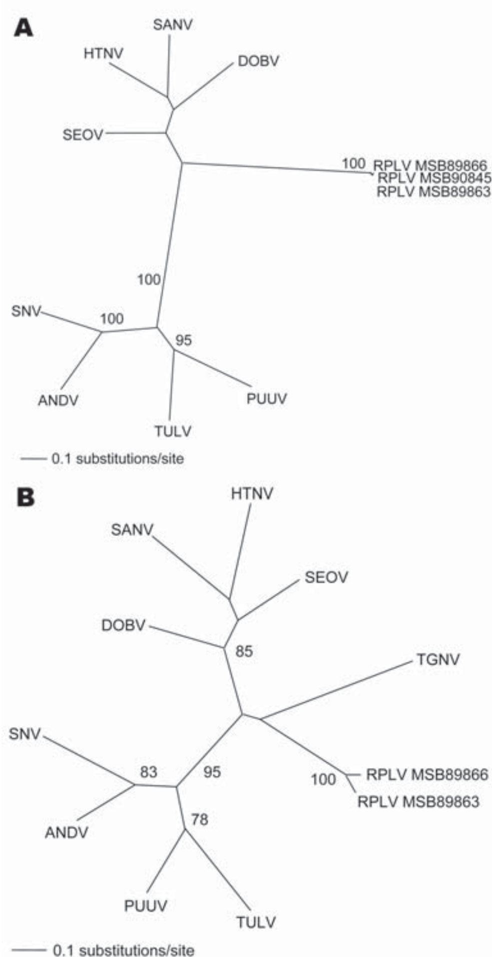

Figure. Phylogenetic trees generated by maximum likelihood method and generalized time reversible + I + G model of evolution as estimated from data on alignment of the partial (A) 1,390-nt medium (M)– and (B) 490-nt large (L)–genomic segments of Camp Ripley virus (RPLV). Phylogenetic positions of RPLV strains MSB89863, MSB89866, and MSB90845 are shown in relationship to representative Murinae rodentborne hantaviruses, including Hantaan virus (HTNV 76–118, NC_005219, NC_005222), Sangassou virus (SANV SA14, DQ268651, DQ268652), Dobrava virus (DOBV AP99, NC_005234, NC_005235), and Seoul virus (SEOV 80 39, NC_005237, NC_005238); Arvicolinae rodentborne hantaviruses, including Tula virus (TULV M5302v, NC_005228, NC_005226) and Puumala virus (PUUV Sotkamo, NC_005223, NC_005225); and Neotominae and Sigmodontinae rodentborne hantaviruses, including Andes virus (ANDV Chile 9717869, NC_003467, NC_003468) and Sin Nombre virus (SNV NMH10, NC_005215, NC_005217). Tanganya virus (TGNV Tan826, EF050454) from the Therese shrew (*Crocidura theresae*) is also shown. Host identification of *Blarina brevicauda* was confirmed by morphologic assessment of voucher specimens and by mitochondrial DNA sequences (data not shown). The numbers at each node are bootstrap support values (expressed as the percentage of replicates in which the node was recovered), as determined for 100 maximum likelihood iterations under the same model of evolution by PAUP version 4.0 (http://paup.csit.fsu.edu). The scale bar indicates 0.1 nt substitutions per site. GenBank accession nos. for RPLV: M (EF540774, EF540775, EF540773) and L (EF540771, EF540772).

ed that RPLV differed from rodentborne hantaviruses by 26.8%–34.1% at the nucleotide level and 20.7%–34.5% at the amino acid level (Table 2). RPLV differed from Tanganya virus (TGNV), a hantavirus detected recently in the Therese shrew (*Crocidura theresae*) in Guinea (*7*), by 27.3%–28.8% and 24.0%–25.0%, respectively. The higher degree of sequence similarity in the L segment between RPLV and other hantaviruses probably signifies the limits of functional preservation of the RNA-dependent RNA polymerase.

Repeated and exhaustive phylogenetic analyses based on nucleotide and deduced amino acid sequences of the M and L genomic segments generated by the maximum-likelihood method indicated that RPLV was distinct from rodentborne hantaviruses (with high bootstrap support based on 100 maximum likelihood replicates) (Figure). Similar topologies were consistently derived by using various algorithms and different taxa (including La Crosse virus) and combinations of taxa, which suggested an ancient evolutionary origin. However, definitive conclusions about the molecular phylogeny of RPLV and its relationship to TGNV and other soricidborne hantaviruses must await complete-genome sequence analyses.

Conclusions

As we had previously encountered in sequencing the entire genome of TPMV (J.-W. Song, R. Yanagihara, unpub. data), the divergent genome of RPLV presented challenges in designing suitable primers for RT-PCR. We were also constrained by the limited availability of tissues from the 3 infected shrews and the need to retain small portions of tissues for future virus isolation attempts. Consequently, we have been hitherto unable to obtain the full-length sequence of RPLV.

The northern short-tailed shrew (family *Soricidae*, subfamily *Soricinae*), 1 of 2 poisonous mammals in North America (*8*), inhabits forests and grasslands within the central and eastern half of the United States, extending north to Canada, west to Montana, and south to Tennessee and Georgia. Cytochrome *b* mitochondrial DNA and 16S rRNA sequence analyses support a monophyletic origin for the genus *Blarina*, with phylogeographic structuring of northern short-tailed shrews into well-defined groups to the east and west of the Mississippi River basin (*9*). Current studies will examine whether RPLV is harbored by the eastern haplogroup of northern short-tailed shrews and by the southern short-tailed shrew (*Blarina carolinensis*), a closely related species, which inhabits the southeastern United States, extending as far north as southern Illinois and south-central Virginia and as far south as central Florida.

Given the sympatric and synchronistic coexistence of northern short-tailed shrews with *Neotominae* and *Arvicolinae* rodents (such as *Peromyscus leucopus* and *Microtus*

Table 2. Nucleotide and amino acid sequence similarities of partial medium (M) and large (L) segments of Camp Ripley virus and other hantaviruses*

Virus	Strain	M Segment		L Segment	
		1,390 nt	436 aa	490 nt	163 aa
Hantaan	76–118	65.6	66.9	71.2	79.3
Soochong	SC-1	65.2	64.4	73.2	77.3
Dobrava	AP99	66.4	67.3	72.5	79.0
Seoul	HR80–39	65.8	67.1	71.3	77.7
Sangassou	SA14	62.6	58.7	69.3	78.8
Puumala	Sotkamo	60.1	55.0	71.6	72.3
Tula	M5302v	61.9	54.6	67.2	69.3
Prospect Hill	PH-1	58.1	52.6	NA	NA
Sin Nombre	NMH10	60.3	57.0	65.9	65.5
Andes	Chile 9717869	60.2	56.3	70.2	69.2
El Moro Canyon	RM97	59.8	56.5	NA	NA
Tanganya	Tan826	NA	NA	72.3	75.5

*Values are percentages. NA, not available.

pennsylvanicus) and their ferocious territorial behavior, hantavirus spillover may be possible. Viruses closely related antigenically to Hantaan virus have been isolated from the Asian house shrew (*Suncus murinus*), greater white-toothed shrew (*Crocidura russula*), and Chinese mole shrew (*Anourosorex squamipes*) (*10–12*), which suggests that shrews are capable of serving as incidental hosts for hantaviruses typically harbored by rodents.

Shrews that harbor genetically distinct hantaviruses pose a compelling conceptual framework that challenges the long-accepted dogma that rodents are the sole reservoirs of hantaviruses. Viewed within the context of the recent detection of TGNV in the Therese shrew in Guinea, the identification of RPLV in the northern short-tailed shrew in the United States indicates that renewed efforts, facilitated by the rapidly expanding sequence database of shrewborne hantaviruses, will lead to the discovery of additional hantaviruses in soricids throughout Eurasia, Africa, and the Americas. Our preliminary studies indicate 3 other novel soricidborne hantaviruses in the Republic of Korea, Vietnam, and Switzerland. To establish if ≥1 of these newly identified hantaviruses is pathogenic for humans will require development of robust serologic assays (*13*) and application of other sensitive technologies, such as microarray analysis (*14*,*15*), for rapid detection of shrewborne hantavirus RNA in human tissues and bodily fluids.

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Imported Fatal Hantavirus Pulmonary Syndrome

To the Editor: Hantavirus pulmonary syndrome (HPS) is characterized by fever, gastrointestinal symptoms, respiratory distress, elevated hematocrit, hypoalbuminemia, and thrombocytopenia. Most cases in North America are acquired from rodent vectors and are caused by the Sin Nombre virus. Person-to-person transmission has been reported for Andes virus (*1*,*2*) but not for Sin Nombre virus (*3*). We describe a patient with fatal hantavirus pulmonary syndrome.

The patient was a previously healthy 15-year-old Canadian girl. In the spring of 2006, she had traveled to the Santa Cruz-San Jose de Chiquitas corridor of Bolivia with her parents and siblings for a 4-week visit (Figure), where they stayed with family and friends on their farms. The family noted rodent droppings outside but no rodents were seen. The patient had no known exposure to rodents or rodent droppings after her return to Canada.

