EMERGING INFECTIOUS DISEASES®

December 2012

Zoonoses

John and Mable Ringling Museum of Art, The State Art Museum of Florida, a division of Florida State University, USA (detail)

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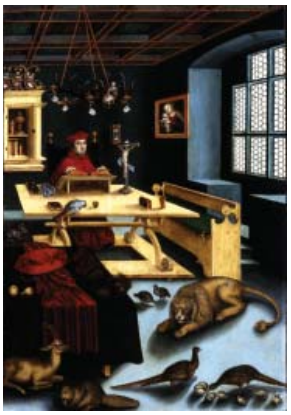

On the Cover

Lucas Cranach the Elder (1472–1553)
Cardinal Albrecht of Brandenburg as St. Jerome
(1526) Oil on wood panel (114.9 cm × 78.9 cm) (detail)

John and Mable Ringling Museum of Art, The State Art Museum of Florida, a division of Florida State University, USA

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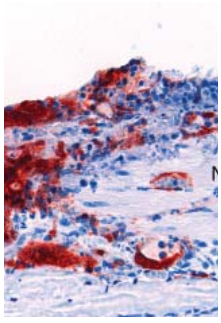

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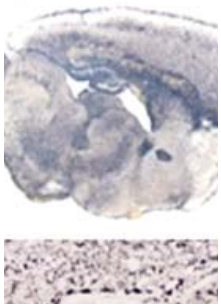

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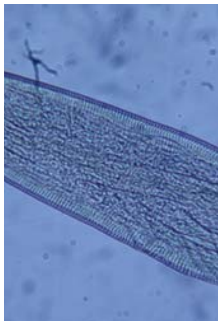

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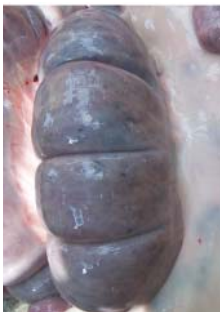

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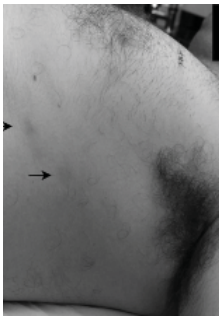

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Farm Animal Contact as Risk Factor for Transmission of Bovine-associated *Salmonella* Subtypes

Kevin J. Cummings, Lorin D. Warnick, Margaret A. Davis, Kaye Eckmann, Yrjö T. Gröhn, Karin Hoelzer, Kathryn MacDonald, Timothy P. Root, Julie D. Siler, Suzanne M. McGuire, Martin Wiedmann, Emily M. Wright, Shelley M. Zansky, and Thomas E. Besser

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Release date: November 14, 2012; Expiration date: November 14, 2013

Learning Objectives

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

- Analyze the epidemiology of salmonellosis.
- Distinguish broad characteristics of patients with salmonellosis in the current study.
- Assess risk factors for bovine-associated salmonellosis in the current study.

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CME Author

Charles P. Vega, MD, Health Sciences Clinical Professor; Residency Director, Department of Family Medicine, University of California, Irvine. *Disclosure: Charles P. Vega, MD, has disclosed no relevant financial relationships.*

Authors

*Disclosures**: Kevin J. Cummings, DVM, PhD; Margaret A. Davis, DVM, MPH, PhD; M. Kaye Eckmann, BS; Yrjö T. Gröhn, DVM, PhD; Karin Hoelzer, PhD; Kathryn MacDonald, PhD, RN; Timothy P. Root; Julie D. Siler; Suzanne M. McGuire, RN, BSN, CCTC; Emily M. Wright, BS; Shelley M. Zansky, PhD;** and **Thomas E. Besser, DVM, PhD, DACVM,** have disclosed no relevant financial relationships. **Lorin D. Warnick, DVM, PhD,** has disclosed the following relevant financial relationships: served as an advisor or consultant for Pfizer Animal Health. **Martin Wiedmann, DVM,** has disclosed the following relevant financial relationships: served as an advisor or consultant for Roka; owns stock, stock options, or bonds from Neogen, Sample6 Technologies.*

Author affiliations: Texas A&M University, College Station, Texas, USA (K.J. Cummings); Cornell University, Ithaca, New York, USA (K.J. Cummings, L.D. Warnick, Y.T. Gröhn, K. Hoelzer, J.D. Siler, M. Wiedmann, E.M. Wright); Washington State University, Pullman, Washington, USA (M.A. Davis, T.E. Besser); Washington State Department of Health, Olympia, Washington, USA (K. Eckmann, K. MacDonald); and New York State Department of Health, Albany, New York, USA (T.P. Root, S.M. McGuire, S.M. Zansky)

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Salmonellosis is usually associated with foodborne transmission. To identify risk from animal contact, we compared animal exposures of case-patients infected with bovine-associated *Salmonella* subtypes with those of control-patients infected with non-bovine–associated subtypes. We used data collected in New York and Washington, USA, from March 1, 2008, through March 1, 2010. Contact with farm animals during the 5 days before illness onset was significantly associated with being a case-patient (odds ratio 3.2, p = 0.0008), after consumption of undercooked ground beef and unpasteurized milk were controlled for. Contact

with cattle specifically was also significantly associated with being a case-patient (odds ratio 7.4, p = 0.0002), after food exposures were controlled for. More cases of bovine-associated salmonellosis in humans might result from direct contact with cattle, as opposed to ingestion of foods of bovine origin, than previously recognized. Efforts to control salmonellosis should include a focus on transmission routes other than foodborne.

Salmonella enterica remains a formidable public health challenge, resulting in ≈1.2 million illnesses and 400 deaths annually in the United States alone (*1*). Disease manifestations include diarrhea, fever, anorexia, abdominal pain, vomiting, and malaise. Although clinical disease generally resolves within 3–7 days, *Salmonella* spp. can also produce potentially fatal invasive infections. The incidence of human salmonellosis has not declined over the past 15 years and is significantly higher than it was during 2006–2008 (*2*). An estimated 94% of *Salmonella* infections are foodborne (*1*); common sources include undercooked eggs, poultry, beef, and pork; unpasteurized dairy products; and raw vegetables (*3–7*). Although some studies have shown that direct contact with infected animals is a risk factor for salmonellosis (*8,9*), the foodborne route is still regarded as the primary transmission route.

Dairy cattle are considered a key source of several *Salmonella* serovars that are a threat to human health, including multidrug-resistant *S. enterica* serovar Newport and *S. enterica* serovar Typhimurium (*8–11*). Foodborne transmission can occur through fecal contamination of beef carcasses at the time of slaughter (*12*) or through contamination of crops, either by manure used as fertilizer or by manure-contaminated irrigation water (*13*). Milk and other dairy products pose less of a public health threat because of commercial pasteurization, although consumption of unpasteurized dairy products persists. Infection by direct contact is an occupational risk for dairy farm workers and veterinarians. The most recent National Animal Health Monitoring System Dairy Study reports that there were >75,000 dairy operations in the United States in 2006, and the American Veterinary Medical Association reports that there were >5,000 veterinarians engaged either predominantly or exclusively in food animal practice as of 2010. Persons who interact with dairy cattle in public settings, such as open farms, petting zoos, and county or state fairs, are also at risk for salmonellosis through direct exposure (*8,9,14,15*).

Our objective was to identify significant risk factors for salmonellosis caused by bovine-associated *Salmonella* subtypes (including those within the Newport and Typhimurium serovars) by using the case–case study design (*16*). We specifically evaluated the role of direct animal contact as a potential route of transmission.

Materials and Methods

Study Population

This case–case study was conducted by using culture-confirmed human salmonellosis cases reported in targeted geographic areas in the states of New York and Washington, USA. Specimens were collected from March 1, 2008, through March 1, 2010.

New York State

Public Health Law in New York requires laboratories and physicians to report all salmonellosis cases to local health departments and to submit isolates to the New York State Department of Health (NYSDOH) Wadsworth Center Public Health Laboratory for diagnostic confirmation and speciation. The local health departments submit all case information, including laboratory data and questionnaire results, to the NYSDOH by a secure electronic data collection system. Within the FoodNet (www.cdc.gov/foodnet/) catchment area of the Centers for Disease Control and Prevention (CDC) Emerging Infections Program, surveillance officers actively monitor clinical microbiology laboratories and contact local health departments to ascertain all laboratory-confirmed salmonellosis cases, and they review case reports for accuracy and completeness. This catchment area includes 34 counties in the Albany, Buffalo, and Rochester areas of New York, representing ≈4.3 million residents (22% of the total state population).

Washington State

As in New York, salmonellosis is reportable in the state of Washington, and clinical laboratories are required to submit all isolates to the Washington State Department of Health (WSDOH) Public Health Laboratories for further characterization. County health departments submit all case information to the WSDOH Communicable Disease Epidemiology Unit. The 6 participating counties in Washington were King, Pierce, Snohomish, Spokane, Whatcom, and Yakima. These included 3 of the most populous counties (King, Pierce, and Snohomish) and 2 counties with the highest concentrations of dairy cattle (Whatcom and Yakima) in Washington. Spokane County comprises an urban population in addition to rural and farming communities. The 6 participating counties represent ≈4.3 million residents (65% of the total state population).

Laboratory Methods

In New York, serotyping and pulsed-field gel electrophoresis (PFGE) were performed on all *Salmonella* FoodNet isolates received by the NYSDOH during the study period. Typing data were forwarded to Cornell University (Ithaca, NY, USA) for PFGE pattern comparison

by BioNumerics software (Applied Maths Inc., Austin, TX, USA). Confirmed *Salmonella* isolates of bovine origin, obtained either from clinical samples submitted to the Cornell University Animal Health Diagnostic Center or from field study samples collected from clinically ill and asymptomatic dairy cattle, were sent to the US Department of Agriculture, Animal and Plant Health Inspection Service, National Veterinary Services Laboratories (Ames, IA, USA) for serotyping by standard protocols. PFGE subtyping of bovine isolates was performed in the Food Science Laboratory at Cornell University. The standard CDC PulseNet protocol (*17*) was used for subtyping all study isolates.

In Washington, serotyping and PFGE were performed on all human clinical isolates submitted to the WSDOH as described for New York. Bovine isolates, obtained either from clinical samples submitted to the Washington Animal Disease Diagnostic Laboratory or from samples collected during dairy cattle field studies, were also sent to the National Veterinary Services Laboratories for serotyping. PFGE subtyping of bovine isolates was performed at Washington State University, again by using the standard CDC PulseNet protocol.

Questionnaire

As part of the routine investigation of foodborne *Salmonella* infections, trained interviewers from local health departments in both states administered a standardized questionnaire to each patient by telephone. The NYSDOH *Salmonella* questionnaire was adapted from a previous version used for investigating all cases of foodborne infection. The standard WSDOH questionnaire was supplemented with an additional set of questions to ensure completeness of exposure data collection and to better align Washington data with New York data. Patient identification data were removed from each dataset before being transferred to the university research group in the respective state. Data collected in each state included demographic information, clinical features, and exposure history during the 5 days before disease onset. Exposure data included animal contacts, food history, food hygiene practices, water use for drinking and recreation, health care or daycare exposures, and travel history. After data collection had ended, both datasets were compiled at Cornell University for analysis.

Case-Patients and Control-Patients

Eligible cases included *Salmonella*–positive patients from the NYS FoodNet catchment area and the 6 participating Washington counties that were identified during the study period. Patients were excluded if they were associated with an obvious outbreak (as noted by the state health departments) or if they had a typhoidal *Salmonella* infection (either Typhi or Paratyphi A). For the case–control analysis, case-patients were defined as patients infected with *Salmonella* isolates that matched contemporary bovine isolates from the respective state by serovar and PFGE pattern. Control-patients were defined as patients infected with *Salmonella* isolates that were not associated with cattle, according to those criteria. Specifically, all patients infected with *S. enterica* serovar Dublin were classified as case-patients because this serovar is host-adapted to cattle (*18*). Patients infected with 6 other serovars (Newport, Typhimurium, Infantis, 4,5,12:i:–, Agona, and Montevideo) were classified as potential case-patients because of the importance of these serovars in bovine and human hosts.

According to a recent comprehensive study on the incidence of salmonellosis among dairy herds in New York and other northeastern states, the first 5 serovars just mentioned were among the leading serovars shed by dairy cattle with clinical *Salmonella* infections (*19*), and Montevideo is consistently one of the most prevalent serovars shed by asymptomatic cattle (*20*). All 6 are among the top 20 serovars isolated from human patients with laboratory-confirmed salmonellosis in the United States (*21*). For patients infected with one of the aforementioned serovars, PFGE patterns from the human isolates were compared with those from cattle. To be considered bovine associated, an isolate had to have a PFGE pattern indistinguishable from that of isolates obtained from ≥2 cattle in the same state from March 1, 2007, through March 1, 2010; patients infected with such isolates were thus classified as case-patients. Human isolates that differed from the most similar bovine isolate by 1–3 visible bands were excluded from the analysis, as were human isolates with a PFGE pattern matching that of just 1 bovine animal. Patients infected with isolates that differed from the most similar bovine isolate by ≥4 visible bands were classified as control-patients.

Patients infected with *Salmonella* serovars other than those previously listed were classified as control-patients if the serovar was not detected in cattle in the same state during that time frame. If the serovar was detected in cattle, the human isolate had to differ from the most similar bovine isolate by ≥4 visible bands in order for that patient to be considered a control-patient; otherwise, the human isolate was excluded from the analysis. A total of 422 bovine isolates from New York and 447 bovine isolates from Washington were used for PFGE pattern comparison.

Data Analysis

Data were imported into a commercially available statistical software program (SAS, version 9.2; SAS Institute Inc., Cary, NC, USA) for variable coding and analysis. Age was converted into a categorical variable

(<5, 5–12, 13–20, 21–40, 41–60, and >60 years of age). Animal, food, and other exposures were analyzed as dichotomous variables (yes/no). The variables "farm animal contact" and "bovine contact" were created to most effectively capture data from 2 state health department questionnaires that were not identical. In the New York dataset, farm animal contact was considered "yes" if the patient reported an occupation of animal farming or a history of farm animal contact; bovine contact was considered "yes" if the patient specified cattle as the type of farm animal. In the Washington dataset, farm animal contact was considered "yes" if the patient reported a history of living or working on a dairy or other farm type or reported a history of contact with cattle, sheep, goats, horses, or pigs; bovine contact was considered "yes" if the patient specified cattle as the type of farm animal.

Analysis was performed to compare exposures between case-patients and control-patients. Univariable descriptive analysis was performed on all explanatory variables. Bivariable analysis with the χ^2 test was used to determine whether each variable was independently associated with case or control status. Multivariable logistic regression models were used to identify risk factors for infection with bovine-associated subtypes; case or control status was used as the dichotomous outcome variable. Initial selection of variables was based on the bivariable analysis screening ($p<0.25$), and a backward elimination approach was used to identify a final multivariable model; values of $p<0.05$ were considered significant. Relevant 2-way interaction terms (involving exposure variables retained in the final model, demographic variables, and state) were also investigated for significance within each model. Consumption of undercooked ground beef and unpasteurized milk in the 5 days before disease onset were included in each model as potential confounders. The population attributable fraction (PAF), defined as the proportion of disease in a population that can be attributed to a given exposure, was calculated for variables retained in each model by using the formula $PAF = P(OR_{adj} - 1)/OR_{adj}$ (where P = the proportion of case-patients exposed to the risk factor and OR_{adj} = the adjusted odds ratio for that factor) (*22*).

Results

From March 1, 2008, though March 1, 2010, the NYSDOH received nontyphoidal *Salmonella* isolates from 835 patients within the NYS FoodNet catchment area. According to our criteria, 40 (4.8%) of these were classified as case-patients and 356 (42.6%) as control-patients. Among case-patients, 20 (50.0%) were female; among control-patients, 215 (60.4%) were female. The median age among case-patients was 31.5 years, whereas that among control-patients was 31 years. Typhimurium was the most common serovar among case-patients (67.5%), and Enteritidis and Typhimurium were equally predominant among control-patients (10.1%; Table 1).

During the study period, 562 patients with nontyphoidal salmonellosis were identified in the 6 participating Washington counties. According to our criteria, 87 (15.5%) of these were classified as case-patients and 428 (76.2%) as control-patients. Among case-patients, 53 (60.9%) were female; among control-patients, 229 (53.5%) were female. The median age among case-patients was 28 years, whereas that among control-patients was 33 years. The most common serovar among case-patients was Typhimurium (51.7%), and the most common serovar among control-patients was Enteritidis (40.4%, Table 2).

The datasets from each state were combined to yield a total of 127 case-patients and 784 control-patients.

Table 1. Distribution of *Salmonella* serovars among 835 patients, New York, USA, March 1, 2008–March 1, 2010

Serovar	No. (%)
Case-patients, n = 40	
Typhimurium	27 (67.5)
Dublin	7 (17.5)
Newport	5 (12.5)
Infantis	1 (2.5)
Control-patients, n = 356	
Enteritidis	36 (10.1)
Typhimurium	36 (10.1)
Heidelberg	32 (9.0)
Newport	21 (5.9)
Braenderup	16 (4.5)
Javiana	14 (3.9)
Saintpaul	13 (3.7)
Hadar	12 (3.4)
B,5:i:–	11 (3.1)
Muenchen	10 (2.8)
Agona	8 (2.2)
Berta	8 (2.2)
Paratyphi B var. L-tartrate+	8 (2.2)
B,5:b:–	7 (2.0)
Poona	7 (2.0)
Stanley	7 (2.0)
Hartford	6 (1.7)
Miami	6 (1.7)
Montevideo	6 (1.7)
Schwarzengrund	5 (1.4)
Bovismorbificans	4 (1.1)
Derby	4 (1.1)
Ealing	4 (1.1)
Manhattan	4 (1.1)
Mississippi	4 (1.1)
Other serovars	67 (18.8)
Excluded patients, n = 439	
Enteritidis	195 (44.4)
Typhimurium	99 (22.6)
Thompson	22 (5.0)
Oranienburg	18 (4.1)
Newport	14 (3.2)
Montevideo	8 (1.8)
Infantis	7 (1.6)
Tennessee	7 (1.6)
Panama	6 (1.4)
Other serovars	46 (10.5)
Not typed	17 (3.9)

Table 2. Distribution of *Salmonella* serovars among 562 patients, Washington, USA, March 1, 2008–March 1, 2010

Serovar	No. (%)
Case-patients, n = 87	
Typhimurium	45 (51.7)
Montevideo	14 (16.1)
Newport	13 (14.9)
4,5,12:i:–	9 (10.3)
Dublin	5 (5.7)
Infantis	1 (1.1)
Control-patients, n = 428	
Enteritidis	173 (40.4)
Typhimurium	29 (6.8)
Paratyphi B var. L-tartrate+	23 (5.4)
Javiana	11 (2.6)
Montevideo	11 (2.6)
Stanley	10 (2.3)
Braenderup	9 (2.1)
Litchfield	9 (2.1)
Thompson	9 (2.1)
4,5,12:i:–	8 (1.9)
Heidelberg	8 (1.9)
Muenchen	7 (1.6)
Senftenberg	7 (1.6)
Virchow	7 (1.6)
Agona	6 (1.4)
Potsdam	6 (1.4)
Saintpaul	6 (1.4)
Oranienburg	5 (1.2)
Other serovars	84 (19.6)
Excluded patients, n = 47	
Typhimurium	7 (14.9)
Heidelberg	6 (12.8)
Montevideo	6 (12.8)
Brandenburg	4 (8.5)
Oranienburg	3 (6.4)
Saintpaul	3 (6.4)
4,5,12:i:-	2 (4.3)
Infantis	2 (4.3)
Newport	2 (4.3)
Panama	2 (4.3)
1,4,5,12:i:-	1 (2.1)
Anatum	1 (2.1)
Enteritidis	1 (2.1)
Hadar	1 (2.1)
Mbandaka	1 (2.1)
Oslo	1 (2.1)
Sandiego	1 (2.1)
Uganda	1 (2.1)
Not typed	2 (4.3)

Bivariable analysis indicated that more case-patients (11.0%) than control-patients (3.8%) reported a history of farm animal contact during the 5 days before disease onset (p = 0.0004). More case-patients (6.3%) than control-patients (1.0%) also reported a specific history of bovine contact during the 5 days before illness (p<0.0001). Attendance at an open farm/petting zoo/fair was more common (p = 0.05) among case-patients (11.8%) than control-patients (7.0%), and more case-patients (12.6%) than control-patients (6.5%) reported a history of contact with animal manure (p = 0.01). Fewer case-patients (3.1%) than control-patients (13.9%) reported a history of international travel before illness (p = 0.0006). Case-patients and control-patients did not differ significantly with respect to sex, age group, eating undercooked ground beef, or drinking unpasteurized milk.