On day 26 after her return from Bolivia, the patient sought treatment at a community hospital at 6:30 AM for malaise and mild fever. During the night before seeking treatment, she had mild confusion. Her initial blood pressure was 99/50, heart rate 97, and oxygen saturation 96% on room air. Her hemoglobin was 192 g/L (reference range 117–149), platelets 82 × 10^9/L (reference range 165–400), and leukocyte count 7.5 × 10^9/L (reference range 3.9–10.2). She was initially treated with 3 liters of normal saline and repeat hemoglobin tests showed a value of 206 g/L. Due to ongoing hypotension and hypoxia, she was intubated and sedated. Rocuronium was used as a paralytic agent to facilititate high pressure mechanical ventilation and maintain patient-ventilator synchrony. Dopamine and epinephrine were given as intravenous drips. Arrangements were made for the patient to be transferred to a tertiary pediatric care center for possible extracorporeal membrane oxygenation. During air transport, she had an asystolic cardiac arrest. While administering cardiopulmonary resuscitation, members of the healthcare team were exposed to a considerable volume of pulmonary edema fluid expelled from the patient's endotracheal tube. Few, if any, were able to maintain adequate protection with face shields or protective eyewear. Resuscitation efforts were unsuccessful, and the patient was pronounced dead on arrival at the tertiary care center at 7:26 PM. Postmortem examination showed evidence of marked pulmonary edema, diffuse alveolar damage, and lymphoid inflammation in the pulmonary interstitium.

Serologic examination of an acute blood sample was immunoglobulin M (IgM) positive for Sin Nombre virus, but low optical densities indicated potential for an infection with a related hantavirus rather than Sin Nombre virus. Subsequently, reverse transcription–PCR (RT–PCR) on blood in EDTA and lung tissue followed by sequence analysis confirmed an Andes-like hantavirus infection. None of the 40 household and healthcare contacts of the patient had symptoms compatible with HPS during an 8-week monitoring period. Two contacts with nonspecific symptoms were tested and found to be negative for hantavirus-specific IgM and IgG and negative by RT-PCR.

Additonally, a seroprevalence survey of close contacts and assessment of level of contact was conducted. Close contacts were defined as persons who lived in the same household as the patient, were in the same enclosed space for >2 hours, or provided healthcare to her while she was symptomatic. Twenty-eight (62%) of 45 close contacts provided serum over the next 5 months. All serum samples were negative for Sin Nombre and Andes IgG and Sin Nombre IgM by ELISA. Fourteen (50%) of the 28 completed a self-administered questionnaire which assessed the type and intensity of contact. Of these, 12 were healthcare workers and 2 were friends. One friend had contact with

Figure. Map of Bolivia with an inset map of North America showing the location of British Columbia (BC) and its relation to Bolivia.

the patient 3 days before she died, a friend and a healthcare worker had contact with her on the day before her death, and the rest of the healthcare workers had contact with the patient on the day she died.

To our knowledge, this is the first imported case and the tenth case of HPS reported in British Columbia, Canada, since 1994 (2006 BC Annual Summary of Reportable Diseases, available from www.bccdc.org/content.php?item=33) (*4*). Six of these 10 cases were fatal. All cases except the 1 described here have been locally acquired Sin Nombre infections. Sin Nombre virus is endemic in the *Peromyscus maniculatus* (deer mice) population in most of British Columbia (*5*).

Worldwide, imported cases of HPS are unusual, although HPS has been reported in countries that are in close geographic proximity or in travelers to disease-endemic areas (*6*–*8*). Fortunately, none of the persons exposed to the patient reported symptoms consistent with HPS during the incubation period, and none who were tested seroconverted. Seroprevalence surveys in Chile among healthcare worker contacts of patients with HPS caused by the Andes virus showed a prevalence of 0% (*9*). A report from Argentina showed that cases due to secondary transmission occurred mostly in nonhealthcare workers after prolonged close contact in the prodromal period (*10*). In conclusion, we describe an imported case of fatal HPS due to an Andes-like hantavirus with no evidence of secondary transmission.

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Disseminated Bocavirus Infection after Stem Cell Transplant

To the Editor: Human bocavirus (HBoV) (*1*) is increasingly recognized as a cause of respiratory infections worldwide. Children and infants appear to be most at risk (*2*–*7*), although HBoV's role in immunocompromised patients remains unclear. We report on a child with disseminated HBoV infection after hematopoietic stem cell transplantation (HSCT). HBoV DNA was detected at high levels in nasopharyngeal aspirates (NPAs) and in blood and stool samples.

A 4.5-year-old boy with dyskeratosis congenita was brought for treatment to our hospital due to severe persistent cytopenia. Allogenic HSCT was performed in August 2006 after conditioning with total body irradiation (200 cGy, day –8 before HSCT surgery), fludarabine (days –7 to –4), antithymocyte globulin (days –4 to –1), and cyclophosphamide (days –3

to –2). He received 7.16×10^8 nucleated bone marrow cells/kg body weight from a 9/10 human leukocyte antigen –matched unrelated donor. Graft-versus-host disease (GvHD) prophylaxis consisted of a short course of methotrexate and cyclosporin A. Neutrophil and platelet engraftment occurred on days 22 and 65 after surgery, respectively. Despite pre- and post-HSCT anti-infective prophylaxis with cotrimoxazole, colistin, acyclovir, and fluconazole, *Enterobacter cloacae* sepsis was diagnosed on day 2. After meropenem treatment, blood cultures remained negative. On day 12, fever reoccurred, elevated C-reactive protein values (229 mg/L) and reduced general health were noted, but no bacterial pathogen was isolated. During this period, the patient received antimicrobial drug therapy with meropenem, tobramycin, vancomycin, and amphotericin B. On day 16, his body temperature peaked to 40.6°C, and a cough and dyspnea without wheezing developed. Chest radiograph results suggested pneumonia with perihilar infiltrates. Reduced oxygen saturation (pO_2 86%) was recorded transcutaneously, and oxygen supplementation (maximum 4 L/min) was started by face mask (online Appendix Figure, available from www.cdc.gov/EID/content/13/9/1425-appG.htm). An NPA sample investigated by multiplex PCR (results provided by W. Puppe and J. Weigl; www.pid-ari.net) was negative for adenovirus, respiratory syncytial virus, human metapneumovirus, parainfluenza viruses 1–4, influenza viruses A and B, coronavirus, reovirus, enterovirus, *Clamydia pneumoniae*, *Mycoplasma pneumoniae*, *Bordetella pertussis*, *B. parapertussis*, and *Legionella pneumophila,* but positive for rhinovirus RNA. Retrospectively, the same NPA sample was reanalyzed for HBoV DNA by real-time PCR (*7*) and showed a viral load of 4.6×10^7 copies/mL (online Appendix Figure); specificity was confirmed by sequencing.

From day 19 on, the patient's general health improved and the chest radiograph results returned to normal. After neutrophil engraftment (day 22) and addition of erythromycin to the antimicrobial drug regimen, body temperature decreased and oxygen supplementation was discontinued. However, rhinitis, cough, and low-grade fever (<38.5°C) persisted until day 50 (online Appendix Figure), and HBoV DNA was detected in NPAs on days 37 and 44 at 2.4×10^{11} and 1.3×10^{14} copies/mL, respectively (online Appendix Figure). The NPA sample on day 37 was still rhinovirus positive.

Concurrent with the increased HBoV load in NPAs, cytomegalovirus (CMV) reactivation was first diagnosed by PCR on day 20 and peaked (58.250 copies/mL whole blood) on day 41 despite gancyclovir therapy. Switching to foscarnet led to temporary control of CMV replication (online Appendix Figure). Additionally, on day 22, acute GvHD grade I with skin manifestations developed. Treatment with steroids until day 60 led to complete resolution.

HBoV infection in this patient was not restricted to the respiratory tract. Diarrheic stool samples obtained on day 21 and, after resolution of respiratory symptoms, on day 75 showed substantial HBoV DNA (2.5×10^6 and 6.0×10^5 copies/mg, respectively; online Appendix Figure). Tests for rotavirus and adenovirus antigens were negative, and no bacterial pathogen was isolated. Moreover, HBoV DNA was detected at lower levels (3.7×10^3 to 7.8×10^4 copies/mL) in 4 EDTA plasma samples taken days 21–47. Subsequent plasma (days 61, 68, 75, 88, 219), NPA (day 219), and stool (day 219) samples were negative for HBoV DNA. However, the ability of HBoV to cause persistent infection, as do other members of the *Parvovirinae* subfamily, cannot be excluded. Future investigations are needed to address this hypothesis.

Here, we report on disseminated HBoV infection in an immunocompromised patient. Whether the clinical course in this case was more severe or prolonged than it would have been for HBoV infections in non-HSCT children remains unknown due to the lack of long-term observations in immunocompetent children. The dramatic increase of HBoV load in NPAs and viral dissemination most likely resulted from progressive impairment of cellular immunity as indicated by simultaneous CMV reactivation. Moreover, the increased viral load might have also been a consequence of steroid addition to immunosuppressive therapy to control GvHD. The contribution of HBoV to respiratory disease remains ambiguous because 2 NPA samples were also rhinovirus positive. Additional studies are required to investigate the pathogenic role of HBoV in double or multiple infections. Association of HBoV with the patient's continued diarrhea is in accordance with previous studies (*8*–*10*). Prolonged fecal shedding has important implications for isolation measures in transplantation units. More studies in immunocompromised patients are required to evaluate the spectrum of pathology caused by this emerging virus.