Multivariable logistic regression analysis showed that a history of farm animal contact during the 5 days before disease onset was significantly associated with being a case-patient (odds ratio [OR] 3.2, 95% CI 1.6–6.4, p = 0.0008), after consumption of undercooked ground beef and unpasteurized milk was accounted for (Table 3). A specific history of bovine contact during the 5 days before illness was also significantly associated with being a case-patient (OR, 7.4, 95% CI 2.6–20.9, p = 0.0002), according to estimates from a separate logistic regression model that also controlled for those food exposures (Table 4). International travel was negatively associated with being a case-patient in each of the models. No significant interaction was found between farm animal/bovine contact and state, sex, or age group in the respective models. The PAF, applied here as the proportion of *Salmonella* infections among the source population of laboratory-confirmed cases that can be attributed to a certain exposure, was calculated to be 7.6% for farm animal contact and 5.4% for bovine contact in particular.

To perform a sensitivity analysis for testing the effect of our strict case definition, we repeated multivariable logistic regression models under 2 extreme scenarios; all potential case-patients that were excluded (because the isolate differed from the most similar bovine isolate by 1–3 visible bands or because its PFGE pattern matched that of just 1 bovine animal) served alternatively as case-patients (scenario 1) and control-patients (scenario 2). The parameter estimates and ORs from these hypothetical models were comparable to those obtained from our original analyses (the ORs under scenarios 1 and 2 were 3.2 and 2.3 for farm animal contact, 6.1 and 4.0 for bovine contact, respectively).

Discussion

The case–case study design proposed by McCarthy and Giesecke is an adaptation of the conventional case–control approach (*16*). It has been used to study risk factors and clinical features associated with particular subtypes of *Salmonella* spp. (*8,23–26*), *Campylobacter* spp. (*27,28*), and *Clostridium difficile* (*29*). One of its main advantages is the removal of selection bias imposed by the surveillance system; case-patients and control-patients were subjected to the same selection process in order to be detected by a state health department as a laboratory-confirmed case. Another advantage is the negation of recall bias (a form of information bias); because case-patients and control-patients had salmonellosis, their recall of exposures should have been similarly affected by attitudes regarding causation.

Table 3. Association between infection with a bovine-associated *Salmonella* subtype and farm animal contact, New York and Washington, USA, March 1, 2008–March 1, 2010*

Variable	Odds ratio (95% CI)	p value
Farm animal contact	3.2 (1.6–6.4)	0.0008
Undercooked ground beef	1.5 (0.7–3.1)	0.3
Unpasteurized milk	0.5 (0.1–4.2)	0.5
International travel	0.2 (0.1–0.6)	0.002

*Estimated by a logistic regression model.

A potential limitation of the case–case study design is that the control-patients might not represent the exposure prevalence in the source population on account of the unique exposures that led them to become infected. However, we believe that we addressed this issue by including a diverse array of serovars and PFGE types in the control group, assuming that their associated exposures were presumably also diverse and thus more representative of the total spectrum of exposures associated with nonbovine *Salmonella* strains. Another possible drawback of this study design is that case-patients and control-patients share a certain subset of exposures that pose a risk for *Salmonella* infections in general; such exposures will therefore remain unidentified or at least be underestimated as risk factors. Although this study design precludes the study of general risk factors for salmonellosis, it is useful for investigating exposures that are serovar or subtype specific.

Other studies have found an association between salmonellosis and having previous contact with either cattle or a farm environment (*8,9,30*). Our study investigated this association with a case–case approach that used a strict case definition. Another strength of this study was the use of sporadic cases of salmonellosis rather than cases associated with outbreaks. Insight regarding the epidemiology of sporadic *Salmonella* infections has traditionally been limited because specific sources of enteric illness are seldom identified when not occurring as part of an outbreak.

Direct contact with dairy cattle or their environment during the 5 days before illness onset was significantly associated with salmonellosis caused by a bovine-matched subtype in New York and Washington. Because there was no interaction between state and the animal contact variables in the models, we concluded that the effect estimate was consistent across the 2 states. The ORs for farm animal contact and specific bovine contact in each state were also similar to those obtained from analysis of the combined dataset (data not shown). In addition, our sensitivity analysis led us to decide that we still would have reached the same conclusions with modified case criteria. These results have important implications for dairy farm workers and their families, veterinarians and veterinary staff, and those who interact with dairy cattle in public settings.

Although attendance at an open farm/petting zoo/fair was not significantly associated with being a case-patient in this study, it is logical to believe that visiting such a facility might increase the risk for salmonellosis, on the basis of our other findings. *S. enterica* is transmitted primarily by the fecal–oral route. Direct contact with the feces of infected cattle can occur through feeding, petting, or otherwise handling them; contaminated clothing or footwear, animal bedding, barriers, or other environmental surfaces can also be sources of infection (*15,31*). This threat is underscored by the recent finding that the median duration of fecal *Salmonella* shedding following clinical disease among dairy cattle is 50 days (*32*).

The negative association between recent international travel and salmonellosis caused by a bovine-matched subtype was anticipated. Although travel outside the United States is a well-known risk factor for *Salmonella* infections (*33*) (observed in a significantly higher proportion of control-patients in this study), it would not be expected to have an association with salmonellosis caused specifically by subtypes shared by dairy cattle in New York and Washington.

The percentage of *Salmonella* infections in the United States that are foodborne was recently estimated at 94% (*1*). The results of our statistical analyses suggest that this percentage might be an overestimate, at least for bovine-associated *Salmonella* subtypes, although our PAF results (which also take into account the frequency of exposure) are more consistent with this estimate. It also must be noted that the effect of animal exposure observed in New York and Washington might not be representative of the rest of the country. Nevertheless, more human infections originating from bovine sources might result from direct contact with cattle (as opposed to foods of bovine origin) than previously recognized. Clear evidence for the role of direct farm animal contact as a source of human salmonellosis indicates that it is imperative for *Salmonella* control efforts to include a focus on transmission routes other than foodborne. The efficacy and public health impact of addressing nonfoodborne transmission of *Salmonella* spp. have been demonstrated by studies of direct contact transmission from pet turtles to humans, particularly children. In response to studies that established turtles as an important source of human salmonellosis (*34,35*), federal legislation in 1975 prohibited the sale and distribution of turtles <4 inches in

Table 4. Association between infection with a bovine-associated *Salmonella* subtype and bovine contact, New York and Washington, USA, March 1, 2008–March 1, 2010*

Variable	Odds ratio (95% CI)	p value
Bovine contact	7.4 (2.6–20.9)	0.0002
Undercooked ground beef	1.6 (0.8–3.2)	0.2
Unpasteurized milk	0.5 (0.1–5.1)	0.6
International travel	0.2 (0.1–0.5)	0.002

*Estimated by a logistic regression model.

carapace length. Still in effect today, this ban coincided with an 18% reduction in *Salmonella* infections among children 1–9 years of age (*36*).

A number of measures can be taken to minimize the likelihood of becoming infected with *Salmonella* spp. from direct contact with farm animals. Washing hands with soap and water is a simple yet highly protective step that can be taken after contact with animals or feces (*15,37*). Children <5 years of age, elderly adults, and immunocompromised persons are at increased risk for invasive salmonellosis (*38,39*) and thus should pay special attention to hygiene or avoid certain animal contacts altogether. Veterinarians should teach cattle owners and farm employees to wash well after work or before eating, to disinfect boots and equipment, and to keep coveralls out of the house. If treating infected cattle, veterinarians must specifically counsel their clients about the risk for zoonoses. In particular, high-risk groups should avoid contact with infected cattle. Veterinarians should instruct their staff members to protect themselves by using appropriate infection control procedures (*40*), especially if working with livestock. Increased physician awareness of the role of direct farm animal contact in transmitting *Salmonella* spp. and other enteric zoonotic pathogens is likewise needed. Physicians should educate their patients, particularly those at increased risk for severe disease, regarding the potential threat posed by animal contact and the importance of hand hygiene after such contact. Patients with diarrheal illness should be questioned about their exposures to cattle, farm environments, and other animal species. It is also essential that those who operate open farms and other animal exhibits adhere to current guidelines by equipping such areas with handwashing facilities, preventing food and drink in these areas, maintaining an adequate cleaning and disinfection protocol, and providing visitors with educational materials on disease prevention (*37*). In conclusion, prevention of salmonellosis should include a focus on safe animal contact in addition to food safety measures.

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Dr Cummings is an assistant professor of epidemiology at Texas A&M University College of Veterinary Medicine and Biomedical Sciences. His research focuses on diseases of importance to public health, in particular *Salmonella* spp. and other foodborne pathogens.

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Address for correspondence: Kevin J. Cummings, Department of Veterinary Integrative Biosciences, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, 4458 TAMU, College Station, TX 77843-4458, USA; email: kcummings@cvm.tamu.edu

Outbreak of Influenza A (H3N2) Variant Virus Infection among Attendees of an Agricultural Fair, Pennsylvania, USA, 2011

Karen K. Wong, Adena Greenbaum, Maria E. Moll, James Lando, Erin L. Moore, Rahul Ganatra, Matthew Biggerstaff, Eugene Lam, Erica E. Smith, Aaron D. Storms, Jeffrey R. Miller, Virginia Dato, Kumar Nalluswami, Atmaram Nambiar, Sharon A. Silvestri, James R. Lute, Stephen Ostroff, Kathy Hancock, Alicia Branch, Susan C. Trock, Alexander Klimov, Bo Shu, Lynnette Brammer, Scott Epperson, Lyn Finelli, and Michael A. Jhung

During August 2011, influenza A (H3N2) variant [A(H3N2)v] virus infection developed in a child who attended an agricultural fair in Pennsylvania, USA; the virus resulted from reassortment of a swine influenza virus with influenza A(H1N1)pdm09. We interviewed fair attendees and conducted a retrospective cohort study among members of an agricultural club who attended the fair. Probable and confirmed cases of A(H3N2)v virus infection were defined by serology and genomic sequencing results, respectively. We identified 82 suspected, 4 probable, and 3 confirmed case-patients who attended the fair. Among 127 cohort study members, the risk for suspected case status increased as swine exposure increased from none (4%; referent) to visiting swine exhibits (8%; relative risk 2.1; 95% CI 0.2–53.4) to touching swine (16%; relative risk 4.4; 95% CI 0.8–116.3). Fairs may be venues for zoonotic transmission of viruses with epidemic potential; thus, health officials should investigate respiratory illness outbreaks associated with agricultural events.

Triple reassortant swine influenza A viruses have circulated in swine herds in North America since 1998 (*1*–*3*). On the rare occasions that these viruses infect humans, they are called influenza A variant viruses (*4*). Viruses resulting from reassortment of swine influenza A (H3N2) virus and influenza A(H1N1)pdm09 (pH1N1) virus have emerged among US swine (*4*–*6*), and similar viruses have been identified among swine outside the United States (*7*,*8*). During August 2011, the first known human infection with influenza A (H3N2) variant [A(H3N2)v] virus containing the pH1N1 matrix (M) gene was reported in the United States (*9*). The pH1N1 M gene is implicated in increasing influenza transmissibility in animal models (*10*,*11*), and there was concern that this new A(H3N2)v virus could be efficiently transmitted among humans. Because these viruses contain a novel combination of genes, little is known about the epidemiologic and clinical characteristics of human infections.

During August 2011, a child who had attended an agricultural fair in Pennsylvania (Fair A) attended by ≈70,000 persons became ill; the Centers for Disease Control and Prevention (CDC) confirmed infection with A(H3N2)v virus in the child 6 days after Fair A closed and immediately began an investigation with the Pennsylvania Department of Health (PA DOH), the Allegheny County Health Department, and the Pennsylvania Department of Agriculture (PDA) to determine the extent of A(H3N2)v virus transmission and to identify illness risk factors among Fair A attendees.

Methods

Case Finding

We identified cases through multiple methods. First, 1–2 weeks after Fair A closed, PDA investigators conducted open-ended interviews with swine exhibitors to determine whether they or their household members had be-

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (K.K. Wong, A. Greenbaum, R. Ganatra, M. Biggerstaff, E. Lam, A.D. Storms, J.R. Miller, K. Hancock, A. Branch, S.C. Trock, A. Klimov, B. Shu, L. Brammer, S. Epperson, L. Finelli, M.A. Jhung); Pennsylvania Department of Health, Harrisburg, Pennsylvania, USA (M.E. Moll, E.E. Smith, J.R. Miller, V. Dato, K. Nalluswami, A. Nambiar, J.R. Lute, S. Ostroff); Allegheny County Health Department, Pittsburgh, Pennsylvania (J. Lando, S.A. Silvestri); and Pennsylvania Department of Agriculture, Harrisburg (E.L. Moore)

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come ill; exhibitors were identified though a list provided by fair organizers. Ill exhibitors or their surrogates were interviewed by CDC/PA DOH to assess whether their illness met suspected case criteria. Second, members of a national children's agricultural club who participated in activities in the county where Fair A occurred were interviewed about illness occurring in their households after attendance at Fair A and/or swine exposure. Third, investigators went to another fair (Fair B), which occurred 3 weeks after Fair A »20 miles away in the same county, to enroll a convenience sample of Fair B attendees. Investigators enrolled Fair B attendees as they visited the health department booth, food service areas, exhibit halls, rides, and games. Fair B enrollees were subsequently asked during a phone interview whether they or their household members had become ill after attending Fair A. Fourth, media sources, including newspapers, television, and websites, encouraged community members to contact PA DOH if they became ill with influenza-like symptoms after attending Fair A. Fifth, clinicians were encouraged to obtain respiratory specimens from patients with suspected influenza virus infection who had recent swine or agricultural fair exposure and to refer such patients to PA DOH for interview. Sixth, state and county influenza surveillance supported prospective detection of persons with test results positive for influenza. Seventh, ill contacts of case-patients were interviewed by investigators.

If close contacts of case-patients became ill, they were offered testing for influenza virus infection regardless of whether they attended Fair A. Testing was done only if specimens could be collected ≤7 days after symptom onset; nasopharyngeal swab samples were used for testing.

Retrospective Cohort Study

We conducted a retrospective cohort study among a systematic random sample of members of a children's agricultural club (Club X) who resided in the county where Fair A occurred and who attended Fair A. Club members were children who conducted projects, such as raising livestock, for exhibition at Fair A. From a list of 994 Club X members, every fourth name was selected, yielding a cohort of 247 children. Using a standard questionnaire, we queried the parents of Club X members about illness occurring since Fair A; animal exposures at Fair A, home, work, and/or school; influenza vaccination history; and underlying medical conditions. If no adult was reached after 3 telephone attempts, the household members were considered nonrespondents. Interviews were conducted 3–4 weeks after Fair A concluded. Levels of swine exposure were categorized as 1) no exposure (attending Fair A but not visiting a swine exhibit); 2) indirect exposure (visiting a swine exhibit but not touching swine at Fair A); and 3) direct exposure (touching swine at Fair A). Risk for illness was estimated from the beginning of Fair A through 7 days after its conclusion.

Case Definitions

A suspected case-patient was a person with ≥1 sign/symptom of influenza virus infection from ≥2 of 4 categories occurring ≤7 days after attending Fair A. Categories were: 1) fever (temperature >38C°) or subjective fever; 2) respiratory (cough, sore throat, or runny nose); 3) gastrointestinal (vomiting or diarrhea); and 4) constitutional (fatigue, muscle aches, or joint pain). At least 1 category was required to be fever or respiratory.

A broad clinical case definition was used because the clinical characteristics of A(H3N2)v virus infections were not well understood. Suspected case-patients were reclassified as noncase-patients if respiratory specimens obtained ≤7 days after symptom onset had real-time reverse transcription PCR (rRT-PCR) or genomic sequencing results negative for A(H3N2)v virus or if convalescent-phase serology results were negative for A(H3N2)v virus infection.

A probable case-patient was a person <4 years of age (explained below) who met suspected case-patient criteria and who was seropositive for A(H3N2)v virus. A confirmed case-patient was a person who had rRT-PCR and genomic sequencing results positive for A(H3N2)v virus infection; RNA from a respiratory specimen was used for genomic sequencing (*12*).

Influenza Diagnostic Testing

Respiratory Specimens

Respiratory specimens were obtained ≤7 days after symptom onset. We used rRT-PCR with the Human Influenza Virus Real-Time RT-PCR Diagnostic Panel (CDC, Atlanta, GA, USA) to test specimens. Specimens positive for influenza A were subtyped, and amplified RNA from specimens with results consistent with A(H3N2)v virus infection (positive for InfA, pdmInfA, and H3 markers) or with indeterminate results underwent partial genome sequencing as described (*12*,*13*).

Serologic Testing

We asked suspected case-patients ≤13 years of age to participate in serologic testing. We chose this age group for testing because it is assumed that children have limited prior exposure to viruses similar to A(H3N2)v virus and therefore fewer cross-reactive antibodies. A convalescent-phase serum sample was obtained from participating suspected case-patients 3–5 weeks after illness onset. Serum samples were tested by microneutralization and hemagglutination inhibition (HI) for antibodies to variant strains A/Minnesota/11/2010 (H3N2) and A/Indiana/08/2011 (H3N2). The outbreak strain could not be used as an antigen because vi-

able virus was not isolated from any of the case-patients. Microneutralization and HI tests were performed as described (*14*).

Preliminary testing of serum samples collected in 2007–2008 and in 2010 indicates that no children <4 years of age have antibodies to A(H3N2)v virus, but some children 4–13 years of age have cross-reactive antibodies (*15*). Therefore, test results for children <4 years of age were considered seronegative if HI titers to the variant strains were <10, indeterminate if titers were 10 to <40, and seropositive if titers were ≥40. Test results for children 4–13 years of age were considered seronegative if titers to the variant strains were <10 and indeterminate if titers were ≥10. Children with seronegative test results were reclassified as noncase-patients, and those with seropositive results were reclassified as probable case-patients. Children with indeterminate results retained suspected case-patient status.

Animal Investigation

PDA veterinarians routinely inspected all swine at Fair A. In addition, veterinarians called Fair A swine exhibitors 1–2 weeks after Fair A closed to ask whether signs of illness developed in any swine during or shortly after Fair A.

Data Analysis

We entered data into a Microsoft Access 2010 database (Microsoft, Redmond, WA, USA) and analyzed it by using SAS version 9.3 (SAS Institute, Cary, NC, USA). Relative risks and exact 95% CIs, determined by using the Farrington-Manning method (*16*), are reported for selected exposures.

Ethical Considerations

This investigation was determined to be a response to a public health threat; in accordance with Federal human subjects' protection regulations, it was not considered to be human subjects research. Participation in interviews was voluntary; parents or guardians were interviewed for subjects <18 years of age. Parents or guardians consented to collection of respiratory and serum samples from subjects <18 years of age. Minors ≥7 years of age assented to collection of respiratory and serum samples.

Results

Case Finding

We identified 3 confirmed, 4 probable, and 82 suspected cases, including the index case. No A(H3N2)v virus infections were identified by state or county influenza surveillance or by clinicians among persons who did not attend Fair A. Of the confirmed, probable, and suspected cases, 19 (21%) were identified from Fair A swine exhibitor households, 29 (33%) were identified from Club X households, 4 (4%) were identified among Fair B attendees who also attended Fair A, 34 (38%) were identified among persons who called PA DOH to report illness, 10 (11%) were identified by another case-patient, and 2 (2%) were detected by state influenza surveillance; persons could be identified by >1 method. The median age of all case-patients was 12 years (range 6 months–60 years); 39 (44%) were male (Table 1). Dates of illness onset ranged from day 0–13, where day 0 was the opening day for Fair A (Figure). Most case-patients had illness onset within 4 days after either the swine show or swine auction, and no cases were identified >6 days after the fair ended. Of 87 case-patients for whom medical history was known, 18 (21%) reported at least 1 underlying medical condition. Case-patients reported spending a median of 6 days (range 1–10 days) at Fair A, and 29 (33%) of 89 reported that their household owned swine. Of 87 case-patients for whom swine exposure was known, 80 (92%) reported direct or indirect swine exposure at Fair A.