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Pure Red Blood Cell Aplasia and Isoniazid Use

To the Editor: Isoniazid is a first-line drug in the treatment of tuberculosis; its uses include prevention as well as cure. Isoniazid is usually well tolerated, although its common side effects include gastrointestinal discomfort, rash, allergy, hepatitis, and peripheral neuropathy. Hematologic disorders such as eosinophilia, thrombocytopenia, and autoimmune hemolytic anemia are rarely reported (*1*). Pure red cell aplasia (PRCA) is an uncommon disorder in adults. PRCA occurs secondary to drug exposure in 5% of patients; ≈30 drugs have been implicated (*2*), and few reports involving isoniazid have been published. We report a case of isoniazid-induced PRCA.

In 2006, a 79-year-old woman sought care at a public assistance hospital in Paris; she reported having had asthenia, breathlessness, and decreased appetite for 2 weeks. She had had node-negative, localized gastric adenocarcinoma 2 years earlier, which had been treated by partial gastrectomy, and pulmonary tuberculosis in 1948, which had been treated by partial pneumonectomy. As a result of a pleuropulmonary tuberculosis relapse diagnosed 6 weeks earlier, she was receiving antituberculous treatment with rifampin (400 mg/day), isoniazid (150 mg/day), ethambutol (800 mg/day), and pyrazinamide (1,000 mg/day). She received no other concomitant medications.

Her initial physical examination showed nothing abnormal. Laboratory analysis showed hemoglobin 6.3 g/dL, leukocyte and thrombocyte counts within normal limits, 1% reticulocytes, and zero schizocytes. Other test results (liver and renal function, serum folate and vitamin B12 levels, lactic dehydrogenase levels, C-reactive protein, serum protein electrophoresis, direct and indirect Coombs tests, and antinuclear antibody tests) were within normal limits, as were viral serologic test results (HIV, hepatitis B virus, hepatitis C virus, parvovirus B19). Upper and lower digestive tract endoscopic examination and carcinoembryonic antigen showed no abnormalities, which lessened the likelihood of a tumor relapse. Bone marrow aspiration from the sternum showed a hypocellular marrow with complete absence of the erythroid series, a normal myeloid series, and megakaryocytes. The marrow findings were consistent with PRCA.

In view of previous reports of isoniazid-induced PRCA (*3–5*), we suspected this drug to be responsible in this case. Isoniazid was withdrawn, and other antituberculous drugs were continued. The patient's hemoglobin level rose to 10.6 g/dL and reticulocyte count to 3% over 10 days; no blood transfusion was required.

Drug-induced PRCA is a rare blood disorder in adults and has already been reported in isoniazid-treated patients (*3–5*). For this patient, other causes for anemia (e.g., drug-induced hemolytic anemia, digestive malignancies, viral causes known to date, hematologic malignancies, and autoimmune disorders) were excluded (*2*).

The favorable outcome after isoniazid withdrawal increased the likelihood that isoniazid was the cause. Although rechallenge with isoniazid could have confirmed isoniazid as the cause, it is not an ethical option because of the hazardous adverse effects.

The exact mechanism for isoniazid-induced PRCA remains unclear, but the demonstration of antibodies reacting with nucleated red blood cells in ≈50% of cases suggests an induction of autoimmunity (*4*,*5*). This hypothesis is supported by previously reported cases in which PRCA relapses occurred when treatment with isoniazid was resumed (*3*,*5*). The intrinsic imputability of isoniazid also relies on the lack of a dose-effect relationship and the delay between the introduction

of isoniazid and the onset of PRCA, which can occur up to 6 months after start of treatment (*3*).

Clinicians treating patients with tuberculosis must be aware of this adverse reaction because failure to identify and discontinue isoniazid in patients with such a condition might lead to their illness and death. Given the ongoing worldwide HIV pandemic and the increase in tuberculosis it induces, such adverse effects are more likely to be reported in the next few years.

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Failure of Isoniazid Chemoprophylaxis during Infliximab Therapy

To the Editor: A patient with ankylosing spondylitis was treated with infliximab, a tumor necrosis factor (TNF) blocker that has been associated with reactivation of latent tuberculosis (TB). Because of reactivity in testing with purified protein derivative, isoniazid chemoprophylaxis was started 2 weeks before infliximab therapy. Four months later, a cavitary lung infection developed in the patient, caused by isoniazid-resistant *Mycobacterium kansasii*.

To our knowledge, this is the first documented case of failure of isoniazid prophylaxis due to the emergence of isoniazid-resistant mycobacteria in patients receiving infliximab therapy. TNF blockers have contributed to the control of rheumatic diseases (*1*). Many of the damaging inflammatory mechanisms that they inhibit are important in maintaining TB in the latent phase. Consequently, drugs that target TNF functions have been associated with an increased risk of TB (*2*). For these reasons, prophylactic chemotherapy should be offered to patients with latent TB (*3*). We show the failure of isoniazid chemoprophylaxis in a patient receiving infliximab therapy in whom lung infection developed, caused by isoniazid-resistant *M. kansasii*.

A 39-year-old man with ankylosing spondylitis was admitted to Jimenez Diaz Foundation hospital, Madrid, because of fever and lung infiltrates. He had been receiving antiinflammatory drug therapy without amelioration of his symptoms. Therefore, treatment with infliximab was considered. Fifteen years before, the patient's father had had pulmonary TB caused by *M. tuberculosis* that was susceptible to first-line antituberculous drugs, and the patient was given chemoprophylaxis with isoniazid, 300 mg/day, during a 9-month period. Before beginning infliximab therapy, the patient was again given chemoprophylaxis with isoniazid, 300 mg/day, because a tuberculin test with 5 units of purified protein derivative showed an induration of 18 mm at 72 hours. Results of chest radiographs were normal, and cultures for mycobacteria were negative. Results of HIV testing were also negative.

After 4 months of infliximab therapy, fever, cough, and sputum production developed. New radiographs showed bilateral upper lung field infiltrates with cavitary lesions. Three acid-fast stains of sputum were positive, and treatment with rifampin, isoniazid, pyrazinamide, and ethambutol was started.

A heavy growth of photochromogenic mycobacteria was detected in 3 sputum cultures. The isolate was identified as *M. kansasii* genotype 1 by using common biochemical tests and PCR–restriction fragment length polymorphism analysis of the *hsp*65 gene (*4*). Susceptibility tests showed resistance to isoniazid ($\geq$5 μg/mL), streptomycin, pyrazinamide, p-amino-salicylic acid, and kanamycin but susceptibility to rifampin, ethambutol, and fluoroquinolones.

Treatment was continued with a combination of rifampin, levofloxacin, and ethambutol. Sputum cultures taken after 4, 6, and 9 months of antimicrobial drug therapy were negative. After 20 months of treatment, the patient was doing well with a partial resolution of lung infiltrates, and new cultures were negative.

Isoniazid chemoprophylaxis can effectively lessen the likelihood of active TB in patients treated with TNF antagonists (*5*). However, at least 1 failure of TB chemoprophylaxis in a severely immunocompromised patient treated with infliximab and methotrexate has been published (*6*). Our patient is unique because the mycobacterial lung infection seemed to emerge as a result of the lack of activity of iso-

niazid chemoprophylaxis due to resistance of the infecting organism.

Decreased susceptibility to isoniazid among *M. kansasii* isolates is common (*7,8*), and this microorganism is naturally resistant to pyrazinamide (*9*). This pattern of resistance is a serious obstacle for the use of these drugs in monotherapy or when combined with rifampin in the prevention of lung disease caused by *M. kansasii* (*10*).

The source of the infection in this patient is unknown. In a large series of infectious diseases associated with infliximab therapy, nontuberculous mycobacteria were isolated in 9% of the patients who had mycobacterial diseases (*2*). As in our patient, these infections developed shortly after initiation of treatment with infliximab, which suggests that reactivation of a latent infection is the most probable origin of the disease. Although a mildly positive tuberculin skin test result can be observed in patients infected with atypical mycobacteria, the strong reaction seen in this patient suggests a latent infection with *M. tuberculosis* (*10*). We could speculate on the possibility of a double infection with *M. tuberculosis* (contracted through household contacts with his father) and *M. kansasii* through environmental exposure. In this scenario, isoniazid chemoprophylaxis could have prevented the former but not the latter.

In summary, failure of isoniazid chemoprophylaxis can be anticipated in patients who initiate treatment with infliximab and who have latent infections due to *M. kansasii*. Despite routine antituberculous chemoprophylaxis, patients receiving infliximab therapy should be carefully evaluated for lung infection caused by atypical mycobacteria.

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Extensively Drug-Resistant *Mycobacterium tuberculosis*, India

To the Editor: India is contributing nearly one third of the world's tuberculosis (TB) cases and has the highest rate of new TB cases (*1*). Prevalence of multidrug-resistant TB (MDR TB) cases is on the rise in India, and proportions of new cases of MDR TB have been observed to vary from 1.1% to 5.3% in most of the reported studies. The proportion of previously treated patients with MDR TB varied from 8% to 67% (*2*). Although these studies have been conducted in different parts of India, they indicate an increasing trend of MDR TB cases.