The first confirmed case occurred in a previously healthy girl <4 years of age who touched swine at Fair A (case-patient 1); fever, cough, and rhinorrhea developed 4 days after she had contact with swine. The second confirmed case occurred in a previously healthy girl in the 4- to 13-year-old age group who exhibited swine at Fair A and had subjective fever and vomiting without respiratory symptoms (case-patient 2). The third confirmed case occurred in a girl in the 4- to 13-year-old age group who had a preexisting medical condition (case-patient 3); the girl had contact with swine at Fair A and was hospitalized for respiratory distress. Of 3 confirmed and 4 probable case-patients, 2 (29%) were male and all were ≤13 years of age. All confirmed and probable case-patients attended Fair A on or after day 3 of the fair (Table 2). All except case-patient 2 had fever and respiratory symptoms, and all recovered. All 7 confirmed and probable case-patients visited the swine exhibit at Fair A, and 6 (86%) touched swine.

Illness developed in contacts of 4 case-patients (3 suspected and 1 confirmed case-patient); the contacts had not attended Fair A ≤7 days before illness onset. Respiratory specimens were obtained from 3 of these 4 contacts, including the contact of the confirmed case-patient, ≤7 days after illness onset; all tested negative for influenza by rRT-PCR, and 1 contact tested positive for rhinovirus. One person declined testing for influenza.

Laboratory Results

Respiratory specimens from case-patients 1 and 3 were positive for InfA, H3, and pdmInfA markers by rRT-PCR, and the specimen from case-patient 2 was InfA positive. Phylogenetic analysis of the 3 specimens showed that the genome contained the M gene from pH1N1 and 7 gene segments (hemagglutinin, neuraminidase, polymerase PB1,

Table 1. Characteristics of case-patients with suspected, probable, or confirmed A(H3N2)v virus infection after attending agricultural Fair A, Pennsylvania, 2011*

Characteristic	A(H3N2)v case status		
	Suspected, n = 82	Probable and confirmed, n = 7	All cases, N = 89
Sex			
M	37 (45)	2 (29)	39 (44)
F	45 (55)	5 (71)	50 (56)
Age, y			
Median (range)	13 (0.5–60)	3 (1–9)	12 (0.5–60)
<4	9 (11)	5 (71)	14 (16)
4–13	34 (41)	2 (29)	36 (40)
14–18	12 (15)	0	12 (13)
19–49	17 (21)	0	17 (19)
≥50	10 (12)	0	10 (11)
Underlying medical condition†	17/80 (21)	1/7 (14)	18/87 (21)
Reported any prior influenza vaccination	49/73 (67)	5/5 (100)	54/78 (69)
No. days spent at fair, median (range)	7 (1–10)	4 (1–8)	6 (1–10)
Family owns swine	27/82 (33)	2/7 (29)	29/89 (33)
Reported swine exposure‡	73/80 (91)	7/7 (100)	80/87 (92)
Signs/symptoms			
Fever/feverish	74/82 (90)	7/7 (100)	81/89 (91)
Cough	58/80 (73)	6/7 (86)	64/87 (74)
Runny nose	38/77 (49)	3/7 (43)	41/84 (49)
Headache	58/72 (81)	0/4	58/76 (76)
Muscle aches	40/69 (58)	0/5	40/74 (54)
Sore throat	44/72 (61)	1/5 (20)	45/77 (58)
Shortness of breath	16/69 (23)	1/7 (14)	17/76 (22)
Vomiting	21/82 (26)	2/7 (29)	23/89 (26)
Diarrhea	27/82 (33)	2/7 (29)	29/89 (33)
Fatigue	55/57 (96)	3/3 (100)	58/60 (97)
Joint pain	18/53 (34)	0/1	18/54 (33)
Illness duration, median days (range)	7 (2–18)§	6 (3–9)¶	6 (2–18)**
Received medical attention	28/81 (35)	3/7 (43)	31/88 (35)
Hospitalized	0/81	1/7 (14)	1/88 (1)

*Data are no. (%) with characteristic or no. with characteristic/total no. case-patients with data (%) unless otherwise specified. A(H3N2)v, influenza A (H3N2) variant virus.
†Includes asthma, chronic lung disease, heart disease, diabetes or metabolic disease, kidney disease, liver disease, immunosuppressive condition, cancer, and neurologic or neurodevelopmental disorders.
‡Includes visiting swine exhibit, walking near swine exhibit, or touching swine at fair.
§Of 60 persons with known dates of illness onset and recovery.
¶Of 5 persons with known date of recovery.
**Of 65 persons with known date of recovery.

polymerase PB2, polymerase PA, nucleocapsid protein, nonstructural protein) similar to those from North American swine H3N2 subtype viruses and variant viruses that previously caused infection in humans (*13*).

Convalescent-phase serum samples were obtained from 6 (40%) of 15 persons <4 years of age who initially met suspected case-patient criteria; 4 (67%) of the 6 samples were seropositive for A(H3N2)v virus (HI geometric mean titers ≥57), and 2 (33%) were seronegative. Convalescent-phase serum samples were obtained from 18 (47%) of 38 persons 4–13 years of age who initially met suspected case-patient criteria; 4 (22%) of the 18 samples were seronegative for A(H3N2)v, and 14 (78%) had indeterminate results.

Retrospective Cohort Study

We were able to contact 139 (56%) of the 247 Club X members; 127 (91%) of those contacted agreed to be interviewed. The median age of Club X members was 13 years (range 4–19 years); 47 (37%) were male (Table 3). Of 124 members, 19 (15%) reported ≥1 underlying medical condition. Members spent a median of 9 days at the fair (range 1–10 days), and 75/125 (60%) exhibited animals at Fair A; 34/125 (27%) exhibited swine. Of 125 families, 83 (66%) owned livestock and 33 (26%) owned swine.

Of 127 Club X members, 15 initially met the suspected case definition. Serologic testing was performed for 3 members: 1 was seronegative for A(H3N2)v virus and was reclassified as a noncase-patient, and 2 had indeterminate results. Thus, 14 (11%) of the 127 Club X members were suspected case-patients. Respiratory specimens were not obtained from any club members.

The risk for suspected case status increased as exposure to swine increased from no exposure (referent) to indirect exposure (relative risk [RR] 2.1; 95% CI 0.2–53.4) to direct exposure (RR 4.4; 95% CI 0.8–116.3; p = 0.07 by Cochran-Armitage trend test); however, these differences were not statistically significant (Table 4). Exhibiting swine was not associated with suspected case-patient status (RR 1.1; 95% CI 0.2–3.2). Suspected case-patient status was more common, but not statistically significantly

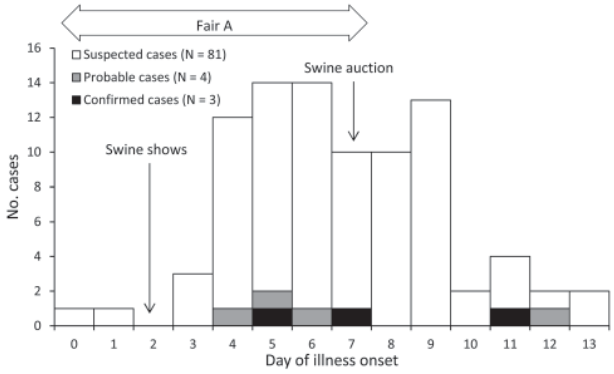

Figure. Epidemic curve, by date of illness onset and case status, for 88 cases of influenza A (H3N2) variant virus infection associated with agricultural Fair A, Pennsylvania, 2011. Day 0 is the first day the fair was open to the public. One suspected case is not shown; the day of illness onset is unknown but ≤7 days after attending the fair.

so, among persons whose families owned swine and among persons who fed or bathed swine or who cleaned the swine pen during the fair (Table 4).

Animal Investigation

The PDA veterinarian inspected >150 swine on day 3 of Fair A. All swine were healthy-appearing at inspection, although fever had developed in 1 pig, and that pig had already been removed from the fair. The febrile pig was housed with other swine at the fair for »24 hours before removal; it was not tested for influenza. Another pig died after a seizure on the last day of the fair; the cause of death was unknown.

No other illness in swine was reported to PDA during the fair. After the fair ended, PDA veterinarians attempted to call 135 households of swine exhibitors and reached 80 (59%). Of those 80 households, 8 (10%) reported that the swine they exhibited had signs of respiratory illness during or shortly after the fair. Ill swine had recovered or had been slaughtered before these interviews. No swine were tested for influenza.

Discussion

We describe an outbreak of respiratory illness, including 3 confirmed infections with a variant influenza A virus not identified in humans before August 2011. The outbreak occurred during a large agricultural fair, where humans and animals had opportunities for repeated and/or prolonged contact.

Outbreaks of variant influenza A viruses at agricultural events have been described, and these events may be key settings for zoonotic influenza transmission (*17*–*24*). Triple reassortant H3N2 subtype viruses containing the pH1N1 M gene were first identified among swine in the United States in 2009 and have been detected among swine in multiple states, including Pennsylvania (*25*,*26*). During July–November 2011, 13 human infections with A(H3N2)v virus containing the pH1N1 M gene were identified, and 5 were linked to agricultural fairs (*24*). Although the frequency of zoonotic influenza transmission at agricultural events is unknown, these events provide opportunities for swine influenza viruses to infect humans who have contact with infected swine. Human and swine influenza viruses may circulate at these events, creating opportunities for virus reassortment and the emergence of novel strains.

This investigation suggests that swine contact during Fair A was a risk factor for illness. Persons reporting direct contact with swine were more likely to report illness. Most case-patients became ill within 4 days after the swine show

Table 2. Characteristics of case-patients with confirmed or probable A(H3N2)v virus infection after attending an agricultural Fair A, Pennsylvania, 2011*

Case-patient no., case status	Age group, y/sex	Underlying condition	Date(s) at fair†	Date of illness onset‡	Symptoms	Clinical outcome
1, confirmed	<4/F	No	3	7	Fever, cough, runny nose	Visited clinic, recovered
2, confirmed	4–13/F	No	0 to 7	11	Feverish, vomiting, abdominal pain	Did not seek medical care, recovered
3, confirmed	4–13/F	Yes	2 to 5	5	Fever, cough, dyspnea, vomiting, diarrhea	Hospitalized, ICU (not mechanically ventilated), recovered
4, probable	<4/M	No	4	6	Fever, cough, runny nose, diarrhea	Visited clinic, recovered
5, probable	<4/F	No	0 to 3, 5, 6	5	Fever, cough	Did not seek medical care, recovered
6, probable	<4/M	No	6	12	Fever, cough, sore throat, runny nose	Did not seek medical care, recovered
7, probable	<4/F	No	–1§ to 2, 4, 5, 7	4	Feverish, cough	Did not seek medical care, recovered

*Case-patients 1–7 visited the swine exhibit, and all except case-patient 4 also touched swine. Case-patient 2 exhibited swine with an agricultural club for children (Club X) and was not randomly selected for the cohort study. A(H3N2)v, influenza A (H3N2) variant virus; ICU, intensive care unit.
†Day 0 is the first day fair was open to the public.
‡Illness onset date represents number of days after fair was open to the public.
§Arrived at fairgrounds 1 day before fair was open to the public.

Table 3. Characteristics of Club X cohort members who attended agricultural Fair A during an outbreak of A(H3N2)v virus infection, Pennsylvania, 2011*

Characteristic	Club X cohort, N = 127
Sex	
M	47/127 (37)
F	80/127 (63)
Case status	
Suspected case-patient	14/127 (11)
Non–case-patient	113/127 (89)
Age, y	
Median (range)	13 (4–19)
<4 y	2/127 (2)
4–13	70/127 (55)
14–18	51/127 (40)
≥19	4/127 (3)
Underlying medical condition	19/124 (15)
Reported any prior influenza vaccination	56/115 (49)
No. days spent at fair, median (range)	9 (1–10)
Exhibited animal at fair	75/125 (60)
Exhibited swine at fair	34/125 (27)
Family owns livestock	83/125 (66)
Family owns swine	33/125 (26)
Reported swine exposure†	100/127 (79)

*Data are no. with characteristic (%) or no. with characteristic/total no. cohort members with data (%) unless otherwise specified. Club X is an agricultural club for children. A(H3N2)v, influenza A (H3N2) variant virus.
†Includes attending swine exhibit, walking near swine exhibit, or touching swine at fair.

or auction, suggesting a temporal relationship between human–swine contact and onset of human illness within ≤4 days. The epidemic curve, which suggests that case-patients were exposed to a common infectious source that was present for several days, is consistent with the hypothesis that infected swine were present for the duration of the fair. Prior investigations of human variant influenza virus infections have documented contact with infected swine (*17*,*21*), and cases have also occurred after contact with apparently healthy swine (*4*,*23*). No swine were tested for influenza during this investigation because swine at Fair A had either been slaughtered or had recovered before the first human case was reported; however, triple reassortant H3N2 subtype viruses containing genetic material from pH1N1 have been detected in swine (*4*–*6*).

Because of limited diagnostic testing, the extent and distribution of illness caused by A(H3N2)v virus among Fair A attendees are unknown; however, two thirds of children <4 years of age who were tested were seropositive for A(H3N2)v virus. This finding suggests that illness in at least some suspected case-patients can be attributed to A(H3N2)v virus infection. Suspected case-patients had illness onset dates and symptoms similar to those for probable and confirmed case-patients. Symptoms were similar to those of seasonal influenza (*27*), but no seasonal influenza was circulating at the time in Pennsylvania.

Although we cannot rule out human-to-human transmission of A(H3N2)v virus at or after Fair A, enhanced surveillance after Fair A through the beginning of the typical influenza season detected no additional cases of A(H3N2)v virus infection in the community; this suggests that the virus did not exhibit efficient or sustained human-to-human transmission. However, A(H3N2)v virus infection has occurred with limited human-to-human transmission among persons who reported no swine contact (*4*).

This investigation is subject to a number of limitations. First, interviews occurred when media sources began reporting "swine flu" linked to Fair A. Persons who became ill after attending Fair A may therefore have been more likely to report swine exposure, thus biasing toward an association between illness and swine exposure. Second, testing for influenza was not conducted for most case-patients. The timing of the investigation allowed for collection of few respiratory specimens and only convalescent-phase rather than paired serum samples. Serologic testing was further limited to young children because cross-reactive antibodies in older age groups made interpretation of test results for convalescent-phase serum samples difficult. Because only convalescent serum samples were obtained and baseline serologic studies for A(H3N2)v were conducted in a differ-

Table 4. Characteristics of Club X cohort members, by case status, who attended agricultural Fair A during an outbreak of A(H3N2)v virus infection, Pennsylvania, 2011*

Characteristic	No. members, N = 127	No. (%) suspected cases	RR† (95% CI)
Sex			
M	47	5 (11)	
F	80	9 (11)	
Age, y			
<4	2	0 (0)	
4–13	70	7 (10)	
14–18	51	7 (14)	
≥19	4	0 (0)	
Underlying medical condition			
No	105	14 (13)	
Yes	19	0 (0)	
Swine exposure‡			
None	27	1 (4)	Referent
Indirect	39	3 (8)	2.1 (0.2–53.4)
Direct	61	10 (16)	4.4 (0.8–116.3)
Exhibited swine			
No	91	10 (11)	
Yes	34	4 (12)	1.1 (0.2–3.2)
Family owns swine			
No	92	7 (8)	
Yes	33	7 (21)	2.8 (0.9–8.6)
Fed swine			
No	78	7 (9)	
Yes	37	7 (19)	2.1 (0.7–6.6)
Bathed swine			
No	80	7 (9)	
Yes	35	7 (20)	2.3 (0.7–7.1)
Cleaned swine pen			
No	84	7 (8)	
Yes	31	7 (23)	2.7 (0.8–8.3)

*Club X is an agricultural club for children. A(H3N2)v, influenza A (H3N2) variant virus. RR, relative risk.
†RR of suspected case status is reported for selected exposures.
‡Direct exposure defined as touching pigs; Indirect exposure defined as attending or walking near the pig exhibit.

ent population, it is possible that elevated HI titers among probable case-patients reflect exposure to A(H3N2)v virus before Fair A. Third, because all members of a household would often attend Fair A together, it was rare to identify ill contacts of case-patients who did not also attend Fair A. This made it difficult to evaluate human-to-human transmission in this population. Fourth, case-patients in the cohort study likely include some persons without A(H3N2)v virus infection, and some persons with mild or asymptomatic A(H3N2)v virus infection may have been considered noncase-patients; the resulting misclassification may have caused underestimation of any association between exposures and illness. One person identified during this investigation had rhinovirus infection identified by rRT-PCR testing, and it is possible that noninfluenza respiratory viruses circulated at Fair A and caused illness in some suspected case-patients. Fifth, the small sample size of the cohort limited our ability to detect statistically significant associations between exposures and case status. Last, we were unable to confirm influenza virus infection among swine at Fair A; therefore, the source of the A(H3N2)v virus cannot be confirmed.

Novel influenza A viruses will continue to emerge sporadically, but steps can be taken to reduce risks to human and animal health. Our findings suggests that swine contact increases risk for A(H3N2)v virus infection; therefore, advising fair attendees, especially those at high risk for complications from influenza, to avoid or limit swine contact may help prevent A(H3N2)v virus infections at agricultural events (*28*). Agricultural club members and others with prolonged swine exposure should also be educated about the risk of zoonotic influenza transmission and actions they can take to reduce transmission risk, such as using personal protective equipment when they or their animals are ill (*29*). We found simultaneous illness in humans and swine at the fair; this finding supports those from prior studies showing that transmission of influenza virus occurs from swine to humans and vice versa (*30*–*32*). Preventing seasonal influenza in humans who have contact with swine (e.g., through annual influenza vaccination) can reduce reassortment opportunities in swine that become co-infected with swine and human influenza viruses. Prompt and thorough investigations should be conducted of all novel influenza virus outbreaks among humans and animals. Investigations can be more timely if patients with influenza-like symptoms inform clinicians of recent swine exposure and if clinicians consider variant influenza virus infection in patients with influenza-like symptoms and recent swine or agricultural fair exposure. Clinicians should work with public health officials to test respiratory specimens by rRT-PCR when they suspect variant influenza virus infection. This investigation was limited by the lack of influenza testing in swine. Representative and timely influenza surveillance among swine, especially during fair season in states where swine are present at agricultural events, would facilitate future investigations.

This outbreak of A(H3N2)v virus infections among persons attending an agricultural fair was likely associated with swine contact. We did not identify sustained human-to-human transmission of A(H3N2)v virus during this investigation; however, the identification of ≈300 human A(H3N2)v virus infections in multiple states during 2011 and 2012 and the occurrence of limited human-to-human transmission in small clusters (*33*,*34*) demonstrate that variant influenza viruses remain a public health concern for animals and humans who may infect each other at venues such as agricultural fairs. Collaboration among public health officials with responsibilities for human and animal health is critical to determining the transmissibility and pandemic potential of variant influenza viruses, such as A(H3N2)v virus, and the epidemiologic features of illnesses caused by them.

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Dr Wong is an Epidemic Intelligence Service officer in the Influenza Division at the Centers for Disease Control and Prevention in Atlanta, Georgia. Her research interests include influenza and infectious disease modeling.

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Address for correspondence: Karen K. Wong, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, MS A32, Atlanta, GA 30329, USA; email: kwong@cdc.gov

Subclinical Influenza Virus A Infections in Pigs Exhibited at Agricultural Fairs, Ohio, USA, 2009–2011

Andrew S. Bowman, Jacqueline M. Nolting, Sarah W. Nelson, and Richard D. Slemons

Agricultural fairs are associated with bidirectional, interspecies transmission of influenza virus A between humans and pigs. We examined pigs exhibited at agricultural fairs in Ohio during 2009–2011 for signs of influenza-like illness and collected nasal swab specimens from a representative subset of these animals. Influenza virus A was recovered from pigs at 12/53 (22.6%) fairs during the 3-year sampling period. Pigs at 10/12 (83.3%) fairs from which influenza virus A was recovered did not show signs of influenza-like illness. Hemagglutinin, neuraminidase, and matrix gene combinations of the isolates were consistent with influenza virus A concurrently circulating among swine herds in the United States. Subclinical influenza virus A infections in pigs at agricultural fairs may pose a risk to human health and create challenges for passive surveillance programs for influenza virus A in swine herds.

Awareness of bidirectional zoonotic transmission of influenza virus A between pigs and humans was heightened by the emergence of the influenza A(H1N1)pdm09 virus, which resulted in an influenza pandemic among humans starting in 2009. Interspecies transmission of influenza virus A is believed to be a principal mechanism contributing to the emergence of novel influenza virus A strains that pose a threat to human and swine health (*1*,*2*). Pig respiratory tracts have receptors for swine-, human-, and avian-origin influenza virus A, which facilitates genomic reassortment among viruses from multiple host species. As a result, swine have been identified as mixing vessels for influenza virus A and a source of emergence for novel viruses (*3*).

Author affiliation: The Ohio State University, Columbus, Ohio, USA

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For >60 years after its identification as a swine pathogen, influenza virus A circulating among North American swine was predominantly the H1N1 subtype (*4*). In 1998, triple-reassortant influenza virus A (H3N2), containing genes originating from swine-, human-, and avian-origin influenza virus A, was identified among swine in the United States (*5*). This lineage quickly became established among North American swine (*6*), and the 6 gene segments coding for internal proteins, including the matrix (M) gene, subsequently served as a common backbone for many new reassortant viruses appearing among pigs (*7*). Various subtype H1N1, H1N2, and H3N2 influenza virus A lineages continue to cocirculate and evolve among North American swine (*6*–*9*).

Swine are a source of novel and existing influenza virus A strains that infect humans (*10*–*13*). These strains pose a pandemic threat if they become capable of being transmitted efficiently from person to person and if limited protective immunity exists in the human population. Bidirectional zoonotic transmission of influenza virus A strains usually involves close contact between humans and swine. The United States has 3 major swine–human interfaces: commercial swine production, abattoirs, and agricultural fairs. Agricultural fairs are unique because they facilitate prolonged commingling of pigs from numerous sources raised under varied management programs with millions of persons who have widely disparate histories of exposure to various influenza viruses. This situation creates an environment conducive to zoonotic transmission of influenza virus A.

More persons come in contact with live swine at agricultural fairs than in any other setting in the United States, and several human cases of influenza A have been linked to swine exposure occurring at fairs. In 1988, a woman died

of infection with a variant influenza virus A (H1N1) that she acquired while attending a Wisconsin fair where numerous pigs showed signs of influenza-like illness (ILI); a follow-up investigation identified more human infections (*14*). In Ohio, human infections with variant influenza virus A after exposure to pigs with ILI were detected at the 1988 Ohio State Fair, 2 weeks before the Wisconsin case was reported (R.D. Slemons, unpub. data), and more recently at the 2007 Huron County Fair (*15*).

Because of dynamic human and swine populations at fairs and the number of human influenza A cases associated with swine exposure that occurs at fairs (*13*–*15*), we hypothesized that influenza virus A infections in swine occur undetected at agricultural fairs. This study was initiated after the emergence of influenza A(H1N1)pdm09 to actively monitor the antigenic and genomic properties of influenza virus A among pigs at agricultural fairs in Ohio, with a goal of protecting the health of swine and the public.

Materials and Methods

Study Sites and Samples

During each year of this 3-year study, 2009–2011, agricultural fairs in Ohio were strategically recruited to participate on the basis of the size of the county's commercial swine industry, the number of 4-H/FFA swine exhibitors, the number of pigs previously exhibited, or the geographic proximity to study sites used for influenza virus A surveillance in wild birds. Selection criteria were chosen to provide a diverse representation of Ohio's exhibition swine and the influenza virus A strains they might harbor. Before visiting the fair, the study team provided the leaders of each participating agricultural fair with an educational fact sheet on swine influenza.

The agricultural fair season in Ohio begins in June and continues into October; the fairs participating in this study occurred throughout the fair season (Figure). Fairs were visited at the end of the swine exhibition period, at which time pigs were visually examined for signs of ILI, and nasal swab specimens were collected from 20 selected pigs representing all areas of the exhibit, without consideration for individual pig health status (healthy or ill). Each nasal swab was placed in an individual vial containing brain–heart infusion broth supplemented with penicillin and streptomycin (*16*). The samples were frozen at -70°C until testing was initiated. The Institutional Animal Care and Use Committee of The Ohio State University approved protocol no. 2009A0134 for the use of animals in this study.

Virus Isolation from Swine Nasal Swab Specimens

Samples were thawed and treated with amphotericin B, gentamicin sulfate, and kanamycin sulfate (*17*); they then underwent centrifugation at 1,200 × *g* for 30 min at 4°C. The brain–heart infusion broth supernatant was added to 24-well plates containing monolayers of Madin-Darby canine kidney (MDCK; catalog no. 84121903, Sigma-Aldrich Co., St. Louis, MO, USA), adapted to and maintained in serum-free medium (A.S. Bowman et al., unpub. data). MDCK monolayers were examied for cytopathic effect (CPE) daily for 3 days after the supernatant was added, at which time the cell culture supernatant was tested for hemagglutination activity by using 0.5% turkey erythrocytes (*18*). All hemagglutinating agents in cell culture supernatant and all MDCK cell cultures showing CPE were tested for the presence of influenza virus A by using Flu DETECT (Synbiotics Corporation, Kansas City, MO, USA). Each cell culture supernatant that had positive test results with Flu DETECT was identified as an influenza A viral isolate.

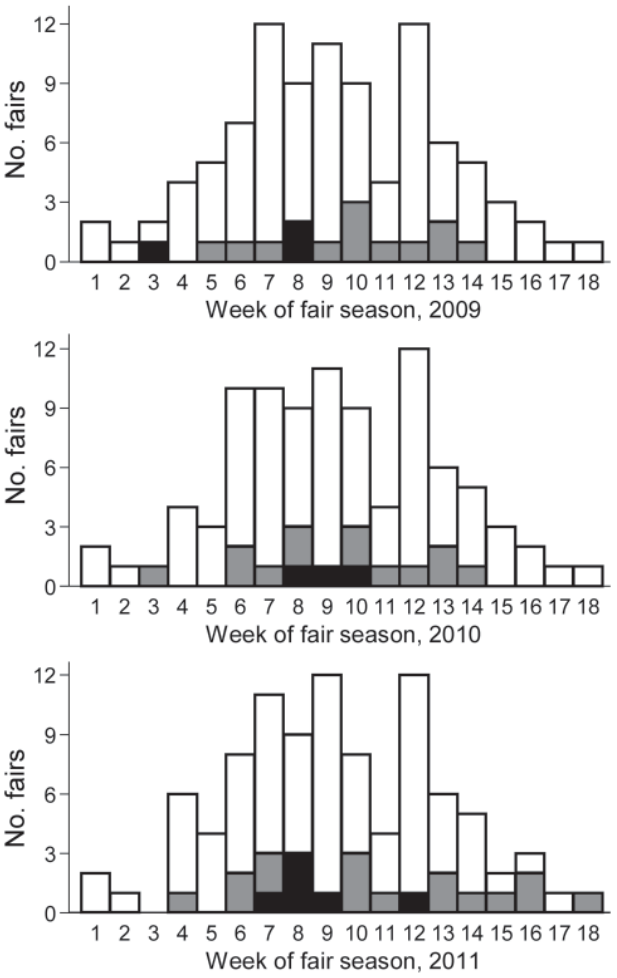

Figure. Frequency distribution of agricultural fairs, by week of the state fair season, Ohio, June–October 2009–2011. Black bar sections, fairs with pigs positive for influenza virus A; gray bar sections, fairs with no pigs positive for influenza virus A; white bar sections, fairs not enrolled in this study.

Influenza virus A isolates were further characterized by using real-time reverse transcription PCR (rRT-PCR) assays.

RNA Extraction and rRT-PCR

RNA was extracted from original samples and influenza virus A isolates by using the PrepEase RNA Spin Kit (Affymetrix, Inc. Cleveland, OH, USA) according to the manufacturer's instructions. Pan–influenza virus A rRT-PCR (*19*,*20*) was used to screen all original samples for influenza virus A. Hemagglutinin (HA) and neuraminidase (NA) subtypes of the influenza isolates were determined by using rRT-PCR assays specific for swine-origin influenza A virus H1 and H3 HA genes and N1 and N2 NA genes by using either a previously published protocol (*21*) modified to laboratory conditions (A.S. Bowman et al., unpub. data) or a commercially available swine influenza virus subtyping assay (Applied Biosystems, Foster City, CA, USA).

The M gene of the influenza A virus isolates was further characterized by differentiating between the North American swine triple-reassortant and the influenza A(H1N1) pdm09 virus M genes by using an rRT-PCR protocol (*22*) adapted to laboratory conditions. The reactions were carried out by using the QuantiFast Multiplex RT-PCR +R Kit (QIAGEN, Valencia, CA, USA) in a 20-μL reaction mixture containing 10 μL 2× quantitative RT-PCR master mix, 7.5 pmol of forward primer, 2.5 pmol of each reverse primer, 0.125 μmol/L EA minor groove binder probe, 0.0625 μmol/L of each NA minor groove binder probe, 0.4 μL 50× ROX reference dye, 0.2 μL of reverse transcription product, and 5 μL of extracted RNA. The reactions were performed on an Mx3000P QPCR System (Agilent Technologies, Inc., Santa Clara, CA, USA) under these thermocycling conditions: 50°C for 20 min, then 95°C for 5 min, followed by 50 cycles of 97°C for 2 s and 60°C for 40 s. Cycle threshold values were calculated for each sample automatically by the QPCR System's software by using the background-based method. Samples with cycle threshold ≤40 were considered positive.

Results

Fifty-three fair events were included in this study: 15 fairs during 2009, 16 fairs during 2010, and 22 fairs during 2011(Figure). Influenza virus A was recovered from ≥1 pigs at 12/53 (22.6%) fair events (Table 1). Results of the pan–influenza virus A rRT-PCR performed on original samples and virus isolation were completely concordant. Pigs with signs of ILI were observed and sampled at 2/53 (3.7%) fair events, and influenza A virus isolates were recovered from pigs at both fairs; pigs without signs of ILI but with positive test results for influenza A virus were found at 10/53 (18.9%) fair events. Therefore, pigs at 10/12 (83.3%) fairs at which pigs had influenza virus A infection did not exhibit signs of ILI.

A total of 1,073 pigs were tested during the 3-year study; influenza virus A was recovered from 155 (14.4%). The frequencies of virus isolation by year were 40/299 (13.4%) during 2009, 34/315 (10.8%) during 2010, and 81/459 (17.7%) during 2011. For the 12 fairs with ≥1 pigs testing positive for influenza A virus, the average frequency of virus isolation from nasal swab specimens was 62.9% (range 5%–100%; Table 2).

Influenza virus A subtypes recovered were H1N2 and H3N2 during 2009, H3N2 during 2010, and H1N2 and H3N2 during 2011 (Table 3). The North American swine triple-reassortant M gene was found in all isolates recovered during 2009 and 2010, whereas the M gene from the influenza A(H1N1)pdm09 virus was found in all of the 2011 H3N2 and H1N2 isolates (Table 3).

Discussion

Our findings highlight the limitations of relying on visual examination for ILI to identify pigs infected with influenza virus A at agricultural fairs. Subclinical influenza virus A infections predominated among the pigs we tested, with subclinical infections detected among pigs at 10/53 (18.9%) participating fairs during 2009–2011. These findings may explain the frequency of variant influenza virus A infections among humans who have only been exposed to apparently healthy swine at fairs.

Agricultural fairs are often the face of agriculture to the general public. The International Association of Fairs and Expositions estimates annual attendance at fairs in North America to be 150 million persons (The Association, pers. comm.). Agricultural fairs have been occurring in the United States since 1811 (*23*) and are special community events with a strong tradition and history of celebrating agricultural heritage and achievement (*24*). As the agricultural workforce has decreased to <2% of the US population (*25*), fairs have added educational programs to showcase advancements in food production systems in an effort to maintain attendance (*26*) and meet societal needs. These much-needed educational efforts often provide an opportunity for attendees to have direct contact with all facets of agriculture, including pork production. Many of these

Table 1. Clinical signs of ILI and influenza virus A recovery from pigs at agricultural fairs, Ohio, USA, 2009–2011*

Year	No. participating fairs	No. fairs with pigs with ILI	No. (%) fairs with ≥1 pig testing positive for influenza virus A	No. (%) fairs with subclinical influenza virus A infection in pigs
2009	15	0	3 (20.0)	3 (20.0)
2010	16	1	3 (18.8)	2 (12.5)
2011	22	1	6 (27.3)	5 (22.7)

*Influenza A virus was recovered from pigs at both fairs where there were pigs with ILI. ILI, influenza-like illness.

Table 2. Frequency of influenza virus A isolation from individual pigs exhibited at agricultural fairs with ≥1 pig testing positive for influenza virus A, Ohio, USA, 2009–2011*

Fair	No. pigs positive/no. tested (%)		
	2009	2010	2011
A	18/20 (80)	0/20	0/20
B	10/20 (50)	0/20	20/20 (100)
C	19/20 (90)	15/20 (75)	20/20 (100)†
D	0/20	1/20 (5)†	19/20 (95)
E	0/20	18/20 (90)	0/20
F	0/20	0/20	3/20 (15)
G		0/20	3/20 (15)
H			16/40 (40)

*Fair G did not participate in 2009; fair H did not participate in 2009 or 2010.
†Fairs where there were pigs with influenza-like illness.

persons would not otherwise have any exposure to swine and the pathogens they harbor, so their close contact with pigs at fairs may play multiple roles in the transmission of influenza A viruses: they may pass human-origin influenza virus A to swine, leading to novel reassortant viruses; they may serve as early sentinels by becoming infected first with a novel swine-origin influenza A virus; or they may disseminate a novel swine-origin influenza virus A in their local communities (*27*).

The long duration of many agricultural fairs (3–10 days) is distinctly different than other swine concentration points or commingling events (i.e., abattoirs, markets, auctions, or shows), where interactions are limited to hours. In addition to their long duration, agricultural fairs also enable the comingling of pigs from multiple locations and various production systems (backyard to intensive commercial) at 1 site. Exhibition swine are commonly a unique population of noncommercial swine, reared by the use of management practices that differ greatly from standard commercial swine production practices (*28*). These pigs likely have varying levels of immunity to influenza virus A and may bring a variety of influenza virus A strains with them to the fair, where the viruses can spread to other pigs, possibly reassort, and potentially transmit to humans.

Swine-to-human transmission of influenza virus A has been sporadically reported worldwide (*11*), but the true incidence of this transmission is unknown. The Centers for Disease Control and Prevention reported that 36 humans were infected with variant influenza virus A in the United States during December 2005–April 2012 (*29*). Of these cases, 15 occurred after July 2011, and 6 cases, all involving infection with influenza A (H3N2) viruses containing the M gene from the influenza A(H1N1)pdm09 virus (H3N2v), were associated with exposure to swine at agricultural fairs. However, none of the implicated fairs reported signs of ILI in the pigs, and influenza virus A could not be isolated from the pigs that were suspected to be the sources because of delays and lack of the availability of the pigs. Nonetheless, it is possible that subclinical influenza infections in pigs at these swine–human interfaces played a key role in zoonotic infections.

The increased swine–human exposure occurring at agricultural fairs may also facilitate human-origin influenza A virus transmission to swine. The earliest reports of introductions of the influenza A(H1N1)pdm09 virus into the US swine herd occurred at the state fairs in Minnesota and South Dakota (*30*,*31*). Human-to-swine transmission is credited as a primary source of the genetic diversity seen in currently circulating swine influenza virus strains (*32*–*34*). Human-to-swine transmission of influenza virus A can be economically devastating for the pork industry because of decreased domestic sales, restrictions imposed by export partners, and production losses due to disease. Agricultural fairs may provide a conduit to introduce human-origin influenza virus A into the US swine herd.

No human cases of variant influenza A associated with any of the agricultural fairs included in this study were reported, even though influenza A (H3N2) viruses containing the M gene from the influenza A(H1N1)pdm09 virus were recovered from pigs at 6 of the participating fairs in 2011. However, the number of confirmed H3N2v cases dramatically increased during the summer of 2012, with most cases epidemiologically linked to swine exposure occurring at agricultural fairs (*35*,*36*).

The HA, NA, and M gene combinations of the influenza virus A isolates recovered from 155/1,073 (14.4%) sampled pigs were consistent with influenza virus A concurrently circulating in the US swine population (*37*,*38*). The high frequency of virus isolation from the pigs at the 12 fairs at which influenza virus A was found is likely due to sample collection occurring at the end of the exhibition period, ≈5–7 days after arrival, which probably coincided with peak viral shedding in the swine population.

A limitation of the study is that extrapolating the findings to other Ohio fairs and fairs in other states may not be possible because of the selection bias and inherent variability among agricultural fairs. Although the fairs where

Table 3. Characterization of HA, NA, and M gene segments of influenza virus A from agricultural fairs with ≥1 pig testing positive for influenza virus A, Ohio, USA, 2009–2011*

Fair	Year		
	2009	2010	2011
A	**H1N2**	Negative	Negative
B	**H3N2**	Negative	H3N2
C	**H3N2**	**H3N2**	H1N2, H3N2
D	Negative	**H3N2**	H1N2, H3N2
E	Negative	**H3N2**	Negative
F	Negative	Negative	H3N2
G		Negative	H3N2
H			H1N2, H3N2

*Fair G did not participate in 2009; fair H did not participate in 2009 or 2010. **Boldface** indicates North American triple-reassortant swine-origin influenza A virus M gene segment; underlining indicates influenza A(H1N1)pdm09 virus M gene segment. HA, hemagglutinin; NA, neuraminidase; M, matrix.

influenza virus A was recovered were diverse regarding the predetermined selection criteria (data not shown), the participating fairs were included in the study because they were ranked relatively high among Ohio fairs within ≥1 selection category. Expanded surveillance efforts for agricultural fairs are underway to more accurately estimate the true prevalence of influenza virus A infections among swine at agricultural fairs in Ohio. Recognized risk factors and accurate prevalence estimates are needed to lay the foundation for studies investigating potential interventions to decrease the probability of swine-to-human and human-to-swine transmission of influenza virus A at agricultural fairs.

The subclinical influenza virus A infections identified in this study would not be detected by the current national swine influenza virus surveillance program (*39*), which is passive and focuses on swine showing signs of ILI and on reacting to reports of variant influenza A cases in humans (*39*). Thus, subclinical influenza virus A infections among pigs are likely underreported. This passive surveillance strategy does not adequately describe the breadth of influenza virus A circulating in swine because it does not identify less virulent strains of influenza virus A (*40*) and does not collect metadata on host, environmental, and agent factors that affect severity of illness. Therefore, to accurately capture the risk influenza virus A in swine populations presents to swine and public health, surveillance efforts should include healthy and clinically ill pigs.

Reducing bidirectional zoonotic transmission of influenza virus A between pigs and humans is crucial to agriculture and biomedicial science. Unfortunately, little scientific evidence exists on which to base changes in policies and management practices to reduce the risk for interspecies transmission of influenza virus A between pigs and humans. This investigation highlights the need for additional studies to quantify the risk for interspecies influenza A virus transmission at fairs and to evaluate interventions to mitigate the risk.

Potential strategies to mitigate the risk for intra- and interspecies transmission of influenza virus A at fairs on the swine side of the human-swine interface include shortening the swine exhibition period, preventing interfair movement of pigs, and vaccinating exhibition swine for appropriate influenza A viruses. Recommendations have previously been made for mitigating risk on the human side of the human–swine interface (www.cdc.gov/flu/swineflu). Expanded risk assessments at agricultural fairs will provide animal and public health officials with scientific data that will enable them to make appropriate decisions to protect animal and public health while still furthering appreciation and understanding of agriculture and ensuring our future food security.

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Dr Bowman is a graduate research associate at The Ohio State University. His research interests include the ecology and epidemiology influenza virus A at the swine-human interface along with developing preventive strategies to protect animal and public health.

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Address for correspondence: Andrew S. Bowman, The Ohio State University, Department of Veterinary Preventive Medicine, 1920 Coffey Rd, Columbus, OH 43210, USA; email: bowman.214@osu.edu

Reservoir Competence of Wildlife Host Species for *Babesia microti*

Michelle H. Hersh, Michael Tibbetts, Mia Strauss, Richard S. Ostfeld, and Felicia Keesing

Human babesiosis is an increasing health concern in the northeastern United States, where the causal agent, *Babesia microti*, is spread through the bite of infected *Ixodes scapularis* ticks. We sampled 10 mammal and 4 bird species within a vertebrate host community in southeastern New York to quantify reservoir competence (mean percentage of ticks infected by an individual host) using real-time PCR. We found reservoir competence levels >17% in white-footed mice (*Peromyscus leucopus*), raccoons (*Procyon lotor*), short-tailed shrews (*Blarina brevicauda*), and eastern chipmunks (*Tamias striatus*), and <6% but >0% in all other species, including all 4 bird species. Data on the relative contributions of multiple host species to tick infection with *B. microti* and level of genetic differentiation between *B. microti* strains transmitted by different hosts will help advance understanding of the spread of human babesiosis.