MDR TB cases threaten the effectiveness of chemotherapy for both treatment and control of TB and require the use of second-line drugs that are more expensive, toxic, and less effective than first-line anti-TB drugs (*3*). The Green Light Committee established by the Stop TB partners (*4*), which ensures the proper use of second-line drugs to prevent increasing drug resistance in MDR TB cases in resource-limited countries, encountered resistance to these drugs. This led to the emergence of new terminology in relation to drug-resistant TB, i.e., extensively drug-resistant TB (XDR TB). XDR TB is defined as TB caused by a *Mycobacterium tuberculosis* strain that is resistant to at least rifampin and isoniazid among the first-line anti-TB drugs (MDR TB) in addition to resistance to any fluoroquinolones and at least 1 of 3 injectable second-line drugs (*5*). A recent report describes the current prevalence of XDR TB worldwide (*6*). Although India has high annual risk for TB cases and increasing prevalence of MDR TB cases, XDR TB has not yet been described in India.

From December 2000 through December 2002, 68 MDR TB isolates

were obtained from sputum samples from pulmonary TB patients, referred to Department of Microbiology, King George's Medical University, Lucknow Uttar Pradesh, India, for culture and sensitivity testing. Drug susceptibility testing for first-line drugs was performed by 1% proportion method against streptomycin (4 μg/mL), isoniazid (0.2 μg/mL), rifampin (40 μg/mL), and ethambutol (2 μg/mL) (*7*). The susceptibility of MDR TB isolates against second-line drugs was done by the absolute concentration method (MIC) for ofloxacin (0.5–16 μg/mL) and kanamycin (2–64 μg/mL) and by 1% proportional sensitivity method for ethionamide (40 μg/mL), p-amino salicylic acid (0.5 μg/ml), clarithromycin (2 μg/mL), and capreomycin (40 μg/mL). Resistance to ofloxacin, kanamycin, and ethionamide was determined by a cut-off of MIC ≥8 μg/mL, ≥64 μg/mL, and ≥128 μg/mL, respectively (*8*). All drugs were procured from Sigma (St. Louis, MO, USA), and quality control for drug susceptibility test was provided by the Tuberculosis Research Center, Chennai, India.

Among 68 MDR strains, 21 were from patients who had never been previously treated, and 47 were from patients whose medical history was positive for anti-tubercular treatment in the past, for at least 4 weeks. All MDR TB isolates were tested for susceptibility to second-line drugs, and high resistance to these drugs was found. MDR strains, which were further resistant to ofloxacin, and to at least 1 of 2 injectable second-line drugs tested (i.e., kanamycin or capreomycin), were classified as XDR TB. A total of 5 (7.4%) of 68 MDR TB strains met criteria for XDR TB. XDR TB isolates were usually resistant to almost all 4 first-line and second-line anti-TB drugs tested (Table). Global data on XDR TB are limited; however, a recent article reported that the problem of XDR TB is worldwide and includes a prevalence of 6.6% XDR TB cases in the studied countries (*6*). The Republic of Korea reports the maximum numbers of such cases, with 200 (15.4%) of 1,298 XDR TB strains tested from MDR TB patients included in the study. On December 1, 2006, World AIDS Day, South Africa reported >300 cases of XDR TB (*9*).

Here, we report, to our knowledge, the first XDR TB cases in India and the emergence of XDR TB in settings like India, where adequate monitoring of treatment regimens for MDR TB in TB control programs is difficult to implement due to a huge population and the high annual risk of acquiring TB is of great concern. A limitation to accurate detection of XDR TB is those existing tests for resistance to second-line drugs is not yet standardized and is less reproducible than results for first-line drugs (*10*). Access to management and treatment of MDR TB cases with second-line drugs, standardized methods, improved diagnostics, and quality assurance for susceptibility testing are needed to ensure reliable testing and the design of appropriate drug regimens.

Our study has some limitations, however. The data are not representative of the whole community and are limited to 1 hospital. Limited numbers of drugs were used in drug susceptibility testing, and the sample size is also not statistically adequate. A community-based, multicenter study, which includes all parts of the country and uses the full spectrum of drugs, is needed to describe the true prevalence of XDR TB in India.

Table. Resistance pattern of XDR TB isolates*

Strain no.	Resistant to first-line drugs	Resistant to second-line drugs
RM 55	S, H, R, E	K, O, CAP, CLA, PAS, ETH
RM 490	S, H, R, E	K, O, CAP, CLA, PAS, ETH
RM 552	S, H, R, E	K, O, CAP, CLA, PAS, ETH
RM 585†	S, H, R, E	K, O, CLA, PAS, ETH
RM 789	S, H, R, E	K, O, CAP, CLA, PAS, ETH

*n = 5; XDR TB, extensively drug-resistant tuberculosis; S, streptomycin; H, isoniazid; R, rifampin; E, ethambutol; K, kanamycin; O, ofloxacin; CAP, capreomycin; CLA, clarithromycin; PAS, p–amino salicylic acid; ETH, ethionamide.
†Sensitive to capreomycin.

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Stray Dogs and Leishmaniasis in Urban Areas, Portugal

To the Editor: In southern Europe, zoonotic visceral leishmaniasis caused by *Leishmania infantum* used to be considered a rural disease, but it is becoming more prevalent in urban areas. Outbreaks in urban/periurban settings are associated with the urbanization of natural zoonotic foci (*1*). The presence of a high number of stray dogs in urban/periurban settlements may contribute to the spread and increase of new infections.

A canine survey was performed twice a month from December 1, 2002, through December 31, 2003. A total of 374 dogs from urban areas of Lisbon were screened for leishmaniasis. Owners voluntarily brought 277 domestic dogs; 97 stray dogs were from public shelters. Indirect fluorescent assay was used for detection of anti-*Leishmania* antibodies using a cut-off of 1/64, and popliteal lymph node aspirates for Novy, Nicolle, and MacNeal cultures were tested (*2*).

A high overall prevalence (19.2%) of canine leishmaniasis was found, despite use of conventional tests only. The infection rate would probably have been higher had more sensitive techniques, such as molecular tools, been used. During the 1980s, Abranches et al. (*2*) performed a similar seroepidemiologic survey and found a prevalence rate of 5.5%.

Our results show an increase of canine leishmaniasis cases in Lisbon. In our study, the prevelance of infection in domestic dogs was 18.4% (51/277), and the prevelance in stray dogs was 21.6% (21/97), with no statistical difference ($p = 0.48$, significance level 95%). These results support the importance of the role of stray dogs in parasite transmission in Lisbon but differ from the 7.8% seroprevalence found in Madrid, where 1,803 stray dogs were studied over a 10-year period (*3*). However, the sample size and duration of both studies are different. In other urban areas of large European cities and Brazil, the existence of a high canine seroprevalence has shown an urbanization of the parasitosis (*4,5*). This is associated with an increase in 1-family homes with gardens in the peripheries of cities. Dogs are commonly kept in these gardens, which can provide good habitats for sandflies. On the other hand, the development of suburban areas can also lead to an increase of solid waste and deficient sanitary conditions, thus attracting infected stray dogs. The difference in percentage of domestic dogs (39.21%) and stray dogs (28.57%) that appeared healthy, although infected, was not statistically significant ($p = 0.39$). The percentage of apparently healthy dogs was lower than expected, as different studies have shown that more than half of the seropositive dogs are asymptomatic (*3,6*). Moreover, stray dogs are more likely to experience deficient health and nutritional conditions, and we thus expected larger differences between the 2 groups of animals. Of note, asymptomatic infected dogs can be a source of infection to the vectors, although symptomatic dogs are more effective reservoirs (*6*).

Along with the canine survey, from June through September a total of 488 sandflies were collected from 99 biotopes selected from the studied areas where canine or human cases have been diagnosed. The vectors were morphologically identified by standard entomologic keys (*7*) as follows: 392 (80.33%) *Phlebotomus perniciosus*, 93 (19.06%) *Ph. ariasi,* and 3 (0.61%) *Ph. sergenti*. Phlebotomine density ranged from 0.08 to 7.70 specimens/CDC trap/night. *Ph. ariasi* was found infected, reflecting an overall infection rate of 1.22 % (1/82).

In Portugal, *Ph. ariasi* and *Ph. perniciosus* are the proven vectors of *L. infantum* (*8*). Although phlebotomine infection was proven in Lisbon, it was low when compared with the canine infection rate, highlighting the need for a more extensive vectorial study in these areas. From 2002 through 2006, 20 new cases of kala-azar in immunocompromised patients (16 children and 4 adults) were diagnosed in our laboratory. In spite of the number of new cases being higher in immunocompromised persons, namely, HIV-infected patients, generally only the cases of immunocompetent persons reflect natural zoonotic transmission. Immunocompromised patients can also experience the reactivation of an old latent infection or be infected by zoonotic transmission or by anthroponotic transmission without a vector. Despite some studies that have shown a direct relationship between the prevalence of leishmaniasis in canine and human populations, canine leishmaniasis is much more prevalent and more widely distributed than visceral leishmaniasis, and it does not strongly correlate with the prevalence in humans (*6*). Moreover, *Ph. ariasi* and *Ph. perniciosus* are known to be preferentially zoophilic.

In domestic dogs, if the owner takes preventive measures, the infection risk may be reduced. Stray dogs, however, are an easier target for infection and sandfly biting due to precarious physical conditions and outdoor living habits that make canine leishmaniasis control much more difficult.

In conclusion, sanitary conditions and animal health must be improved to prevent the transmission risk of leishmaniasis by this group of animals. The absence of surveillance or preventative measures and equilibrium rupture in

the ecologic system could contribute to the emergence of human leishmaniasis in urban areas.

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TaqMan Assay for Swedish *Chlamydia trachomatis* Variant

To the Editor: *Chlamydia trachomatis* (CT) is the most prevalent bacterial sexually transmitted infection worldwide. Recently, a new variant of CT (swCT) has been reported in Halland County, Sweden. A total of 12 swCT specimens were sequenced and found to have the same deletion, a 377-bp deletion in the cryptic plasmid (*1*). Because the deletion was found in the target area of 2 commercial CT nucleic acid amplification tests (Roche, Basel, Switzerland, and Abbott Laboratories, Abbott Park, IL, USA), screening tests have produced false-negative results for patients infected with this new Swedish variant (*1*). In specific regions of Sweden, the proportion of all detected CT cases attributable to swCT ranges from 13% to 39%; a considerable number of chlamydia infections have escaped detection by commonly used test systems (*1*).

Although the first 2 studies to monitor potential spread of the swCT variant outside Sweden (Ireland and the Netherlands) did not detect swCT, a third study (Norway) did identify this variant (*2–4*). Subsequently, the European Surveillance of Sexually Transmitted Infections network and the European Center for Disease Prevention and Control launched an initiative, consisting of a short questionnaire, to learn more about this swCT variant problem outside Sweden (*5*).

However, quick monitoring of the spread of the swCT variant has been hampered by lack of a direct test to detect this swCT variant and by lack of a readily available positive control. We therefore constructed a positive control by using a clinical specimen of the swCT variant in which the deletion was present (forward swCT 5′-TCC GGA TAG TGA ATT ATA GAG ACT ATT TAA TC-3′ reverse swCT 5′GGT GTT TGT ACT AGA GGA CTT ACC TCT TC-3′) (*2*). The specimen was obtained in Sweden (by B.H.) and confirmed as swCT by the method described by Ripa and Nilsson (*6*). The obtained 98-bp amplicon was subsequently cloned in a pGEM-T Easy Vector (Promega Benelux b.v., Leiden, the Netherlands) and transformed in *Escherichia coli* DH5α. After extraction the plasmid was verified for the correct insert by sequencing and quantified as described (*7*). This positive control is available for researchers and clinicians free of charge.

Subsequently, we developed a real-time PCR (TaqMan assay) that specifically detects the swCt variant by using a probe that spans the 377-bp left and right gap border sequences: probe- swCT 5′-^{FAM} GGA TCC GTT TGT TCT GG ^{MGB} -3′. One copy of cloned positive swCT control could be detected in our swCT assay. We selected 10 copies per PCR as positive swCT control for each run. A total of 239 recent samples known to be CT positive and identified with techniques detecting the swCT variant were retrospectively analyzed with our new swCT real-time PCR for 3 cohorts: 1) 30 real-time PCR CT-positive clinical samples (CT prevalence in the population, 1.8%) from the Department of Medical Microbiology and Infection Prevention, VU University Medical Center, Amsterdam, the Netherlands; 2) 57 Becton Dickinson (Franklin Lakes, NJ, USA) CT-positive samples (CT prevalence in the sexually transmitted disease population, 7.3%) from the Department of Infectious Diseases, South Limburg Public Health Service, Heerlen, the Netherlands; and 3) 152

CT-positive culture samples (CT prevalence in the population, average 15% [*8*]) from the Faculty of Medicine, St. Petersburg State University, St. Petersburg, Russia, and from the Laboratory of Microbiology, D.O. Ott Research Institute of Obstetrics and Gynaecology, St. Petersburg, Russia.

Cohort 1 consisted of cervical swabs in 2-sucrose-phosphate (2SP) transport medium, stored at –80°C. Cohort 2 consisted of frozen dry swabs that had been shaken for 10 s in 1 mL 2SP transport medium before sample preparation. Cohort 3 consisted of positive cultured samples. DNA extraction used 200 μL 2SP and was performed with the NucliSens easyMAG (bioMérieux, Boxtel, the Netherlands); the DNA was eluted in 110 μL 2SP (*7*). Presence of CT DNA was reconfirmed for all samples with our in-house PCR. Sensitivity of this assay was determined by using a previously described serial dilution of lymphogranuloma venereum (LGV) strain L2 and was assessed at 0.01 inclusion-forming units (*9*). Amplification and detection were performed with an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) by standard PCR conditions of the manufacturer, with 45 cycles. The Swedish variant was found in none of the 3 cohorts tested. Sensitivity and specificity were confirmed by using 12 swCT variant samples from Sweden, which were all positive according to our swCT TaqMan assay.

Our new swCT TaqMan assay, combined with the positive control (which can be obtained by contacting S.M.), will be a helpful tool for determining whether this Swedish CT variant is present outside Sweden, other than in the 2 case-patients identified in Norway. We did not find any evidence of the swCT variant in the Netherlands or St. Petersburg, Russia, each of which is near Scandinavia (Table). Recently, the *C. trachomatis* LGV strain was discovered in the Netherlands in a population of men who have sex with

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Table. Published studies and the current study on screening for the swCT variant*

Location	Ct+, no. detected	swCT variant, no. detected	Reference
Amsterdam, the Netherlands	75	ND	(*3*)
Dublin, Ireland	750	ND	(*4*)
Oslo, Norway†	47	2	(*5*)
St. Petersburg, Russia	152	ND	This study
Heerlen, the Netherlands	57	ND	This study
Amsterdam, the Netherlands	30	ND	This study

*swCT, Swedish *Chlamydia trachomatis* variant identified in Halland County, Sweden; Ct+, *C. trachomatis* DNA; ND, not detected.
†2 female patients: 1 originally from Sweden, 1 from Norway.

men. In this instance, the real-time TaqMan assay also proved helpful in determining spread (*10*).

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Highly Pathogenic Porcine Reproductive and Respiratory Syndrome, China

To the Editor: Since April 2006, a highly pathogenic disease caused by unknown agents and characterized by high fever and a high proportion of deaths in pigs of all ages, emerged in some swine farms in Jiangxi Province, People's Republic of China. The morbidity rate was 50%–100% and mortality rate was 20%–100%. In the next several months, the disease spread rapidly to most provinces of China. In almost all affected swine herds, the following clinical signs were observed: high and continuous fever, anorexia, red discolorations in the bodies, and blue ears; in the late phase of the disease, diarrhea and other clinical signs might be seen due to the secondary infections. Clinical samples (from lungs, kidneys, liver, and lymph nodes) were collected from animals in different provinces and sent for laboratory diagnosis. DNA and RNA were extracted from the tissue homogenate and PCR or reverse transcription–PCR (RT-PCR) was conducted to detect porcine reproductive and respiratory syndrome virus (PRRSV), classic swine fever virus, porcine circovirus, and pseudorabies virus, respectively (*1*). In clinical samples, only PRRSV was found to be the dominant virus (48 of 50 samples were PRRSV posi-

tive). PRRSVs were then isolated successfully on MARC-145 cells with an obvious cytopathologic effect, characterized by cell congregation, contraction, and brushing off at passage 2; immunofluorescence assay using PRRSV NP-, M- and GP5-specific monoclonal antibodies confirmed that the isolated viruses were PRRSV (*2*,*3*). Full-length genomic sequencing of 1 of the isolates (HuN4 strain) showed extensive amino acid (aa) mutations in GP5 protein and 2 deletions in Nsp2, 1 aa deletion at 482, and 29 aa deletions at 533–561, compared with the previous Chinese isolates CH-1a and BJ-4.

The newly isolated PRRSV was used to examine the pathogenicity in 60-day-old PRRSV-free piglets, under closed and biosafety (P2) conditions. Each of the piglets (N = 5) received intranasally $10^{5.0}$ 50% tissue culture infecting dose of the isolated virus propagated in MARC-145 cells (*4*,*5*). The animals were kept in separate rooms throughout the experiment. Clinical observations of respiratory signs, behavior, rectal temperature, and coughing were recorded daily. Blood samples were collected every 2 days and tested for PRRSV-specific antibodies by ELISA (*6*,*7*). Tissue samples (from heart, lungs, kidneys, spleen, and lymph nodes) from all animals that died during the experiment were collected and detected by histopathologic examination (*8*) and virus isolation. Results showed that the clinical manifestations of all pigs were similar to those that appeared in the field investigation (including high and continuous fever, anorexia, red discolorations in the bodies, and blue ears). The specific antibodies to PRRSV were detected at 8 days postinfection, and the high antibody level lasted until the animal's death, and all infected pigs died at either 7, 8, 12, 16, or 21 days postinoculation, respectively. Furthermore, viruses reisolated from the dead pigs showed an identical homology with the inoculated PRRSV in genes coding for GP5 and partial Nsp2 (2,535–3,307 nt). The results showed that the emerging PRRSV, characterized by deletions in Nsp2, is highly pathogenic to pigs.

To investigate whether the emerging PRRSV was the causative agent of the pandemic diseases on swine farms, an extensive virus survey was conducted. More than 48 samples collected from different swine farms in12 provinces were found to be PRRSV positive by RT-PCR, based on open reading frame (ORF) 5 and Nsp2 (Figure). Sequence analysis of ORF5 and partial Nsp2 showed that these PRRSVs are highly homologous to each other (98.5%–100% for GP5; 98.2%–100% for Nsp2) and share the same deletions at the same positions of Nsp2 gene with HuN4 strain. Sequence comparison of ORF5 indicated that the HuN4 strain shares 93%, 86%, and 88% nucleotide identities with CH-1a (Chinese isolate), BJ-4 (Chinese isolate), and VR2332 (American isolate), respectively. All the newly isolated PRRSVs belong to the North American type.