Human babesiosis is a growing public health concern, especially in the northeastern United States. Babesiosis is a zoonotic, malaria-like illness that can be particularly severe and sometimes fatal in elderly, asplenic, or immunocomprised persons (*1*). In the lower Hudson Valley region of New York State, 5 locally acquired cases of babesiosis were documented in 2001 (*2*), and incidence has increased 20-fold from 2001 through 2008 (*3*).

The causal agent of human babesiosis, *Babesia microti* (Apicomplexa: Piroplasmida), is a protozoan blood parasite that is transmitted in nature by the bite of an infected tick. In the northeastern United States, the vector of this disease is *Ixodes scapularis*, the black-legged tick, which is also the primary vector of *Borrelia burgdorferi* and *Anaplasma phagocytophilum,* the causal agents of Lyme disease and human granulocytic anaplasmosis, respectively. *B. microti* is not known to be transmitted transovarially on the basis of available evidence (*4–7*), indicating that it is not passed from infected adult ticks to eggs, and therefore must be acquired from a blood meal on an infected host. Larval ticks bite infected animals and obtain the pathogen, molt into nymphs, and overwinter. The following year, they then can infect additional hosts when seeking a second blood meal as nymphs or a third blood meal as adults. *B. microti* has been detected in questing adult and nymphal ticks in studies across the northeastern United States (*8–12*). In the case of Lyme disease, which is transmitted by the same vector, the nymphal stage is most relevant to human health because bites from nymphs often go undetected and provide greater opportunities to transmit the pathogen (*13*,*14*). Babesiosis can also be acquired through blood transfusions, another growing concern for public health (*15*).

B. microti can infect a range of animal species, but the reservoir competence of many wildlife hosts in the northeastern United States is not well known. We define reservoir competence as the mean percentage of ticks infected by any individual host of a given species. Furthermore, many studies test for the presence of or exposure to the pathogen in host blood or tissue, which provides useful information about host infection, but not information on how often infected animals transmit the pathogen to tick vectors. White-footed mice (*Peromyscus leucopus*) and meadow voles (*Microtus pennsylvanicus*) have been established as reservoir hosts on the basis of seroprevalence (*16*,*17*), and *P. leucopus* mice are known to transmit the pathogen to ticks (*18*). Other rodent species, including species in other genera of mice (*Apodemus* and *Sicista*) and voles (*Eothenomys, Lagurus,* and *Myodes*) are known hosts in Europe (*19–21*) and Asia (*22–24*). *B. microti* or *B. microti*–like infection has been observed in other common eastern US mammal species, such as short-tailed shrews (*Blarina brevicauda*) (*25*), eastern cottontail rabbits *(Sylvilagus floridanus*) (*17*), eastern chipmunks (*Tamias striatus*) (*17*), raccoons (*Procyon lotor*) (*26*) and

Author affiliations: Bard College, Annandale-on-Hudson, New York, USA (M.H. Hersh, M. Tibbetts, M. Strauss, F. Keesing); and Cary Institute of Ecosystem Studies, Millbrook, New York, USA (M.H. Hersh, R.S. Ostfeld)

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foxes (*27*), and congeneric species in other regions or countries, including *Sciurus* spp. squirrels (*28*) and *Sorex* spp. shrews (*29*). Birds have not been extensively tested for *B. microti,* but in Europe, evidence of *B. microti* infection has recently been discovered in engorged larval ticks that had been feeding on birds of several species (*30–32*). Determining the role that any of these species play in *B. microti* dynamics in nature requires information on the rate at which hosts transmit *B. microti* to tick vectors.

Few systematic surveys have been conducted that compare reservoir competencies of multiple potential host species for *B. microti*. Testing a broad sample of wildlife species would enable us not only to identify which species transmit the pathogen, but also which species act as weakly competent or incompetent hosts, providing blood meals to ticks but rarely or never transmitting the pathogen (*33*). Infection of nymphs by other tickborne pathogens, such as *B. burgdorferi*, is affected by the presence of strongly and weakly competent reservoir hosts in the same communities (*34*,*35*). On Nantucket Island, an area well studied for *B. microti*, multiple hosts transmit the pathogen but differ in infection prevalence (*17*). Although several studies have extensively sampled small mammal communities for seroprevalence of *B. microti* (*19–23*), comprehensive surveys of more diverse host communities, for either seroprevalence or reservoir competence, are rare.

In this study, we sought to determine the level of reservoir competence for *B. microti* for as many host species as possible in a babesiosis-endemic region (Dutchess County, New York) where human babesiosis cases are rapidly increasing (*3*). We designed and tested a real-time PCR method to determine whether ticks were infected with *B. microti*. To determine the relative levels of reservoir competence in as many potential host species as possible, we applied this method to sample newly molted nymphal ticks that fed as larvae on a range of potential wildlife hosts. We tested the hypothesis that white-footed mice (*P. leucopus*) were the predominant host of *B. microti* in this community. We also compared these results to levels of prevalence in questing nymphal ticks from the same region. Our overall goal was to improve understanding of the role of multiple wildlife host species in *B. microti* transmission.

Methods

Field Methods

Hosts were trapped on the property of the Cary Institute of Ecosystem Studies (Millbrook, NY, USA) during the peak abundance of larval black-legged ticks (*I. scapularis*) in July–September in 2008, 2009, and 2010. Hosts included 10 mammal and 4 bird species (Table 1). We focused our sampling efforts on common forest-dwelling terrestrial mammals and ground-dwelling songbirds known to host *I. scapularis* ticks. Hosts were held for 3 days in cages with wire mesh floors suspended over pans lined with wet paper towels so that ticks could feed to repletion, drop from hosts, and be collected. Our ideal was to sample 10–25 ticks/individual host, but our ability to meet this depended on host tick loads. If hosts did not drop enough ticks within 3 days, we increased sample size when possible for selected individuals by infesting them with unfed larval ticks according to the methods of Keesing et al. (*35*). Larval ticks were either collected in the field or hatched from eggs in the laboratory. Larvae hatched from eggs in the laboratory were the offspring of locally collected adult ticks fed on uninfected rabbits. Because transovarial transmission of *B. microti* is not known to occur (*4–7*), these infestations did not affect host exposure to the pathogen. Hosts that had been infested were held for an additional 4 days and engorged ticks were collected each day. All engorged larval ticks were held in moistened glass vials at constant temperature and humidity until they molted into the nymphal stage. Newly molted nymphs were flash-frozen in liquid nitrogen and stored at −80°C. All procedures were conducted with approval from the Cary Institute of Ecosystem Studies Institutional Animal Care and Use Committee.

To provide a context for assessing the reservoir competence of hosts at the study site, we also sampled questing nymphal ticks in June 2010 (13 sites) and June 2011 (5 sites) in the towns of Washington, New York, and adjacent Pleasant Valley, New York. We collected questing nymphal ticks and estimated nymphal density by drag sampling (*36*). Corduroy cloths (1 m^2) were dragged along 400-m transects in each site once or twice in a given year during the annual peak in nymphal questing activity. Ticks were counted and collected every 15–30 min. Questing nymphs were flash frozen upon collection and stored as described above. All sites sampled for questing nymphs were in oak-dominated eastern deciduous forests as described by Ostfeld et al. (*36*) either on the grounds of or within 12 km of the Cary Institute of Ecosystem Studies, where trapping occurred. For each site, the total density of nymphs was multiplied by the proportion of infected nymphs to provide an estimate of the density of infected nymphs.

DNA Extraction and Amplification

Only ticks from hosts that produced a minimum of 10 newly molted nymphs were tested for infection, with the exception of flying squirrels (*Glaucomys volans*), masked shrews (*Sorex cinereus*), and American robins (*Turdus migratorius*), which had low tick loads. For these species, we tested ticks from hosts with >4 newly molted nymphs, but considered these data provisional given low sample sizes per individual host.

Table 1. Host species tested for *Babesia microti* reservoir competence, southeastern New York, USA, 2008–2010*

Host species	Common name	No. hosts tested	No. ticks tested	Mean no. ticks sampled per host (range)
Mammals				
Blarina brevicauda	Northern short-tailed shrew	28	534	19.1 (12–25)
Didelphis virginiana	Virginia opossum	24	464	19.3 (11–25)
Glaucomys volans	Northern flying squirrel	5	84	16.8 (6–25)
Mephitis mephitis	Striped skunk	2	31	15.5 (10–21)
Peromyscus leucopus	White-footed mouse	17	308	18.1 (11–25)
Procyon lotor	Raccoon	21	396	18.9 (10–25)
Sciurus carolinensis	Eastern gray squirrel	18	333	18.5 (10–25)
Sorex cinereus	Masked shrew	6	41	6.8 (4–10)
Tamias striatus	Eastern chipmunk	15	245	16.3 (10–25)
Tamiasciurus hudsonicus	Eastern red squirrel	15	295	19.7 (11–25)
Birds				
Catharus fuscescens	Veery	15	310	20.7 (10–25)
Dumetella carolinensis	Gray catbird	13	240	18.5 (10–24)
Hylocichla mustelina	Wood thrush	18	318	17.7 (10–25)
Turdus migratorius	American robin	17	293	17.2 (8–23)

*Number of ticks tested per host can include samples from either natural tick loads or experimental infestations and are not representative of total tick loads.

To obtain DNA from the ticks, we extracted total genomic DNA using either the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) or the Gentra Puregene Tissue Kit (QIAGEN). Each DNA extraction included a negative control of unfed larval ticks. We designed 2 primers to amplify a 133-bp fragment of the 18S rDNA region in the *B. microti* species complex (including all clades within *B. microti*) (*37*), smbaJF (5′-GCG TTC ATA AAA CGC AAG GAA GTG T-3′), and smbaKR (5′-TGT AAG ATT ACC CGG ACC CGA CG-3′).

We then amplified DNA in a real-time PCR by using SYBR green technology in a C1000 Thermal Cycler with CFX96 Optical Reaction Module (Bio-Rad, Hercules, CA, USA). The reaction mixture included 12.5 μL iQ SYBR Green Supermix (Bio-Rad), 1.25 μL of 10 μmol/L solutions of each primer, 7.5 μL autoclaved or filter-sterilized ultrapure water, and 2.5 μL of template (undiluted tick extracts). Reaction conditions were 10 min at 95°C, followed by 40 cycles for 10 s at 95°C, 20 s at 68°C, and 40 s at 72°C. As a positive control, we used *B. microti* isolates from human patient provided by the New York State Department of Health (Albany, NY, USA). DNA extractions from unfed larval ticks and ultrapure water were used as negative controls to account for potential contamination during the extraction and PCR. To prevent contamination between samples, barrier pipette tips were used throughout the process. Three replicate PCRs were run per tick.

We incorporated melting curve analysis after amplification to distinguish true-positive samples from false-positive samples or mispriming. PCR products were heated from 72°C to 90°C; temperature was increased by 0.5°C every 30 s. Positive controls consistently had melting point maxima of 84°C or 84.5°C. To confirm PCR results, a subset of 197 real-time PCR products were sequenced, including samples with melting point maxima close to the range of standards (83.5°C–84.5°C) and representative samples of products amplifying at different melting point maxima from different host species (Table 2). PCR products were purified by using a QIAquick PCR Purification Kit (QIAGEN) and sequenced by using an ABI 3730XL Autosequencer (Applied Biosystems, Carlsbad, CA, USA). Sequences were edited manually by using FinchTV (Geospiza, Seattle, WA, USA), and identity of sequences was confirmed by using basic local alignment search tool (BLAST) searches (National Center for Biotechnology Information, Bethesda, MD, USA) of GenBank and the blastn algorithm (*38*). Identical molecular protocols were used for analysis of questing nymphs.

Ticks were considered positive for *B. microti* if any 1 of 3 replicate samples amplified and had a melting point maximum of 83.5°C–84.5°C. Ticks with marginal results (positive replicates with double peaks, positive replicates with a melting point maximum of 83°C, or replicates that amplified and had a melting point maximum of 83.5°C–84.5°C but low fluorescence) were run a second time. If results of the second run met the criteria for positivity described above, ticks were considered positive for *B. microti*. If results of the second run were marginal or negative, ticks were considered negative for *B. microti*. Reservoir competence for each host species was calculated

Table 2. Sequenced PCR products with different melting point maxima for *Babesia microti* real-time PCR and primers smbaJF and smbaKR, southeastern New York, USA, 2008–2010

Melting point maximum, °C	No. *B. microti*–positive samples/no. tested
≤82	0/70
82.5	1/3
83	1/4
83.5	20/20
84	42/43
84.5	14/16
85	0/5
≥85.5	0/36

as the average percentage of ticks infected per individual host. Hosts were considered infected if they produced ≥1 infected tick.

Results

We sampled 3,892 ticks from 214 individual hosts for *B. microti* by real-time PCR (Table 1). We used melting curve analysis and DNA sequencing to confirm efficacy of the real-time PCR. False-positive and false-negative results were rare (Table 2). Of the 79 replicates sequenced with melting point maxima ranging from 83.5°C to 84.5°C, 76 (96.2%) were confirmed as *B. microti* by sequencing. Of the 118 samples sequenced with melting point maxima outside that range, only 2 (1.7%) samples were confirmed as *B. microti* by sequencing. Among samples positive for *B. microti*, 38 of 38 sequences from ticks fed on raccoons (collected from 7 hosts), 3 of 4 sequences from ticks fed on opossums (3 hosts), and 1 sequence from a tick fed on a wood thrush had 2 single bp differences from all other *B. microti*–positive samples sequenced (1 substitution and 1 insertion) (Table 3, Appendix, wwwnc.cdc.gov/EID/article/18/12/11-1392-T3.htm). One sequence from a tick fed on a raccoon had 1 additional substitution; this sample had a melting point maximum of 82.5°C.

We assessed levels of reservoir competence in 14 host species (10 mammals and 4 birds) (Table 1). White-footed mice, raccoons, short-tailed shrews, and chipmunks had mean levels of reservoir competence >17% (Figure). All other hosts with >10 individuals tested, including opossums, gray and red squirrels, and all 4 species of birds tested (veery [*Catharus fuscescens*], gray catbird [*Dumetella carolinensis*], wood thrush [*Hylocichia mustelina*], and American robin), had mean levels of reservoir competence <6%. Variance in reservoir competence differed significantly among these 11 species ($\chi^2 = 73.6973$, df = 10, $p = 8.525 \times 10^{-12}$, by Fligner-Killeen test of homogeneity of variances). Flying squirrels, striped skunks, and masked shrews all transmitted *B. microti* to ticks, but sample sizes were smaller than those of the other 11 species, leaving us unable to make firm conclusions about relative levels of reservoir competence. There were no host species that did not transmit *B. microti* to any larval ticks (Figure, Table 4). The percentage of hosts infected ranged from 85.7% (raccoons) to 11.8% (robins), and the average percentage of ticks infected by infected hosts ranged from 4.5% (gray catbirds) to 41.8% (white-footed mice), not including host species with a sample size of <10 individuals (Table 4).

In addition, we tested 414 questing nymphs from 18 field sites for *B. microti* (mean 23 ticks/site, range 12–34 ticks/site). Mean (SD) prevalence of *B. microti* was 16.8% (12.2%). Variation in infection prevalence among sites was high, ranging from 0% to 41.4%. Mean (SD) nymphal density among all sites was 6.49 questing nymphs/100 m^2 (5.13 questing nymphs/100 m^2) and ranged from 0.67 to 16.5 nymphs/100 m^2. On the basis of these data, the mean (SD) density of infected nymphs at these 18 sites was 1.21 nymphs/100 m^2 (1.62 nymphs/100 m^2, range 0–6.83 infected nymphs/100 m^2).

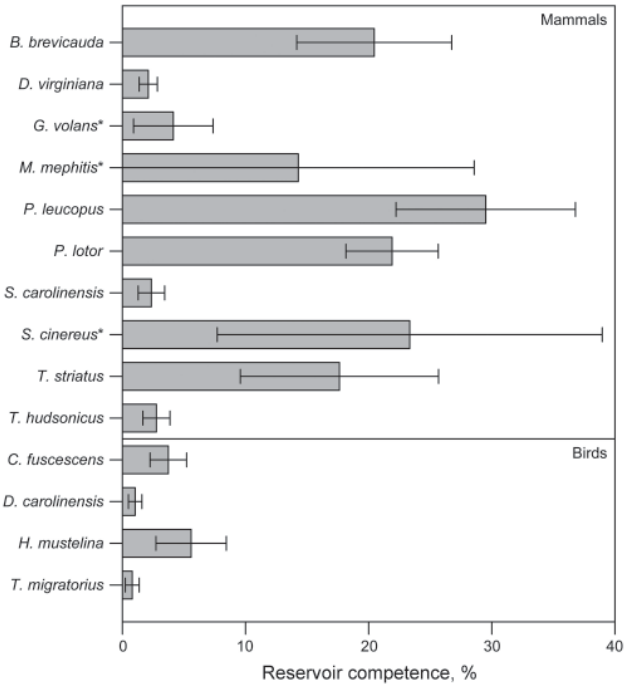

Figure. Mean reservoir competence of 14 host species (10 mammals and 4 birds) for *Babesia microti*, southeastern New York, USA, 2008–2010. Error bars indicate SE. Reservoir competence is defined as the mean percentage of ticks infected by any individual host of a given species. Host species with <10 individual hosts sampled are indicated by an asterisk. See Table 1 for sample sizes. Single-letter abbreviations for genera along the left indicate *Blarina*, *Didelphis*, *Glaucomys*, *Mephitis*, *Peromyscus*, *Procyon*, *Sciurus*, *Sorex*, *Tamias*, *Tamiasciurus*, *Catharus*, *Dumetella*, *Hylocichla*, and *Turdus*, respectively.

Discussion

Our broad survey of 14 commonly parasitized, co-occurring mammals and birds in a babesiosis-endemic zone showed variation in *B. microti* reservoir competence among host species. As expected, white-footed mice (*P. leucopus*) act as a competent reservoir for *B. microti,* infecting an average of 29.5% of larval ticks. However, other small mammals (*B. brevicauda* shrews and *T. striatus* chipmunks) and raccoons (*P. lotor*) are also relatively competent reservoirs (Figure), showing mean reservoir competence ranging from 17.6% to 21.9%. All other wildlife species with >10 individuals tested had low levels of *B. microti* transmission; no individual host species infected >6% of ticks on average. Depending on their tick loads and relative

Table 4. Host species infected with *Babesia microti*, southeastern New York, USA, 2008–2010*

Host species	No. (%) infected hosts	No. (%) infected ticks	Mean % infected ticks/infected host (range)
Mammals			
Blarina brevicauda	15 (53.6)	103 (19.3)	38.2 (5.6–100.0)
Didelphis virginiana	7 (29.2)	10 (2.2)	7.2 (4.3–10.5)
Glaucomys volans†	2 (40.0)	2 (2.4)	10.3 (4.0–16.7)
Mephitis mephitis†	1 (50.0)	6 (19.4)	28.6
Peromyscus leucopus	12 (70.6)	90 (29.2)	41.8 (4.0–90.9)
Procyon lotor	18 (85.7)	93 (23.5)	25.6 (4.3–52.6)
Sciurus carolinensis	5 (27.8)	9 (2.7)	8.5 (4.2–16.0)
Sorex cinereus†	2 (33.3)	12 (29.3)	70.0 (50.0–90.0)
Tamias striatus	7 (46.7)	42 (17.1)	37.8 (4.3–90.9)
Tamiasciurus hudsonicus	5 (33.3)	9 (3.1)	8.3 (4.0–10.5)
Birds			
Catharus fuscescens	6 (40.0)	12 (3.9)	9.3 (4.0–18.8)
Dumetella carolinensis	3 (23.1)	3 (1.3)	4.5 (4.2–4.8)
Hylocichla mustelina	7 (38.9)	18 (5.7)	14.4 (4.0–50.0)
Turdus migratorius	2 (11.8)	2 (0.68)	6.8 (5.3–8.3)

*Infected hosts are those that transmitted *B. microti* to ≥1 *Ixodes scapularis* tick larvae. For sample sizes, see Table 1.
†Host species with <10 individual hosts sampled.

abundances, these species have the potential to lower rates of disease risk (i.e., infection prevalence) by feeding ticks but not transmitting this pathogen (*35*). Within a host species, individual differences such as age, reproductive status, sex, time since infection, and degree of co-infection may affect transmission of *B. microti*. Our approach focused on sampling a breadth of species under a range of natural conditions rather than sampling any single species in sufficient depth to take potential causes of intraspecific variation into account. Future studies examining the mechanisms controlling individual variation in reservoir capacity are warranted because we observed considerable variation in reservoir capacity between individual species (Table 4).

The average level of *B. microti* infection in questing nymphal ticks was intermediate between more and less competent reservoir species, consistent with the fact that larval ticks are feeding on a combination of hosts with varying levels of reservoir competence. Variation in *B. microti* prevalence in questing ticks among sites could reflect differences in host community composition or differences in infection prevalence among hosts at different sites. Site-specific differences in the density of questing nymphs at each site also contribute to variation in the density of infected nymphs among sites. Nymphal densities measured fall within the range documented in previous studies (*36*). Future studies should focus on sampling questing nymphs more broadly across landscapes to link levels of infection in questing ticks and the density of infected nymphs to host communities and other landscape-scale habitat variation. Monitoring of babesiosis-endemic areas for temporal changes in nymphal infection prevalence may also help in understanding mechanisms for the regional increase in human babesiosis cases.

Ground-dwelling bird species have not been sampled frequently for their capacity to transmit *B. microti* to ticks, but they might play a role in disease transmission and spread because they are exposed to *I. scapularis* ticks. Of the 4 species tested in this study, wood thrushes (*H. mustelina*) had the highest reservoir competence of all birds tested (5.6%), but the other 3 bird species were also able to transmit the pathogen, albeit at low levels (<4%). Transmission of *B. microti* has been detected in other bird species; positive *I. ricinus* tick larvae have been found feeding on European robins (*Erithacus rubecula*) (*32*) and other birds (*30*,*31*).

The real-time PCR developed for this study was effective in amplifying *B. microti* DNA and had a high level of specificity for this pathogen. The measured rates of false-positive and false-negative results were <5% (Table 2). The low false-positive rate is dependent on melting curve analysis after amplification. False-negative results tended to occur at melting point maxima just below what we considered diagnostic for *B. microti* (82.5°C–83°C), and may represent strain variation within *B. microti*. In future studies, it may be necessary to directly sequence samples with these melting point maxima.

On the basis of the short fragments that we sequenced, our samples of *B. microti* suggest some degree of host specialization (Table 3), consistent with previous work showing divergent clades of *B. microti* (*37*,*39*,*40*). Variation found in raccoon and opossum isolates matches variation in other raccoon isolates (GenBank accession nos. AY144701 and AB197940). We stress the preliminary nature of this result given the short sequence length and the relative paucity of *B. microti* sequences available in GenBank. The role of raccoons and opossums in human babesiosis dynamics depends on the zoonotic potential of the *B. microti* strains they carry. Phylogenetic analyses of 18S and β-tubulin genes (*37*) and the chaperonin-containing t-complex polypeptide l gene (*39*) of *B. microti* showed a distinction between raccoon isolates and known zoonotic

strains. Given that all host species sampled transmitted *B. microti*, further studies detailing the level of genetic differentiation between *B. microti* samples isolated from different hosts are critical, as is a thorough assessment of the genetic diversity of *B. microti* infections of humans. Improved understanding of *B. microti* strain diversity is necessary to consider the public health implications of the role of different hosts in *B. microti* dynamics.

In this study, we demonstrated that *P. leucopus* mice are a competent reservoir host for *B. microti*, but other small mammals and raccoons have comparable reservoir competence and might play a critical role in disease transmission, depending on their tick loads and relative abundance (*34*,*35*). At least 1 individual of all wildlife host species sampled transmitted *B. microti* to ≥1 *I. scapularis* tick, even when host sample sizes were relatively low. Tick vectors that transmit this pathogen interact with strongly and weakly competent reservoir hosts, and this variation in reservoir competence within host communities should be considered when predicting risk for infection with *B. microti* based on animal community composition. To explain the recent emergence of human babesiosis, the community ecology of *B. microti* needs to be understood in greater depth.

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Dr Hersh is a postdoctoral researcher at Bard College, Annandale-on-Hudson, NY, and the Cary Institute of Ecosystem Studies, Millbrook, NY. Her research interest is linkages between biodiversity and disease.

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Address for correspondence: Michelle H. Hersh, Department of Biology, Eastern Michigan University, 441 Mark Jefferson Science Complex, Ypsilanti, MI 48197, USA;; email: hersh.mh@gmail.com

Diagnostic Assays for Crimean-Congo Hemorrhagic Fever

Jessica Vanhomwegen, Maria João Alves, Tatjana Avšič Županc, Silvia Bino, Sadegh Chinikar, Helen Karlberg, Gülay Korukluoğlu, Miša Korva, Masoud Mardani, Ali Mirazimi, Mehrdad Mousavi, Anna Papa, Ana Saksida, Batool Sharifi-Mood, Persofoni Sidira, Katerina Tsergouli, Roman Wölfel, Hervé Zeller, and Philippe Dubois

Crimean-Congo hemorrhagic fever (CCHF) is a highly contagious viral tick-borne disease with case-fatality rates as high as 50%. We describe a collaborative evaluation of the characteristics, performance, and on-site applicability of serologic and molecular assays for diagnosis of CCHF. We evaluated ELISA, immunofluorescence, quantitative reverse transcription PCR, and low-density macroarray assays for detection of CCHF virus using precharacterized archived patient serum samples. Compared with results of local, in-house methods, test sensitivities were 87.8%–93.9% for IgM serology, 80.4%–86.1% for IgG serology, and 79.6%–83.3% for genome detection. Specificity was excellent for all assays; molecular test results were influenced by patient country of origin. Our findings demonstrate that well-characterized, reliable tools are available for CCHF diagnosis and surveillance. The on-site use of such assays by health laboratories would greatly diminish the time, costs, and risks posed by the handling, packaging, and shipping of highly infectious biologic material.

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne zoonotic disease caused by a virus (CCHFV) belonging to the *Nairovirus* genus (*1*). The disease is asymptomatic in infected animals but can develop into severe illness in humans, with case-fatality rates as high as 50% in some outbreaks (*2*,*3*). The incubation period is typically 3–7 days, with sudden onset of myalgia, headache, and fever that can develop into a severe hemorrhagic syndrome (*4*,*5*). CCHFV is transmitted by tick bite (from mainly *Hyalomma* spp. ticks) or by contact with blood or tissues from infected livestock or patients with CCHF (*2*,*6*).

Sporadic cases of CCHF and community and nosocomial outbreaks have been increasingly reported, and the disease's geographic distribution is the most extensive among tick-borne diseases. Currently, CCHFV is enzootic in southeastern Europe (Bulgaria, Albania, Kosovo, and Greece), southern Russia, and several countries in the Middle East, Africa, and Asia (*7*–*9*). Given the abundance of vectors, potential hosts, favorable climate and ecology, and intensified human travel, emergence and rapid establishment of new CCHF foci in other countries are substantial risks (*10*). Emergence or reemergence of CCHF poses a serious public health threat because it is highly contagious and highly lethal, has the potential to cause nosocomial infection, and is difficult to treat, prevent, and control. In addition to enhanced surveillance and development of therapeutics, access to early, sensitive, and specific laboratory diagnosis is a key factor in increasing preparedness in Europe and other countries at risk (*11*–*13*).

Although viral isolation is the standard for CCHF diagnosis, because it has to be done in high-containment biosafety level 4 facilities, the number of laboratories that can perform this technique is limited. Moreover, because cell cultures lack sensitivity and usually only detect the relatively high viremia level encountered during the first 5 days of illness, viral isolation is not without error or uncertainty. As a consequence, reference laboratories have been using the best available practicable methods to determine the presence or absence of infection (*11*).

Author affiliations: Institut Pasteur, Paris, France (J. Vanhomwegen, P. Dubois); European Centre for Disease Control, Stockholm, Sweden (J. Vanhomwegen, H. Zeller); National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal (M.J. Alves); Faculty of Medicine, Ljubljana, Slovenia (T. Avšič Županc, M. Korva, A. Saksida); Institute of Public Health, Tirana, Albania (S. Bino); Pasteur Institute of Iran, Tehran, Iran (S. Chinikar); Swedish Institute for Infectious Disease Control, Solna, Sweden (H. Karlberg, A. Mirazimi, M. Mousavi); Refik Saydam National Public Health Agency, Ankara, Turkey (G. Korukluoğlu); Shahid Beheshti University of Medical Sciences, Tehran, Iran (M. Mardani, B. Sharifi-Mood); Aristotle University of Thessaloniki, Thessaloniki, Greece (A. Papa, P. Sidira, K. Tsergouli); and Bundeswehr Institute of Microbiology, Munich, Germany (R. Wölfel)

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These methods include conventional and real-time quantitative reverse transcription PCR (RT-PCR and qRT-PCR) for detection of the viral genome (*14–18*) and indirect immunofluorescence assays (IFAs) or ELISAs for detection of specific IgM and IgG antibodies (*19–22*). No consensus on the most efficient molecular and serologic testing method has been reached.

In this context, a working group of experts from reference laboratories was constituted under the initiative of the European Network for Diagnostics of Imported Viral Diseases to take part in a multicenter study of CCHF diagnostic tests. The aim of this study was to evaluate and compare the performance of, and review the operational characteristics of, available CCHF diagnostic tests by using panels of well-characterized, archived serum samples from patients from geographically diverse settings.

Materials and Methods

Study Participants and Diagnostic Tools

Experts from 5 institutions participated in this study: Bundeswehr Institute of Microbiology, Munich, Germany; Department of Microbiology, Aristotle University of Thessaloniki, Thessaloniki, Greece; Center for Vectors and Infectious Diseases Research, National Institute of Health, Águas de Moura, Portugal; Institute of Microbiology and Immunology, Medical Faculty, Ljubljana, Slovenia; and Center for Microbiological Preparedness, Swedish Institute for Infectious Disease Control, Solna, Sweden. Diagnostic methods that could be performed in standard laboratory facilities were selected on the basis of a systematic review of the literature and the experiences of the members of the working group. During April 2010, an extensive search of available CCHF diagnostic tools was performed by using both generic (Google) and scientific (PubMed) Internet-based search engines. To meet the selection criteria, assays had to be commercially available or in the prerelease phase at the time of our assessment (or have quality assessed reagents and well-defined protocols for noncommercial assays); yield early and rapid results (within 5 hours); not require the purchase of specific equipment; and have demonstrated sufficient scope to detect diverse CCHFV variants or antibodies. The reporting of results was conducted according to Standards for Reporting of Diagnostic Accuracy criteria (www.stard-statement.org; online Technical Appendix Table 1, wwwnc.cdc.gov/EID/pdfs/12-0710-Techapp.pdf).

Patient Status Definition and Samples

Because no reference test for CCHV is universally accepted, patients with clinically suspected CCHF were confirmed on the basis of results of serologic and molecular diagnostic tests that were in use in the CCHF reference laboratories at the time of the study (Table 1). These cases were defined by the either positive rule: detection of CCHFV genome or CCHFV-specific IgM, IgG, or both, during either the acute or convalescent phase of the disease. Each participant in the working group contributed a panel of archived serum samples that had tested positive for CCHFV by IgM, IgG, or both, and a panel of archived serum RNA extracts from which CCHFV genome had been detected; samples were collected from patients with laboratory-confirmed CCHF infection. Negative controls were samples from healthy persons who originated from disease-endemic or at-risk areas (e.g., blood donors) and samples from febrile patients with a diagnosis of 1 of the conditions that can produce symptoms similar to those of CCHFV infection (e.g., hemorrhagic fever with renal syndrome, leptospirosis, West Nile fever, chikungunya). All samples were included in the study with the consent of the patients.

Assay Methodology and Data Collection

Tests were performed according to the manufacturers' instructions or according to validated protocols provided by the developer. Working group members tested the selected assays in duplicate on their respective sample panels within their facilities. Results of qualitative assays (IFAs and low-cost, low-density [LCD] arrays) were read by 2 independent readers. Results were collected at the end of each testing session by using a standard report datasheet and combined into a final database.

Table 1. Reference methods used by the reference laboratories that participated in evaluation of serologic and molecular assays for diagnosis of CCHF

Laboratory	Reference IgM serologic tests	Reference IgG serologic tests	Reference molecular tests
1	In-house IFA (CCHFV-infected cells)	In-house IFA (CCHFV-infected cells)	Nested RT-PCR (*23*) and qRT-PCR (*24*)
2	In-house IgM capture ELISA (CCHFV strain IbAr10200 antigen)	In-house sandwich ELISA (CCHFV strain IbAr10200 antigen)	Nested RT-PCR (*25*) and qRT-PCR (*17*)
3	In-house IFA (CCHFV strain ArD39554 infected cells)	In-house IFA (CCHF strain ArD39554 infected cells)	qRT-PCR (*18*)
4	In-house IFA (CCHFV infected cells)	In-house IFA (CCHF infected cells)	qRT-PCR (*18*)
5	Testing performed by external reference laboratory	Testing performed by external reference laboratory	qRT-PCR (*18*)

*CCHF, Crimean-Congo hemorrhagic fever; IFA, immunofluorescence assay; CCHFV, CCHF virus; RT-PCR, reverse transcription PCR; qRT-PCR, quantitative RT-PCR.

Data Analysis

Results obtained for each selected diagnostic tool were compared in a 2×2 table with results from the reference in-house diagnostic to estimate indices of sensitivity, specificity, and corresponding 95% CIs. In addition, test results were compared with the results of a composite reference standard (positive if detection of specific CCHFV genome or specific CCHF IgM or IgG antibodies by in-house reference methods; negative otherwise) to confirm the specificity estimates and corresponding 95% CIs. Statistical analysis was performed in STATA/SE version 12.0 software (StataCorp, College Station, TX, USA). CIs were calculated by using binomial exact methods. A univariate analysis was conducted by using the Fisher exact test to identify factors influencing test sensitivity (i.e., patient country of origin, severity of disease, number of days after illness onset that sample was collected, and sample storage time before testing); $p<0.05$ was considered significant. A multinomial exact logistic regression analysis was performed to identify independent factors influencing sensitivity, including all variables associated with sensitivity in the univariate analysis ($p<0.1$). Data on operational characteristics of each test (i.e., ease-of-use, level of staff training required, time, ease of interpretation) were gathered through a questionnaire. Tests were scored on operational characteristics.

Results

Selected Diagnostic Methods

Six diagnostic assays met the criteria for inclusion in the study. For specific CCHF serodiagnosis, a commercial IgM and IgG ELISA (Vector-Best, Novosibirsk, Russia) and a commercial IgM and IgG IFA (Euroimmun, Luebeck, Germany) were selected. For detection of the CCHFV genome, a commercial real-time RT-PCR (Altona-Diagnostics, Hamburg, Germany) and a low-cost, low-density macroarray (*26*) were used. Characteristics of the selected tests are shown in Table 2. After selection, assays were purchased directly from the manufacturers and shipped according to their instructions to the working group members by express delivery.

Characteristics of Study Population and Sample Panels

The serum panel constituted for the evaluation of the serologic tests consisted of 66 stored serum samples from acute-phase CCHF patients (those who recovered or died) and patients with confirmed CCHF diagnosis who had an early recovery; 32 samples from febrile patients who had symptoms compatible with CCHFV infection; and 41 samples from healthy persons. Molecular tests were evaluated by using a panel of RNA extracts from acute-phase patient serum samples: 54 samples from patients with confirmed CCHF diagnosis, 16 samples from febrile patients who had symptoms compatible with CCHFV infection, and 5 samples from healthy persons. Characteristics of

Table 2. Characteristics of selected assays compared in study of CCHF diagnostic tools

Characteristics	IgM ELISA	IgG ELISA	IgM IFA	IgG IFA	qRT-PCR	LCD array
Assay (manufacturer, location or reference)	VectoCrimea-CHF ELISA (Vector-Best, Novosibirsk, Russia)		Crimean-Congo Fever Mosaic 2 IFA (Euroimmun, Luebeck, Germany)		RealStar CCHFV RT-PCR Kit 1.2 (Altona-Diagnostics, Hamburg, Germany)	CCHF2006 1.5 LCD Kit (*26*)
Reference no.	D-5054	D-5056	FI 279a-1010-2M	FI 279a-1010-2G	181203	NA
Target	CCHFV-specific IgM	CCHFV-specific IgG	CCHFV-specific IgM	CCHFV-specific IgG	CCHFV S segment	CCHFV S segment
Shelf life, mo	9	9	18	18	12	Unknown
Storage temperature, °C	2 to 8	2 to 8	2 to 8	2 to 8	–15 to –25	2 to 8, –20
Quoted accuracy, %						
Sensitivity	100	100	97.2	89.5	Unknown	100
Specificity	100	100	97.5	100	Unknown	100
Sample type	Serum, plasma	Serum, plasma	Serum, plasma	Serum, plasma	RNA extract from serum or blood	RNA extract from serum or blood
Sample volume, µL	10	10	5	5	10	10
Minimum kit format (no. reactions)	12 strips × 8 tests (96)	12 strips × 8 tests (96)	10 slides × 5 tests (50)	10 slides × 5 tests (50)	8 tubes × 12 tests (96)	4 slides × 8 tests (32)
Price, Euros†						
Per kit	139.2	139.2	326	326	1,200	Unknown
Per reaction	1.45	1.45	6.51	6.51	12.50	Unknown
Estimated run time, min	175	175	70	70	58	175

*CCHFV, Crimean-Congo hemorrhagic fever virus; IFA, immunofluorescence assay; qRT-PCR, quantitative reverse transcription PCR; LCD, low-cost, low-density; NA, not applicable; S segment, small segment.
†Does not include shipping costs.

the patient population and the sample panels are shown in Table 3. Confirmed CCHF case-patients originated from Iran, Kosovo, Albania, Turkey, and sub-Saharan Africa; most had moderate CCHF. Patients with symptoms compatible with CCHF infection included patients who had a diagnosis of leptospirosis, chikungunya fever, hemorrhagic fever with renal syndrome (HFRS), Q fever, tularemia, brucellosis, and West Nile encephalitis. Serum samples were collected 5–49 days after onset of symptoms; RNA extracts were obtained from serum samples collected 2–14 days after onset of symptoms. Storage time until testing ranged from 1 to 23 years for serum samples and 1 to 4 years for RNA extracts.

Performances of Selected CCHF IgM Serology Assays

A total of 138 and 90 samples from the collected patient serum panels were tested for CCHFV-specific IgM by the Vector-Best ELISA and the Euroimmun IFA, respectively. Because of limited sample amounts, IFA could not be performed on all collected samples. When compared with the reference IgM serology tests, the sensitivity of the IgM ELISA ranged from 75.0% to 100.0% for different laboratories, with an overall sensitivity of 87.8% (95% CI 78.6%–96.9%). For the IgM IFA, sensitivity ranged from 75.0% to 100.0%, with an overall sensitivity of 93.9% (95% CI 85.8%–100.0%). Overall specificity was estimated to be 98.9% (95% CI 96.7%–100.0%) for the IgM ELISA and 100% for the IgM IFA (Table 4). When a composite reference standard (described in the Methods section) was used as reference, the observed specificity was 100% for both tests.