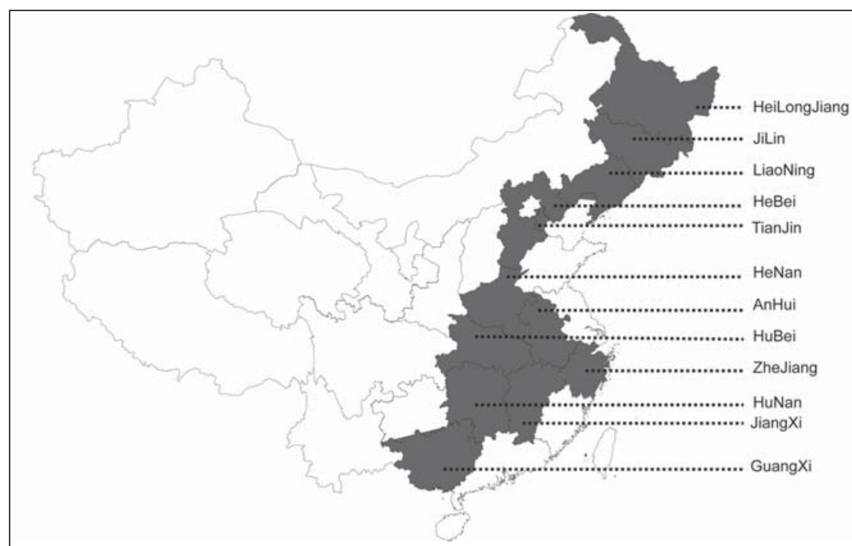

Figure. Geographic distribution of porcine reproductive and respiratory syndrome viruses (PRRSVs) examined in the study. Shaded areas indicate the provinces where the PRRSVs characterized by deletions in Nsp2 were detected.

Although the cause of the emerging pandemic disease of pigs with a high proportion of deaths in 2006 is unknown, we found high correlation between PRRSV isolation rate and the diseased pigs. The regression test in its natural animal showed that the newly isolated PRRSV was much more virulent than earlier PRRSV isolates. Also, sequence analysis demonstrated a substantial diversity from the PRRSVs isolated during 1996–2005. Further study is needed to answer the question: What role did the newly isolated PRRSV play in the 2006 outbreaks on many of the swine farms in China?

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Recurrent American Cutaneous Leishmaniasis

To the Editor: Leishmaniasis recidivans is an unusual clinical Old World disease primarily associated with *Leishmania tropica* (*1*). Recurrence of previously cured cutaneous leishmaniasis (CL) lesions is found in American CL, for which a specific nosologic form known of disease known as leishmaniasis recidiva cutis (LRC) has been identified. Although <30 cases of LRC have been reported from Brazil, Colombia, Peru and Ecuador; these cases were caused mainly by *L. braziliensis*, *L. amazonensis*, and *L. panamensis* (*2*). We report 7 cases of recurrent American CL caused by *L. guyanensis* in French Guiana.

Forty-eight military personnel who lived in France spent 3 months in French Guiana in 2004 and took part in a military training program in the rainforest for 15 days. Despite similar exposure conditions, American CL, confirmed by positive direct examination of Giemsa-stained tissue smears, developed in 21 persons. These patients were treated with 1 or 2 courses of either 3 intravenous or 2 intramuscular injections of pentamidine isethionate (4 mg/kg on alternate days). All lesions were cured 1–3 months after treatment had ended. Recurrence of the CL lesion was observed in 7 patients after a disease-free interval of 3–6 months (Table).

New lesions appeared on the edge of a healed scar for each patient, regardless of the location of the primary lesion (Table), and were diagnosed at Rennes University Hospital (positive direct examination or culture) in 2005. For positive cultures, *L. guyanensis* was identified by genomic and isoenzymatic characterization at the Centre National de Référence des *Leishmania*, Université de Montpellier, Montpellier, France. Patients were treated with 4 intravenous injections of pentamidine isethionate (4 mg/kg every other day) and were cured without recurrence within 2 years. No differences in age or underlying diseases were noted in patients with recurrent CL.

L. (*Viannia*) *guyanensis* is highly prevalent in several leishmaniasis-endemic areas of Brazil, Colombia, French Guiana, Guyana, Surinam, Peru, and Ecuador. This organism accounts for >95% of the 5 *Leishmania* species found in French Guiana, commonly causes localized LCL, and occasionally causes disseminated CL and mucocutaneous leishmaniasis (*3*). Dedet et al. reported that 6.8% of patients with CL caused by *L. guyanensis* had a recurrent lesion at the site of a previously cured lesion, which occurred after a mean interval of 7.3 months (*4*). A total of 33% of our patients had a cured primary infection in <3 months but they had a recurrence after a disease-free interval 3–6 months after treatment.

Additional information on such a recurrent form of CL is needed. Clinical symptoms in our patients were suggestive of LRC as described by Berlin (*1*), i.e., a recurrence at the site of an original ulcer, generally within 2 years and often on the edge of a scar. LRC may not be uncommon in the New World but rather underreported (*2*). Few cases of LRC have been reported; these were caused by *L. braziliensis*, *L. amazonensis*, and *L. panamensis* (*2,5,6*). In CL caused by *L. guyanensis*, borderline clinical symptoms prevent clear distinction of the recurrent form of LRC from early treatment failures or reinfections. In our patients, the risk for reinfection was excluded because the military personnel lived in France and left French Guiana several months before the recurrence.

Although pentavalent antimony is the recommended treatment for American CL, pentamidine isethionate is widely used in French Guiana. Retrospective analysis showed that 5%–25% of early treatment failures

occurred after 1 or 2 intramuscular injections of 4–7 mg/kg of pentamidine isethionate, depending on different risk factors (*7,8*). Our observational study was not designed to evaluate treatment efficiency; we observed 2 (9.5%) of 21 early relapses and 7 (33%) of 21 late-onset recurrences in this series. Although we lacked statistical power because of small numbers, a difference was observed in recurrence by treatment method in 7 (44%) of 16 with recurrent disease who received 3 intravenous pentamidine isethionate injections compared with 0 (0%) of 5 who received 2 intramuscular injections. Re-treatment with a regimen of 4 intravenous injections of pentamidine, under medical surveillance because of possible adverse effects, cured the disease. Physicians in countries in which *L. guyanensis* is endemic should be aware of these complicated forms of CL after treatment, forms that prompt long-term follow-up and evaluation of specific therapeutic protocols.

Finally, the mechanism of late-recurring leishmaniasis is poorly understood. Mendonça et al. suggested that clinical cure of American CL is rarely associated with sterile cure (i.e., elimination of the parasite) (*9*). Immunologic data based on skin hypersensitivity and histopathologic and immunohistochemical findings support the concept that LRC is a late-onset reactivation after persistence of living parasites around or in cured leishmaniasis by as-yet unknown stimuli such as local trauma (2 of our patients reported chronic lesions of the chin caused by a razor blade and 2 others had chronically scratched the scar lesion on their ears) or corticosteroid, and after an incomplete host immune response to an earlier episode (*2, 5,10*). Further immunologic studies of patients and identification of genetic characteristics of *L. guyanensis* are needed to address this question.

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Table. Epidemiologic characteristics, strain identification, and treatment of 21 cases of American cutaneous leishmaniasis, French Guiana, 2004–2005*

	Primary infection				Leishmaniasis recidiva cutis			
Case no.	Lesion(s), location (no.)	PI treatment†	Time to healed lesions, mo	Disease-free interval, mo	Direct examination	Strain identification	PI treatment	Outcome
1	Thorax (1)	IV, 4 mg/kg on alternate days × 3	1	4	+	*Leishmania guyanensis* MON-45	IV, 4 mg/kg on alternate days × 4	Cured
2	Right forearm (1)	IV, 4 mg/kg on alternate days × 3	3	6	+	*L. guyanensis* MON-45	IV, 4 mg/kg on alternate days × 4	Cured
3	Left leg (1) and chin (3)	IV, 4 mg/kg on alternate days × 3	1	4	+	Negative culture	IV, 4 mg/kg on alternate days × 4	Cured
4	Chin (3)	IV, 4 mg/kg on alternate days × 3	1	4	+	Negative culture	IV, 4 mg/kg on alternate days × 4	Cured
5	Behind left ear (1)	IV, 4 mg/kg on alternate days × 3	1	3	+	*L. guyanensis* MON-131	IV, 4 mg/kg on alternate days × 4	Cured
6	Left ear (1)	IV, 4 mg/kg on alternate days × 3	1	6	+	Negative culture	IV, 4 mg/kg on alternate days × 4	Cured
7	Right ankle (5)	IV, 4 mg/kg on alternate days × 3	2.5	4	+	Negative culture	IV, 4 mg/kg on alternate days × 4	Cured
8–21	Various	IV, 4 mg/kg on alternate days × 3 (for 9 patients) or IM, 4 mg/kg on alternate days × 2 (for 5 patients)†	1–3	–	–	–	–	–

*PI, pentamidine isethionate; IV, intravenous; IM, intramuscular.
†IV versus IM administration of pentamidine isethionate was not associated with recurrent forms.