Performances of Selected CCHF IgG Serology Assays

A total of 137 and 92 samples from the collected patient serum panel were tested for CCHFV-specific IgG by the Vector-Best ELISA and the Euroimmun IFA, respectively. When compared with the reference IgG serology tests, the estimated sensitivity for the IgG ELISA ranged from 75.0%

Table 3. Patient characteristics and sample storage information for samples tested for CCHFV

	Sample panel 1, serology, no. (%)		Sample panel 2, genome detection, no. (%)	
Characteristics	CCHFV positive, n = 66	CCHFV negative, n = 73	CCHFV positive, n = 54	CCHFV negative, n = 21
Conditions				
CCHF	66 (100.0)	0	54 (100.0)	0
Brucellosis	0	2 (2.7)	0	2 (9.5)
Chikungunya	0	1 (1.4)	0	1 (4.8)
HFRS	0	13 (17.8)	0	7 (33.3)
Leptospirosis	0	13 (17.8)	0	3 (14.3)
Q fever	0	1 (1.4)	0	1 (4.8)
Tularemia	0	1 (1.4)	0	1 (4.8)
West Nile fever	0	1 (1.4)	0	1 (4.8)
Healthy or non-CCHF	0	41 (56.2)	0	5 (23.8)
Patient country of origin				
Albania	9 (13.6)	0	8 (14.8)	0
Germany	0	23 (31.5)	0	7 (33.3)
Greece	0	9 (12.3)	0	9 (42.9)
Iran	32 (48.5)	0	31 (57.4)	0
Kosovo	21 (31.8)	20 (27.4)	7 (13.0)	4 (19.0)
Portugal	0	20 (27.4)	0	0
Sub-Saharan Africa	4 (6.1)	0	0	0
Turkey	0	1 (1.4)	8 (14.8)	1 (4.8)
CCHF disease severity				
Moderate	49 (74.2)	0	37 (68.5)	0
Severe	11 (16.7)	0	2 (3.7)	0
Fatal	3 (4.5)	0	6 (11.1)	0
Asymptomatic	3 (4.5)	0	7 (13.0)	0
Unknown	0	0	2 (3.7)	0
Length of illness, d				
<15 d	46 (69.7)	0	43 (79.6)	0
≥15 d	12 (18.2)	0	0	0
Asymptomatic	3 (4.5)	0	7 (13.0)	0
Unknown	5 (7.6)	0	4 (7.4)	0
Sample storage time, y				
<10	49 (74.2)	64 (87.7)	54 (100.0)	10 (47.6)
≥10	17 (25.8)	0	0	2 (9.5)
Unknown	0	9 (12.3)	0	9 (42.9)
Sample storage temperature, °C				
−80	34 (51.5)	49 (67.1)	9 (16.7)	4 (19.1)
−70	32 (48.5)	0	45 (83.3)	9 (42.9)
−20	0	24 (32.9)	0	8 (38.1)

*CCHF, Crimean-Congo hemorrhagic fever; CCHFV, CCHF virus; HFRS, hemorrhagic fever with renal syndrome.

Table 4. Overall performance of assays compared in study of CCHF diagnostic tools

	IgM serology		IgG serology		Genome detection	
Parameter	ELISA	IFA	ELISA	IFA	qRT-PCR	LCD array
No. samples tested	138	90	137	92	71	70
No. true positive	43	31	41	31	39	40
No. false negative	6	2	10	5	10	8
No. true negative	88	57	86	56	21	21
No. false positive	1	0	0	0	1	1
Sensitivity, % (95% CI)	87.8 (75.2–95.3)	93.9 (79.8–99.3)	80.4 (66.9–90.2)	86.1 (70.5–95.3)	79.6 (65.7–89.8)	83.3 (69.8–92.5)
Specificity, % (95% CI)	98.9 (93.9–100.0)	100.0 (93.7–100.0)†	100.0 (95.8–100.0)	100.0 (93.6–100.0)	95.5 (77.2–99.9)	95.5 (77.2–99.9)

*CCHF, Crimean-Congo hemorrhagic fever; IFA, immunofluorescent assay; qRT-PCR, quantitative reverse transcription PCR; LCD, low-cost, low-density.
†One-sided 95% CI.

to 100.0%, with an overall sensitivity of 80.4% (95% CI 69.5%–91.3%). For the IgG IFA, sensitivity ranged from 40.0% to 100.0%, with an overall sensitivity of 86.1% (95% CI 74.8%–97.4%). Specificity was estimated to be 100% for both assays (Table 4).

Performances of Selected CCHF Molecular Assays

A total of 71 and 70 samples, respectively, from the collected panel of serum RNA extracts were tested for the presence of the CCHF genome by the Altona-Diagnostics CCHFV qRT-PCR and the CCHF LCD array. When compared with the results of the reference genome detection methods, sensitivity ranged from 42.9% to 100%, with an overall sensitivity of 79.6% (95% CI 68.3%–90.9%) for the qRT-PCR and from 25.0% to 100% with an overall sensitivity of 83.3% (95% CI 72.8%–95.5%) for the LCD array. Both assays demonstrated a specificity of 95.5% (95% CI 90.6%–100%) (Table 4), which increased to 100% when the results of a composite reference standard were used as reference.

Factors Influencing Diagnostic Sensitivity

The influence of several patient and sample characteristics on the sensitivity of the selected assays was analyzed by univariate analysis (online Technical Appendix Table 2). The country of origin of the patient was found to be significantly associated with the sensitivity of the IgG IFA ($p = 0.02$), the qRT-PCR ($p<0.001$), and the LCD array ($p = 0.02$). However, after multivariate analysis, this association only remained significant for the qRT-PCR assay ($p<0.001$). In particular, the qRT-PCR was found to be less sensitive for samples from patients originating from Turkey (adjusted odds ratio [OR] 0.04, 95% CI 0.00–0.87) and from Albania (adjusted OR 0.02, 95% CI 0.00–0.16).

Operational Characteristics of the CCHF Diagnostics

Scores for operational characteristics are summarized in Table 5. The ELISA test obtained a higher overall score (8.5/10) compared with the IFA (6.7/10). The IFA scored lowest in the ease of interpretation of results (1.3/2) and in the requirement for specific technical training (0.3/1). The observed scores for molecular tests were within the same range (6.0–6.3/10). Both molecular assays demonstrated low scores for technical complexity (1.3–1.5/2) and training requirements for equipment and technique (0.3–0.5/1).

Discussion

A number of published studies have described major epidemics, community and nosocomial outbreaks, and the ecology of CCHF (*4*,*7*,*8*,*27–30*). These reports have shown that a distinct epidemiologic situation can arise in regions where the virus is endemic but also that new foci can emerge (*10*,*31*). The World Health Organization has listed CCHF among the emerging diseases for which control and prevention measures should be renewed and intensified (*13*,*32*). In addition, an assessment of the importance and magnitude of vector-borne diseases initiated by the European Centre for Disease Prevention and Control identified CCHF as a priority disease for the European Union (*33*).

A strong laboratory capacity, in particular standardized approaches for diagnostic methods and assay validation, has been identified as a short-term priority in CCHF-endemic areas and regions where CCHFV could be expected to circulate (*11*,*12*). The aim of this study was to identify and evaluate easily available, simple-to-perform CCHF diagnostic methods considered most suitable for widespread use in CCHF-endemic areas and countries at risk.

We assessed the performances of 2 commercially available IgM and IgG serologic tests, the Vector-Best CCHF ELISA and the Euroimmun CCHF IFA, and 2 assays for viral genome detection, the Altona-diagnostics CCHF qRT-PCR and a CCHF LCD array. The IgM and IgG ELISAs showed a sensitivity of 88% and 80%, respectively, lower than the numbers given by the manufacturer. These assays were validated by the manufacturer by using serum panels from CCHF cases originating from southwestern Russia (S. Suchkov, pers. comm.). Therefore, lower sensitivity for certain serum samples tested in this study

Table 5. Operational characteristics of selected CCHF diagnostic assays*

Operational characteristic	Mean score			
	VectoCrimea-CHF ELISA†	Crimean-Congo Fever Mosaic 2 IFA‡	RealStar CCHFV RT-PCR Kit 1.2§	CCHF^{2006} 1.5 LCD Kit (*26*)
Equipment¶				
Maintenance of equipment required	0.8	0.3	0.3	0.3
Training for equipment required	0.8	0.7	0.3	0.5
Additional equipment required	0.8	1.0	0.5	0.8
Technique				
Clarity of instructions¶	1.0	0.7	1.0	1.0
Technical training required¶	0.8	0.3	0.3	0.3
Technical complexity#	2.0	1.7	1.5	1.3
Interpretation				
Training required for result interpretation¶	1.0	0.7	0.8	0.8
Ease of interpretation of results#	1.5	1.3	1.5	1.5
Total score	8.5/10	6.7/10	6.0/10	6.3/10

*CCHF, Crimean-Congo hemorrhagic fever; IFA, immunofluorescent assay; CCHFV, CCHF virus; RT-PCR, reverse transcription PCR; LCD, low-cost, low-density.
†Vector-Best, Novosibirsk, Russia.
‡Euroimmun, Luebeck, Germany.
§Altona-Diagnostics, Hamburg, Germany.
¶A score of 1 was attributed when instructions were sufficiently clear or when no specific training, no additional equipment, or no regular equipment maintenance were necessary.
#Score was attributed according to the degree of simplicity of the technique or interpretation: 2 if it was considered easy, 1 if it was considered acceptable, and 0 if it was considered difficult.

may reflect antigenic variation among CCHFVs circulating in other countries.

Observed sensitivities of the IgM and IgG IFA were 93.9% and 86.1%, respectively. Although these estimates are higher than those observed for the IgM and IgG ELISAs, these results may have had a sampling bias because not all serum samples tested by ELISA could be tested by IFA. This bias was, however, minimized, because the tested serum panel included 15/16 false-negative samples observed for the ELISAs.

The sensitivity of the selected molecular assays was found to be more modest (79.6% for qRT-PCR and 83.3% for LCD array) than for serologic methods and to be associated with the patient country of origin. This result is consistent with the finding that the application of molecular assays in different settings is hampered by the high diversity of the CCHFV genomes, whereas serologic methods can have a broader use due to cross-reactivities. In particular, the qRT-PCR seems to be less sensitive for patients originating from the Balkans region and Turkey than for patients from other countries compared with in-house reference molecular methods. The in-house methods developed by reference laboratories are optimized for detection of strains circulating in that area, which may result in a lower detection limit when compared with methods that cover a broader spectrum. However, other factors, such as RNA degradation due to freezing and thawing cycles or inhibition of PCR reactions because of inhibitory compounds in the samples, may have contributed to decreased sensitivity of molecular methods compared with serologic methods.

The observed specificity was excellent for all assays, ranging from 95.5% to 100% when compared with the reference method and equal to 100% when compared with a composite reference standard. However, predictive positive and negative values for the different assays could not be calculated because precise prevalence data are not available for most CCHF-endemic areas, and these data cannot be predicted for areas where the virus could emerge.

The CCHF IFA demonstrated a higher complexity in equipment, technique, and interpretation. However, the interpretation of fluorescence patterns may enable trained users to differentiate positive from cross-reactive serum samples, whereas such false positives may not be avoided with the ELISA. The operational characteristics of the molecular assays were comparable. Both methods required regular equipment maintenance and specific training for appropriate use of the equipment; however, this was more apparent for the real-time qRT-PCR method. The LCD array technique was considered to be complex but acceptable. Training for technique and result interpretation was recommended for each method.

All participating laboratories are reference centers for CCHF laboratory diagnosis and surveillance in their respective countries, and some are World Health Organization collaborating centers. The protocols from reference methods in use at each site have been extensively validated previously (*16–20,24*). In addition, the laboratories participated in a recent international external quality assessment (*34*). Therefore, local conditions at the participating sites and validity of reference methods were considered comparable.

The multicenter design of this study enabled the testing of a large sample size, representative of ≥1 population, so findings could be more generally applicable. In addition, sample panels were constituted without any selection for

disease severity, and negative controls included not only healthy patients but also patients who had a wide range of other conditions, thereby avoiding inflated estimates of diagnostic accuracy.

Our study has some limitations. Because archived samples were used for the study, specimen quality could have been affected; however, statistical analysis demonstrated that sample storage time and temperature did not influence sensitivity. Also, the use of different sample panels for serologic and molecular testing did not enable calculation of the added value of combining the serologic and molecular CCHF diagnostic methods evaluated.

The results of this study give additional guidance on the type of CCHF diagnostic tools that could be used in different contexts. During a large outbreak, easily interpretable tests for simultaneous analysis of numerous samples, such as the ELISA and real-time qRT-PCR, might be considered useful tools to identify CCHF cases. Methods available in smaller format size and demonstrating a long shelf life, such as the IFA and LCD array, could be used to identify sporadic cases or to confirm single cases as part of a larger outbreak.

This study demonstrates that efficient, well-characterized serologic and molecular assays and protocols are available for CCHF diagnosis. The on-site use of such assays by outbreak assistance laboratories would greatly diminish the risks posed by the handling, packaging, and shipping of highly infectious samples. Moreover, acquiring diagnostic reagents would be more time- and cost-effective for laboratories than would the organization of compliant packaging and shipment of hazardous biologic material to reference laboratories abroad. Nevertheless, laboratory personnel should receive the appropriate training to perform the different assays (e.g., during international workshops or network meetings). Collaborative evaluations of diagnostic methods remain essential to guide decision-making, especially with emerging diseases, where a standard is frequently missing, and laboratory expertise is rare.

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Dr Vanhomwegen is a research fellow at Institut Pasteur, Paris. Her research interests include the development and validation of tools for rapid detection and characterization of emerging pathogens.

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Address for correspondence: Jessica Vanhomwegen, CIBU, Institut Pasteur, 25-28 Rue du Dr. Roux, 75015 Paris, France; email: jvhomweg@pasteur.fr

Borrelia, *Rickettsia*, and *Ehrlichia* Species in Bat Ticks, France, 2010

Cristina Socolovschi, Tahar Kernif, Didier Raoult, and Philippe Parola

Argas vespertilionis, an argasid tick associated with bats and bat habitats in Europe, Africa, and Asia has been reported to bite humans; however, studies investigating the presence of vector-borne pathogens in these ticks are lacking. Using molecular tools, we tested 5 *A. vespertilionis* ticks collected in 2010 from the floor of a bat-infested attic in southwestern France that had been converted into bedrooms. *Rickettsia* sp. AvBat, a new genotype of spotted fever group rickettsiae, was detected and cultivated from 3 of the 5 ticks. A new species of the *Ehrlichia canis* group, *Ehrlichia* sp. AvBat, was also detected in 3 ticks. Four ticks were infected with *Borrelia* sp. CPB1, a relapsing fever agent of the *Borrelia* group that caused fatal borreliosis in a bat in the United Kingdom. Further studies are needed to characterize these new agents and determine if the *A. vespertilionis* tick is a vector and/or reservoir of these agents.

Ticks are obligate hematophagous arthropods that are considered to be second only to mosquitoes as vectors of agents that cause diseases in humans (*1*). Ticks parasitize every class of vertebrates in most regions of the world and occasionally bite humans (*1*). *Argas vespertilionis* (also known as *Carios vespertilionis*) ticks parasitize several bat species around the world, except in the Americas (*2*,*3*). Bats are adequate hosts for blood ingestion by ticks because they lack dense fur and have a supply of large blood vessels just below the dermis. In addition, a larval *A. vespertilionis* tick was collected from a dog in Sweden (*3*). Common habitats for these ticks include cracks and crevices in the walls of bat-infested caves and buildings, tree holes, and any niche occupied by the host. Tick nymphs and adults can bite persons in caves (*4*,*5*). *A. vespertilionis* tick populations in Europe and South Africa exist in temperate climates with pronounced seasonal changes and moderate to heavy rainfall.

The role of *A. vespertilionis* ticks as vectors or reservoirs of bacterial, viral, or protozoal pathogens is poorly understood; however, several pathogens have been detected in these ticks. In 1966, *Coxiella burnetii*, the agent of Q fever, was detected in *A. vespertilionis* ticks collected from southern Kazakhstan (*6*), and in 1973, an arbovirus named Issyk-Kul virus was isolated from bats and *A. vespertilionis* ticks in Kyrgyzstan (*7*). A few years later, Issyk-Kul virus was isolated from a scientist who had become infected while conducting field work in the Kumsangir district of southern Tajikistan (*8*). *Candidatus* Babesia vesperuginis showed potential pathogenicity to a bat in the United Kingdom, and the study authors hypothesized that the *A. vespertilionis* tick could be a vector for these protozoa (*9*). *Borrelia burgdorferi* sensu lato, the infectious agent of Lyme disease, was detected in *A. vespertilionis* ticks that were collected during 1896–1994 and housed at the Natural History Museum in London. Thus, 13/13 ticks collected from bats (mostly pipistrelles) and 12 (75%) of 16 ticks collected from human dwellings had results positive for *B. burgdorferi* s.l. when tested by nested PCR targeting the *ospA* gene (*10*). However, PCR contaminations were not excluded for these results. It has been stated that this tick is a vector of spirochetes in bats, but no conclusive evidence has supported this hypothesis (*2*).

The WHO (World Health Organization) Collaborative Center for Rickettsial Diseases and other Arthropod-Borne Bacterial Diseases receives human samples and arthropod specimens from all parts of the world for tick-borne dis-

Author affiliations: Unité de Recherche en Maladies Infectieuses et Tropicales Emergentes, Marseille, France (C. Socolovschi, T. Kernif, D. Raoult); and World Health Organization Collaborative Center for Rickettsial Diseases and Other Arthropod-Borne Bacterial Diseases, Marseille (P. Parola)

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ease diagnosis. The aim of this study was to analyze *A. vespertilionis* ticks for the presence of *Borrelia*, *Rickettsia*, *Bartonella*, and *Ehrlichia* spp. and for *C. burnetii* by using molecular and culture tools.

Materials and Methods

Tick Collection

On July 12, 2010, the owners of a bat-infested home in Astien, France (42°56′18.25″N, 1°03′54.57″E; elevation 542 m) found 6 live ticks on the floor of their attic, which had been converted into bedrooms (Figure 1). Astien is located in southwestern France in the Ariège region of the Pyrenees Mountains. The ticks were sent to our laboratory at the WHO Collaborative Center for Rickettsial Diseases and Other Arthropod-Borne Bacterial Diseases (Marseille, France), where we used standard taxonomic keys to identify them morphologically as adult *A. vespertilionis* (Latreille, 1802) ticks (online Video, wwwnc.cdc.gov/EID/article/18/12/12-1237-V1.htm) (*2*).

DNA Extraction

Five of the 6 ticks were washed in a 10% water solution of commercial disinfectant–detergent (Amphomousse; Hydenet S.A., Sainghin-en-Melantois, France), rinsed in sterile water, and placed in a 1% solution of sodium hypochlorite for 10 minutes. The ticks were then rinsed with distilled water and incubated in 70% ethanol for 15 minutes, after which they were rinsed in sterile phosphate-buffered saline, dried on sterile filter paper in a laminar flow hood, and individually crushed in sterile tubes (Eppendorf; Hamburg, Germany). DNA was extracted from one half of each of the 5 ticks by using the QIAamp Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The genomic DNA was stored at 4°C until used as a template in PCR assays. The remaining portion of each tick was kept at –80°C for further analysis. DNA extraction and the molecular identification of all ticks were efficiently achieved by using one molecular system: PCR amplification with sequencing of the 338-bp region of the 12S RNA gene, as described (*11*). The sixth tick was kept in the tick collection of the WHO Collaborative Center for Rickettsial Diseases and other Arthropod-Borne Bacterial Diseases, Marseille, France.

Detection of *Rickettsia* spp.

We used quantitative real-time PCR (qPCR) with the 1029 system based on the RC0338 hypothetical protein gene to screen DNA samples from the 5 ticks for all spotted fever group (SFG) rickettsiae (*12*) (Table). Reactions were performed by using LightCycler 2.0 equipment and software (Roche Diagnostics GmbH, Mannheim, Germany). Master mixes were prepared according to the manufacturer's instructions. We confirmed rickettsiae-positive results by using conventional PCR with the GeneAmp PCR System 2400 thermal cycler (PerkinElmer, Waltham, MA, USA). We used primers CS2d–CS877f and Rp CS.409p–Rp CS.1258n to amplify and sequence the full-length citrate synthase gene (*gltA)* found in all rickettsiae (*20*), and we used primers Rr. 190.70 and Rr. 190.701 to amplify and sequence a fragment of the outer membrane protein A (*ompA*) gene (629–632 bp), which encodes a 190-kDa protein (*17*). We used 2 negative controls for all PCR reactions: 1) PCR mix alone and 2) PCR mix with noninfected *Rhipicephalus sanguineus* tick DNA (free of *Rickettsia*, *Ehrlichia*, *Anaplasma*, *Bartonella*, and *Borrelia* spp. and *C. burnetii*). We used DNA extracted from *R. montanensis* as a positive control for detection of rickettsiae. Amplification products were analyzed after electrophoresis on a 1% agarose gel stained with ethidium bromide.

The second half of each tick was placed in sterile 1.5-mL plastic tubes, where they were triturated with a sterile micropestle in 600 μL of Rinaldini solution (6.8 g NaCl, 0.4 g KCl, 0.156 g NaH_2PO_4, 2.2 g $NaHCO_3$, 1.0 g glucose, and 1.0 mg phenol red in 1,000 mL sterile double-distilled water). To isolate *Rickettsia* spp. from the tick solution,

Figure 1. Bat-infested home in Astien, southwestern France, in the Ariège region of the Pyrenees Mountains. *Argas vespertilionis* ticks were collected from the floor of the attic, which had been converted into bedrooms (right).