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Leprosy as Immune Reconstitution Inflammatory Syndrome in HIV-positive Persons

To the Editor: More than 2 decades ago, when HIV was first detected, many investigators predicted the rise of leprosy secondary to opportunistic infection (*1*). Recently, the phenomenon of immune reconstitution inflammatory syndrome (IRIS), or leprosy reversal response, has received attention. IRIS often occurs secondary to initiating highly active antiretroviral therapy (HAART). The first indications of an interaction between HIV and *Mycobacterium leprae* occurred only recently, with the identification of IRIS after initiation of HAART in patients with HIV and previously undetected leprosy. A review by Pustianowski et al. discusses the paradox of HIV and leprosy with IRIS (*2*). In addition, Lawn et al. described the first case of IRIS after the onset of HAART in a patient who had tuberculoid leprosy that was never confirmed by molecular analysis (*3*).

Multiple reports (*4*–*7*) unmasked subclinical Hansen disease (*M. leprae* infection) occurring with HAART or spontaneously (*8*). In case reports by Lu et al. (*6*) and Sharma et al. (*7*), leprosy was associated with erythema nodosum leprosum. Pereira et al. discovered that patients known to have HIV and leprosy, when treated with HAART manifested a type 1 reversal reaction, acute leprosy inflammatory episode (*4*), or IRIS. We describe the first, to our knowledge, 2 cases in the United States of HIV and leprosy infections in which IRIS has occurred after HAART initiation and which has been confirmed by molecular analysis.

Three skin-biopsy samples, 2 from patient 1 and 1 from patient 2, were analyzed to confirm the presence of *M. leprae*. Patient 1 met the diagnostic criteria for leprosy according to biopsy result; patient 2's case was compatible with such criteria. Each patient was treated for leprosy, and each responded favorably. The purpose of our case study was to confirm *M. leprae* DNA in skin samples. The skin specimens were paraffin-embedded slides. DNA was extracted by standard molecular biologic methods that used xylene. PCR amplified the *M. leprae* heat shock protein 65 gene (*hsp65*). After amplification, restriction fragment-length polymorphism (RFLP)–polyacrylamide gel electrophoresis (PAGE) was performed with *Hae*III (*6*).

Patient 1 was a 60-year-old Hispanic man who was first evaluated in Los Angeles, California, with skin lesions covering >50% of his body. He reported having erythematous scaly plaques that had been waxing and waning for several months. Several skin biopsy samples were taken, and an HIV test was conducted; results showed that he had lepromatous leprosy and was HIV positive. Biopsy specimens were both Fite stain positive for numerous acid-fast bacilli. Three months after HAART was initiated, repeat skin biopsy samples were taken from nodules that had recently developed on his right arm and torso. Histologic assessment showed Fite stain–positive granulomatous dermatitis with many foamy cells. He was treated for leprosy and is continuing HAART.

Patient 2 was a 37-year-old West African black man from Burkina Faso who was evaluated in New York for gram-negative bacteremia. He was admitted and treated for disseminated salmonellosis and was found to be HIV positive. His T-lymphocyte count was 7/µL. He was promptly prescribed HAART and responded well to treatment: his T-cell count rose to 112/µL during 5 months and is currently >700/µL. Within 2 years of HAART initiation, multiple anesthetic, hypopigmented skin macules that failed to resolve over 6 months

developed. These macules developed further into nodules. Punch biopsy results were consistent with granulomatous dermatitis. Fite stain was negative for acid-fast bacilli, but leprosy was diagnosed on the basis of anesthesia localized to his skin lesions. When the biopsy samples were taken, the patient was receiving dapsone in addition to HAART. Rifampin treatment was started subsequent to biopsies.

PCR amplification for *M. leprae hsp65* was positive for all 3 samples. Thus, mycobacterial DNA was present in both patients. The RFLP analysis results are shown in the Figure. The *hsp65* RFLP pattern for patient 1 was identical to those described by Martiniuk et al. (*9*) and for the wild-type pattern for patient 2, as shown by Lu et al. (*6*), thus demonstrating the presence of *M. leprae* DNA in these samples.

Previous studies have highlighted the low rate of HIV and leprosy co-infection. For example in Ethiopia, Frommel et al. noted that, before HAART was available in resource-poor settings, increased HIV seropositivity did not alter the natural course of leprosy nor increase the number of patients with *M. leprae* (*10*). Nevertheless, positive reports of IRIS and leprosy after initiation of HAART have been reported from other nations (*3–5*). If this syndrome can be detected even in the mildly leprosy–endemic United States (*8*), an increase in similar cases in areas where HIV and leprosy occur in higher frequency can be anticipated.

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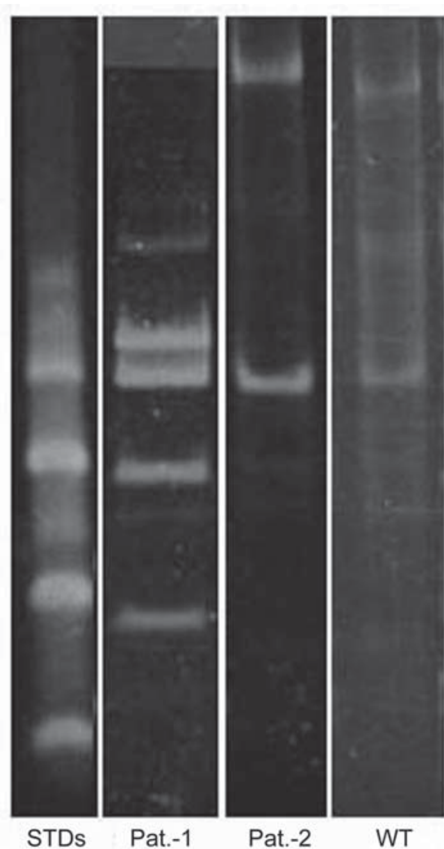

Figure. Polyacrylamide gel electrophoresis–restriction fragment length polymorphism of PCR amplicons digested with *Hae*III with standards. STDs, sexually transmitted diseases; Pat., patient; WT, wild type.

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Endocarditis after Use of Tongue Scraper

To the Editor: Tongue scraping is advocated as a therapy for managing halitosis and as a technique for preventing dental caries by reducing bacterial counts in the mouth (*1*). The practice has been in existence for centuries (*2*). A Cochrane review has concluded that tongue cleaning is marginally and temporarily more effective than use of a toothbrush in reducing a measurable marker for halitosis, exhaled volatile sulfur compounds (*3*). The use of tongue scrapers may not be limited to those with clinical halitosis, as 10%–30% of Americans report bad breath (*4*), and websites offer to solve the problem of "your bad breath" for a price. We report the case of a woman in whom infective endocarditis followed the use of a tongue scraper.

A 59-year-old woman with a known history of mitral valve prolapse with associated valvular regurgitation had onset of progressive malaise, fever, sweats, myalgia, and headache; the symptoms lasted 10 days. Two months previously she had begun cleaning her tongue with a plastic tongue scraper purchased at her local pharmacy. She had not undergone recent dental work. Her medical background included migraines, hypertension, mild quiescent psoriasis, and previous depression. Her medications were venlafaxine and candesartan.

When seen at her local hospital, she reported severe headache and myalgia, with fever. The same day, she had a rigor at home and reported chest tightness and mild dyspnea. Physical examination showed no focal findings other than the mitral valve prolapse. A provisional diagnosis of bacterial meningitis was made. Emergency treatment comprised intravenous dexamethasone, ceftriaxone, and benzylpenicillin. Cerebrospinal fluid analysis performed shortly after showed no cells and normal glucose and protein levels. Culture of the cerebrospinal fluid was negative. No further antimicrobial agents were administered. Multiple blood cultures were drawn but remained culture negative. Serologic tests for Q fever, *Bartonella* spp., and endemic rickettsiae were negative. She continued to be febrile.

A transthoracic echocardiogram showed dilatation of the mitral valve annulus with bi-leaflet prolapse and vegetation attached to the anterolateral commissure. She was referred to a tertiary care center, and therapy with penicillin, flucloxacillin, and gentamicin was begun for culture-negative endocarditis. Transesophageal echocardiography and visual examination at the time of valve replacement confirmed the presence of large valvular vegetations. After infected tissue was excised, a prosthetic mitral valve was placed. Extended culturing of the blood failed to identify a pathogen. Histopathologic examination of the explanted valve identified fibrinopurulent vegetations with destruction of the valve leaflet. The excised material was split into sections and submitted for culture; all demonstrated a scant growth of *Haemophilus parainfluenzae*. This finding was identified by a Remel RapID NH Panel (Remel, Lenexa, KS, USA) and confirmed by 16S rRNA gene sequencing. The patient was treated with ampicillin and gentamicin for 2 weeks. She then had 4 further weeks of therapy with daily ceftriaxone at home. She is now well.

This patient's endocarditis was most likely caused by bacteremia from tongue scraping, and the abnormal valve is likely to have been a predisposing factor. The link between oral flora and endocarditis has long been recognized (*5*), and guidelines for prophylactic use of antimicrobial agents before dental manipulation are established. A literature review did not show any previous reports of endocarditis associated with use of a tongue scraper. There are numerous reports of endocarditis after tongue piercing, with a variety of organisms including viridans streptococci, *H. aphrophilus, Neisseria mucosa*, and methicillin-resistant *Staphylococcus aureus* (*6*–*9*). Most of these articles reported a pre-existing valvular abnormality, as in our case. Bacteremia caused by routine tooth brushing does not appear to be clinically important, and there are conflicting data about its frequency (*10*). The inoculum of bacteria transmitted into the bloodstream with brushing may be smaller than that with tooth extraction. Given the frequency of routine tooth brushing, antimicrobial prophylaxis is impractical in any case. The practice of tongue scraping, however, has not been well studied, and both the magnitude and frequency of bacteremia may be greater than with routine tooth brushing.

We propose that our patient's infective endocarditis was most likely a consequence of bacteremia from her use of a tongue scraper. Persons with abnormal cardiac valves and intravascular devices such as pacemakers may be at particular risk. Patients with previous infective endocarditis and high-risk cardiac valve defects should be informed that tongue scraper use is not prudent.

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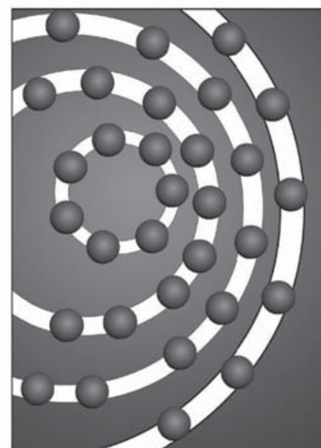

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Physician's Guide to Arthropods of Medical Importance, 5th Edition

Jerome Goddard
CRC Press/Taylor Francis, Boca Raton, Florida, USA, 2007

ISBN: 0849385393
Pages: 480; Price: US $159.95

The 21st century is well under way, and along with the new century has come the emergence and reemergence of arthropodborne diseases. Because of the growing number of challenges these diseases pose to human health, arthropods have garnered heightened concern in the medical community. The concept of symptomatic treatment at the local level may no longer be sufficient to treat certain major types of arthropod-transmitted diseases, such as West Nile virus encephalitis. Contemporary information is a critical element of primary healthcare, but specific therapy has yet to be recommended for most arthropodborne diseases. In the primary care setting, relating the arthropodborne disease to the type of lesion (e.g., bite or sting) is important, especially if vigorous supportive measures would be helpful. Now, more than ever, raising awareness, knowledge, and treatment competency of the healthcare workforce are needed so that these diseases can be quickly diagnosed and effectively managed.

The fifth edition of Physician's Guide to Arthropods of Medical Importance has been thoroughly updated to ensure authoritative coverage of this complex and fast-moving discipline of arthropodborne diseases, and serves as a reliable reference covering all of the major aspects of entomology. Throughout the book, the author's vast experience and dedication are evident. The uniqueness of this book lies in its portrayal of all arthropod pests, with and without human impact, and in its emphasis on arthropods as the cause of disease. A specific arthropod is described in each section, which begins with illustrations for identification, life cycle drawings (if applicable), and succinct text regarding the arthropod's general importance, geographic distribution, and behavior. The text also covers all harmful effects on human health and highlights current management recommendations. Written in a clear and readable style, the text presents complex, academic information on the biodiversity of arthropod pests that even a lay person can understand. As such, it is an excellent resource for clinicians, public health workers, and the public. Educational awareness of signs and symptoms of arthropodborne diseases will reduce unnecessary panic in the event of outbreaks and assist clinicians in differential diagnosis and treatment. This guide, an interdisciplinary contribution to the field of vectorborne diseases, bridges entomology, primary care, and public health.

The following minor changes could be incorporated in the next edition of this outstanding text: 1) on page 7, the end of the second paragraph should be "... oxygen free radicals and several proteins ..."; 2) the CD-ROM included has technical problems—under BugCoach, none of the 4 interactive links (Bugs, Conditions, Useful Links, Identification Helps) can be accessed successfully; and 3) in Chapter 9 on signs and symptoms of arthropodborne disease, photographs would help clinicians with diagnosis and proper treatment and the public about when or if to seek medical attention. The author and the publishers are highly commended for presenting such a comprehensive, highly useful volume on the status of arthropods in current medical concerns.

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Paolo Veronese (Paolo Caliari, 1528–1588). Venice Receives from Juno the Doge's Hat (1555) (detail). Palazzo Ducale, Venice, Italy/The Bridgeman Art Library Nationality/copyright status: Italian/out of copyright

"Nature Hath Fram'd Strange Fellows in Her Time"

—William Shakespeare, The Merchant of Venice

Polyxeni Potter*

Painters take the same license as poets and madmen, Paolo Veronese told the Inquisition Tribunal in Venice during an interrogation. Buffoons, drunkards, exotic creatures, and anachronisms in his Last Supper were placed there "so they might be of service because it seemed to me fitting ..." in creating the scene, not as irreverence (*1*). The dispute was resolved by changing the name of the painting to Supper in the House of Levi. Veronese was not interested in piety or historical accuracy. Large banquets were opportunities to create feasts for the eyes, monumental gatherings framed in architectural detail, bathed in sumptuous color.

The son of a stone mason known only as Gabriele, the painter adopted the name Caliari and later became known as Veronese from his birthplace. A precocious child entirely uninterested in stone cutting, he was quickly recognized for facility with the brush and was trained by local masters Antonio Badile and Giovanni Caroto. Then, according to chronicler Giorgio Vasari, architect and engineer Michele Sanmicheli took him under his wing and "treated him like a son" (*2*).

He painted his first works in Verona and Mantua, but when called to Venice on a commission, he remained there for the rest of his life, becoming a preeminent master of the late Renaissance, along with Titian and Tintoretto. In the Doge's palace, the Church of San Sebastiano, the Villa Barbaro at Maser with the great architect Andrea Palladio, and churches and palaces all over the city, he extolled youth, beauty, and prodigious harvests in frescoes and oil paintings of enduring charm. A kind and amiable man, Veronese was well liked and appreciated, one of the first painters whose work was sought by collectors during his lifetime (*3*).

Early training in the mannerist style, which emphasized the decorative, was transformed by the styles of Venice, an innate sense of composition, and his genius as draftsman. He reveled in rich textures and patterns and captured luminescence in flesh and fabric, lace or wool. He was "the greatest colorist who ever lived," wrote French critic Théophile Gautier, "greater than Titian, Rubens, or Rembrandt" because he created light without violent contrasts and maintained the strength of hue and shadow, which French master Eugène Delacroix (1798–1863) said, "We are always told is impossible" (*4*).

An expert illusionist, Veronese overcame the problems of applying linear perspective to the concave surfaces of church domes, overriding the architecture, simulating limitless space. With *sotto in su* techniques, he created foreshortened figures to be seen from below as floating above the viewer. He moved adventurously between secular and religious themes, incorporated classical and mythologic figures, crafted allegorical pageants, mingled the sacred with what some thought the profane.

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Venice, la Serenissima or Most Serene Republic, and the myths surrounding her mercantile empire lent themselves to the theatrical, apotheotic exuberance of Veronese's style. The city, described by Petrarch in 1364 as "rich in gold but richer in renown," mythologized herself—Venetia, Queen of the Andriatic, at once pagan and medieval, her heritage not so much of classical Rome but the Byzantine East (*5*). He painted her effortless grandeur in gowns of gold brocade, seated on clouds, trumpeted by angels, showered with jewels from the gods.

Venice inspired generations of poets and writers from William Shakespeare and Lord Byron to Thomas Mann. And Veronese influenced the course of European art—in the 17th century through Rubens and Velazquez, in the 18th, through Giovanni Battista Tiepolo and others.

Juno, the Roman goddess bestowing gifts on Venice in Veronese's brilliant allegory on this month's cover, was none other than Greek goddess Hera, powerful wife of Zeus. In antiquity, her giving was legend, for havoc as well as gifts. She ruined foes but sanctioned marriage, her generosity even celebrated by Shakespeare, "Honour, riches, marriage-blessing, / Long continuance, and increasing, / Hourly joys be still upon you! / Juno sings her blessings on you" (*6*). In this, another of her less bellicose appearances, Juno rains gold and crowns on Venice, grooming her for greatness and prosperity. Afloat in sensuous color, she glances down at her. An olive branch, signifying honor, acknowledges a city "mighty in her resources but mightier in virtue" (*7*).

The extravagance of Juno's gesture and its gracious acceptance bespeak the mythic greatness and splendor of Venice. Poetry and utopian texts, as well as the art of Veronese's time, attributed this greatness in part to topography—though lapped by the waves, Venice maintained close ties with the northern mainland and amassed a land empire, the *terraferma* (dry land). Another link to greatness was harmonious interaction with nature and the cosmos. Venetian humanist Pietro Bembo proposed "ideal love" as key to this interaction. Likewise, Jacopo Sannazzaro in L'Arcadia (1500) attributed moral and spiritual perfection to human connection with the natural world and its rhythms (*7*).

La Serenissima succumbed in the late 1700s, becoming a *ville crépusculaire* (city like any other). Like the original Arcadia, she had existed largely in the imagination. Connection with nature, indispensable to the myth, survived the fall of the empire; poets, painters, and scientists still seek it in Venice and elsewhere.

Bejeweled crowns from above, royal coronas, seem far removed from nature. Yet, nature disperses her own, less conspicuously but with far more bountiful abundance than Juno. Coronaviruses, common viruses of animals and humans, are named for their crownlike appearance. Recently, they came under the spotlight, when an obscure animal coronavirus left its wildlife reservoir to cause SARS, a lethal disease in humans. Nature's gift that keeps on giving, these viruses continue to emerge, in more species, more places, and now perhaps in North American bats, which could become involved in future emergence in humans or other animals (*8*).

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Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

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.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.