Table. Primers and probes used to detect and confirm the presence of bacteria in *Argas vespertilionis* ticks collected in 2010 from an attic in France*

Bacterial species	Gene target (ref)	Primers and probe
		Screening
Rickettsia	RC0338 (*12*)	1029-F1: 5′-GAM AAA TGA ATT ATA TAC GCC GCA AA-3′
		1029-R1: 5′-ATT ATT KCC AAA TAT TCG TCC TGT AC-3′
		1029–1P: 6FAM-CTC AAG ATA AGT ATG AGT TAA ATG TAA A-TAMRA
Borrelia	16S rRNA (*13*)	Bor_16S_3_F: 5′ AGC CTT TAA AGC TTC GCT TGT AG 3′
		Bor_16S_3_R: 5′ GCC TCC CGT AGG AGT CTG G 3′
		Bor_16S_3_P: 6FAM- CCG GCC TGA GAG GGT GAA CGG TAMRA
Coxiella burnetii	IS*1111* (*14*)	IS 1111F: 5′-CAA GAA ACG TAT CGC TGT GGC-3′
		IS 1111R: 5′-CAC AGA GCC ACC GTA TGA ATC-3′
		IS1111P: 6-FAM-CCG AGT TCG AAA CAA TGA GGG CTG-TAMRA
Bartonella	ITS (*15*)	Barto ITS3 F: 5′-GAT GCC GGG GAA GGT TTT C-3′
		Barto ITS3 R: 5′-GCC TGG GAG GAC TTG AAC CT-3′
		Barto ITS3 P: 6 FAM-GCG CGC GCT TGA TAA GCG TG-TAMRA
Ehrlichia	16S rRNA (*16*)	**Erli_16S_F: 5′-GGT-ACC-YAC-AGA-AGA-AGT-CC-3′**
		Erli_16S_R: 5′-TAG-CAC-TCA-TCG-TTT-ACA-GC-3′
		Confirmation
Rickettsia	*gltA* (*17*)	**409D: 5′-CCT ATG GCT ATT ATG CTT GC-3′**
		1258R: 5′-ATT GCA AAA AGT ACA GTG AAC A-3′
Rickettsia	*ompA* (*17*)	**190–70: 5′-ATG GCG AAT ATT TCT CCA AAA-3′**
		190–701: 5′- GTT CCG TTAATGGCAGCA TCT-3′
		190–180: 5′- GCA GCG ATA ATG CTG AGT A-3′
Borrelia	16S rRNA (*13*)	**BF1: 5′-GCT GGC AGT GCG TCT TAA GC-3′**
		BR1: 5′-GCT TCG GGT ATC CTC AAC TC-3′
Borrelia	Flagellin (*18*)	Bor1: 5′- TAA TAC GTC AGC CAT AAA TGC-3′
		Bor2: 5′- GCT CTT TGA TCA GTT ATC ATT C-3′
C. burnetii	IS*30* (*14*)	IS30a F: 5′-CGC TGA CCT ACA GAA ATA TGT CC-3′
		IS30a R: 5′-GGG GTA AGT AAA TAA TAC CTT CTG G-3′
		IS30a P: 6-FAM-CAT GAA GCG ATT TAT CAA TAC GTG TAT GC-TAMRA
Bartonella	ITS (*15*)	**Urbarto1: 5′-CTT-CGT-TTC-TCT-TTC-TTC-A-3′**
		Urbarto2: 5′-CTT-CTC-TTC-ACA-ATT-TCA-AT-3′
Ehrlichia	*gltA* (*19*)	**HER-CS133F: 5′-GGW TTY ATG TCY ACT GCT GC-3′**
		HER-CS778R: 5′-GCN CCM CCA TGM GCT GG-3′

*Ref, reference. Primers in **boldface** were used for sequencing.

we used a shell vial cell culture assay, as described (*21*). In brief, we inoculated 300 µL of the rickettsia PCR–positive ticks into 7 shell vials containing 1-cm^2 coverslips on which cell culture lines had been grown. Of the 7 shell vials, 3 contained a coverslip with a monolayer of mouse fibroblasts (L929 cells); 3 contained coverslips with a monolayer of human embryonic lung fibroblasts; and 1 contained a coverslip with cell line XTC-2, derived from *Xenopus laevis*. We did not include antimicrobial drugs in the medium. After the vials were incubated for 8, 15, and 21 days, we performed Gimenez staining and indirect immunofluorescence assays to detect rickettsial organisms in cell culture as described (*21*). Cultures were considered rickettsiae-positive if staining and assay results were positive. We sampled culture supernatants to identify isolates by standard PCR as described (*17,20*).

Detection of *Borrelia* spp.

We used qPCR targeting the 16S rRNA gene, as described (*13*), to screen DNA samples from the 5 ticks for all *Borrelia* spp. (Table). Samples with borreliae-positive results were confirmed positive by conventional PCR with primers Bf1-Br1 and Bor1-Bor2, which enabled amplification of the 16S rRNA gene fragment and *flaB* gene, respectively (*18,22*). We sequenced the amplified product as described above. Positive control reactions for each assay incorporated DNA extracted from *Borrelia crocidurae*. We injected the solution of *Borrelia* spp. PCR–positive ticks into 2 tubes with 10 mL of BSKH medium (Sigma-Aldrich, Taufkirchen, Germany) (*23*) and a 100-µL solution of antimicrobial drugs (product no. A1956 [2 mg phosphomycin (fosfomycin), 5 mg rifampin, and 250 µg amphotericin B per mL in 20% DMSO]; Sigma-Aldrich). Samples were cultivated at 33°C, and once a week we used dark-field microscopy to examine them for the presence of spirochetes. We considered samples to be negative for borreliae if no growth was detected after 8 weeks of incubation.

Detection of *Bartonella* spp.

We used qPCR to screen DNA samples for a fragment of the *Bartonella* spp. intergenic spacer region between the 16S and 23S rRNA genes (Table). Conventional PCR that amplifies a fragment of the 732-bp intergenic spacer region of *Bartonella* spp. was used to confirm bartonellae-positive results (*15*). DNA extracted from *B. elizabethae* served as a positive control for detection of bartonellae.

Detection of *C. burnetii*

We initially detected bacterial DNA by qPCR with *C. burnetii*–specific primers and a probe designed to amplify the IS*1111* gene (*14*). We used qPCR with primers and a probe designed for the amplification of IS30A spacers to confirm *C. burnetii*–positive results (*14*). Amplification of both spacers indicated a positive result. In each test, DNA extracted from *C. burnetii* served as a positive control.

Detection of *Ehrlichia/Anaplasma spp.*

We detected *Ehrlichia*/*Anaplasma* spp. DNA by conventional PCR using primer set EHR16SR– EHR16SD, which amplify a 345-bp fragment of the 16S rRNA gene of ehrlichiae (*16*). A second PCR that amplified a fragment of the citrate synthase gene of *Ehrlichia* spp. was used, as described (*19*), to confirm positive amplification results (Table). The amplified products for both genes were sequenced as described above. Two negative controls and 1 positive control (DNA from *A. phagocytophilum)* were included in each test.

Sequence Analysis

All obtained sequences were assembled and edited by using Auto Assembler software version 1.4 (PerkinElmer). We analyzed sequences by using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) and compared them with sequences in the GenBank database. We performed multiple sequence alignments by using the ClustalX program (www.clustal.org/clustal2/). Phylogenetic trees were constructed by using the test minimum-evolution tree algorithm in the MEGA5 program (http://megasoftware.net/). Support for the tree nodes was calculated with 100 bootstrap replicates.

Results

Tick Identification

The 12S RNA gene from 4 of the 5 *A. vespertilionis* ticks in our study showed 81.4% (253/311 bp) sequence similarity with the *Carios capensis* tick (GenBank accession no. AB075953) and 77.7% (303/390 bp) sequence similarity with the *Ixodes granulatus* tick (GenBank accession no. DQ003012). We submitted the 12S RNA sequence to GenBank (accession no. JX233821); no other 12S sequence for *A. vespertilionis* ticks was available in GenBank.

Detection of *Rickettsiae* spp.

Of the 5 ticks we tested, 3 (sample nos. 62494, 62497, and 62498) were positive for genus-specific rickettsiae DNA by qPCR. Subsequent sequencing of *gltA* gene amplicons from all positive PCR samples showed that the closest sequences available in GenBank were those for *R. peacockii* (accession no. CP001227), *R. africae* (accession no. CP001612), and *R. conorii* (accession no. AE006914), which showed 99.6% (1,257/1,262 bp), 99.52% (1,256/1,262 bp), and 99.52% (1,256/1,262 bp) sequence identity, respectively. The closest sequences available in GenBank for the *ompA* gene fragment were those for *R. africae* ESF-5 (accession no. CP001612), *Rickettsia* sp. strain S (accession no. RSU43805), and *R. mongolitimonae* BJ-90 strain (accession no. AF179365), which

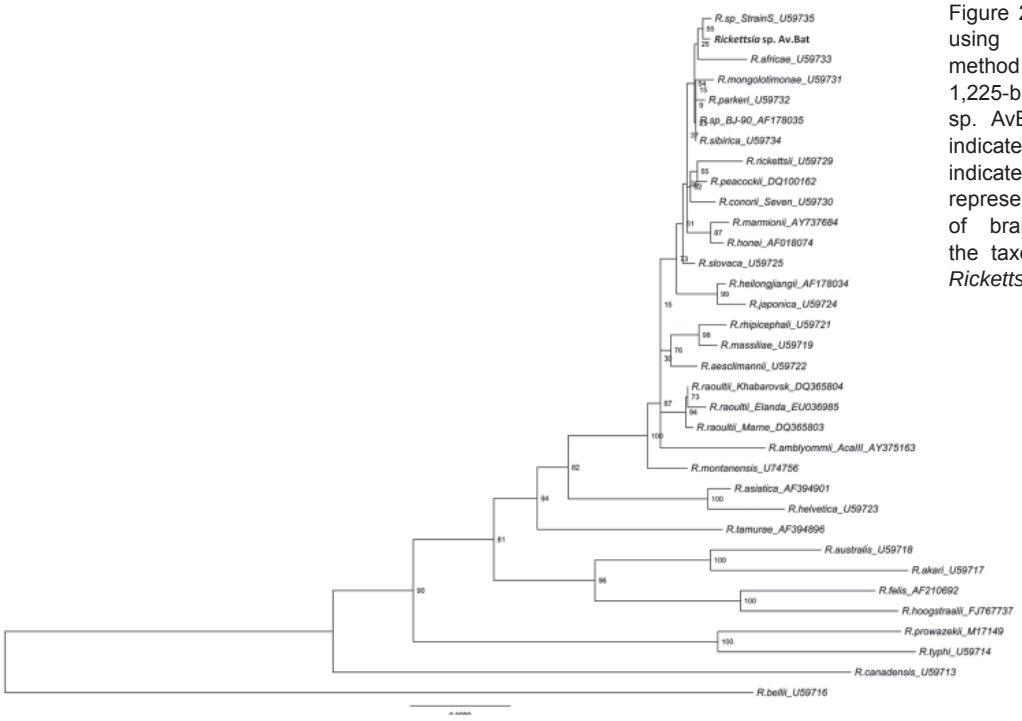

Figure 2. Phylogenetic tree drawn using the minimum evolution method from an alignment of the 1,225-bp *gltA* gene of *Rickettsia* sp. AvBat. Bootstrap values are indicated at the nodes. Scale bar indicates the degree of divergence represented by a given length of branch. **Boldface** indicates the taxonomic position of a new *Rickettsia* sp.

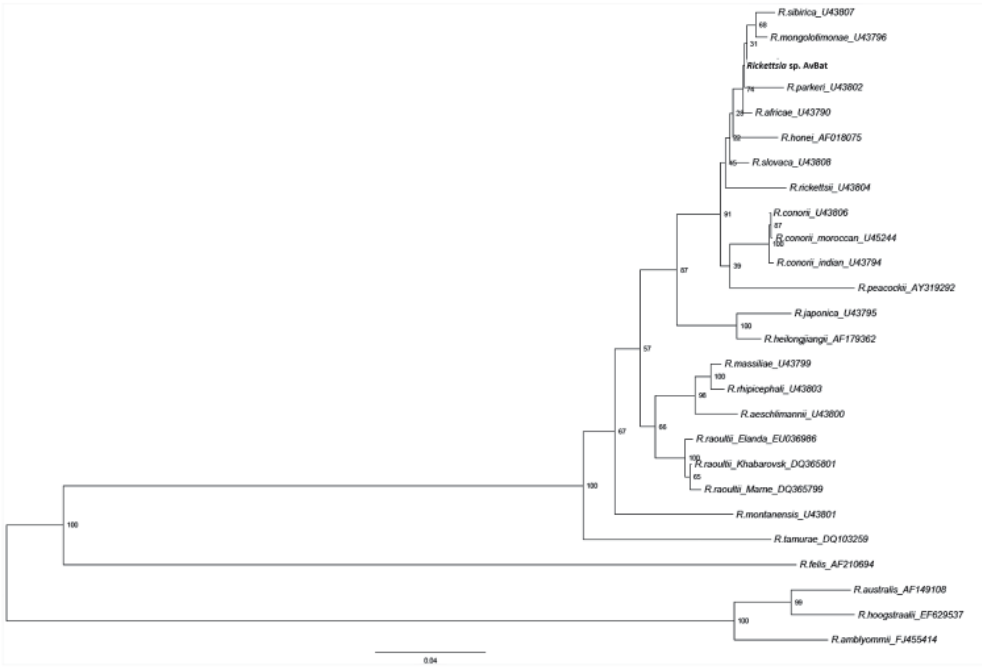

Figure 3. Phylogenetic tree drawn, using the minimum evolution method, from an alignment of the 611-bp *ompA* gene of *Rickettsia* sp. AvBat. Bootstrap values are indicated at the nodes. Scale bar indicates the degree of divergence represented by a given length of branch. **Boldface** indicates the taxonomic position of a new *Rickettsia* sp.

showed 99.34% (611/615 bp), 99.5% (587/590 bp), and 98.67% (594/602 bp) sequence identity, respectively. The sequences for all 3 ticks were identical.

The positive and negative controls produced the expected results. The nucleotide sequence of the full-length *gltA* gene of *Rickettsia* sp. was deposited in GenBank (accession no. JN038177) and named *Rickettsia* sp. AvBat. Maximum parsimony and neighbor-joining analysis of the 1,225-bp *gltA* gene and the presence of the *ompA* gene suggest that *Rickettsia sp.* AvBat be classified as an SFG rickettsiae, with the closest relationships to *Rickettsia* sp. strain S (U59735) and *R. africae* (U59733) (Figures 2, 3) (*24*). On the basis of the guidelines for the classification of new *Rickettsia* spp. (*24*), the bacterial species we identified shared <99.9% similarity with the full-length *gltA* gene and >98.8% similarity with the partial *ompA* gene.

On day 15 of incubation, the shell vial cultures for 2 PCR rickettsiae-positive ticks had Gimenez staining and immunofluorescence assay results positive for *Rickettsia* spp. (Figure 4). The 2 isolates were established in the L929 and XTC-2 cells, respectively (3 passages/cell line).

Detection of *Borrelia* spp.

Of the 5 *A. vespertilionis* ticks tested for the presence of *Borrelia* sp. by qPCR, 4 were positive: tick numbers 62494, 62495, 62497, and 62498. We used standard PCR to amplify the *Borrelia* 16S rRNA gene fragment from all 4 positive ticks. For all samples, DNA sequence analyses of the PCR products showed 100% (1,206/1,206 bp) similarity with the 16S rRNA sequence of *Borrelia* sp. CPB1 (GenBank accession no. FJ868583) and 100% (736/736 bp) similarity with the flagellin gene sequence of *Borrelia* sp. CPB1 (GenBank accession no. FJ868584) (*25*). The bacterial cultures of the borrelia PCR–positive samples did not grow borreliae. Phylogenetic analysis of 2 genes (Figures 5, 6) showed that this *Borrelia* sp. is close to, but distinct from, a cluster containing *B. recurrentis*, *B. duttonii*, *B. microtii*, *B. latyschewii*, and *B. crocidurae*.

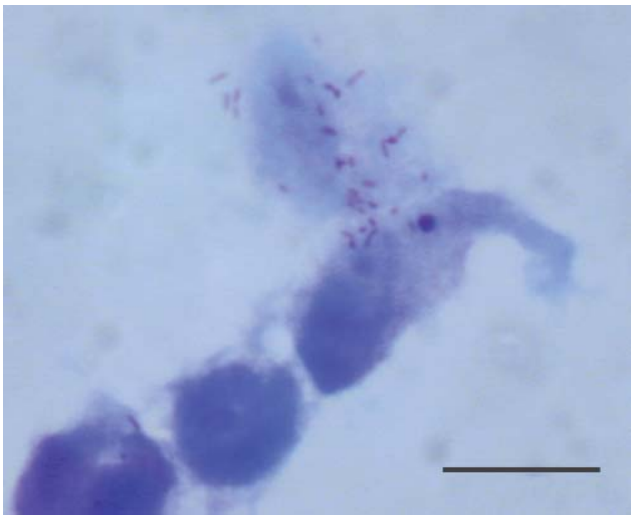

Figure 4. *Rickettsia* sp. AvBat in XTC-2 cell culture with Gimenez staining. Scale bar = 20 μm. A color version of this figure is available online (wwwnc.cdc.gov/eid/article/18/12/11-1237-F4.htm).

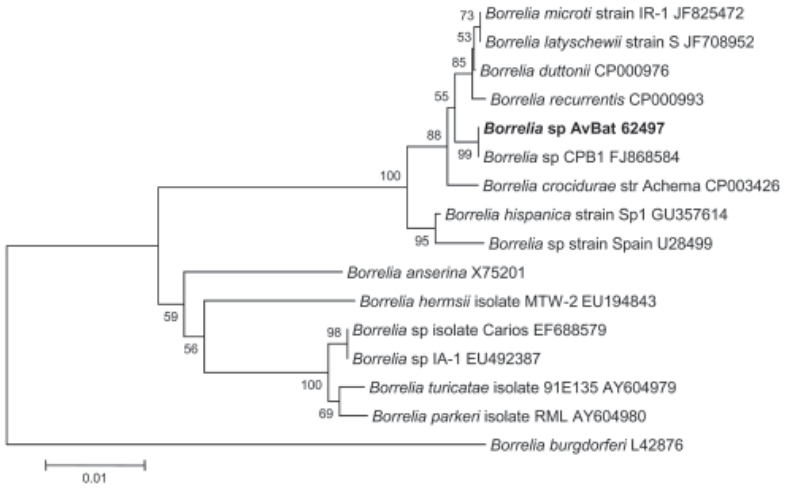

Figure 5. Phylogenetic trees drawn from an alignment of the 736-bp *flaB* gene specific to *Borrelia* spp. by using the minimum evolution method. Bootstrap values are indicated at the nodes. Scale bar indicates the degree of divergence represented by a given length of branch. **Boldface** indicates the position of *Borrelia* sp. AvBat in the phylogenetic tree.

Detection of *Ehrlichia/Anaplasma* spp.

Five ticks were tested by standard PCR for the 16S rRNA and *gltA* genes specific for *Ehrlichia*/*Anaplasma*; results were positive for 3 ticks (nos. 62495, 62496, and 62497). Sequence analyses showed 99.4% (344/346 bp) similarity with the 16S rRNA gene of uncultured *Ehrlichia* sp. clone Khabarovsk 1931 (GenBank accession no. FJ966354) and 98.3% similarity with *E. muris* isolate Kh-1550 (GenBank accession no. GU358692). For these 3 ticks, the closest matches to a *gltA* gene fragment in GenBank were with those of *Ehrlichia* sp. HF (accession no. DQ647319), *Ehrlichia* sp. Yamaguchi (accession no. AF304145), and *E. muris* (accession no. AF304144), which had 88.93% (225/253 bp), 88.53% (224/253 bp), and 88.53% (224/253 bp) sequence similarity, respectively. Sequences of the 16S rRNA and *gltA* genes from all 3 ticks were identical.

We deposited nucleotide sequences for the 16S rRNA and *gltA* genes of this *Ehrlichia* sp. in GenBank (accession nos. JN315412 and JN315413, respectively). In phylogenetic trees based on 257 bp of the *gltA* gene and 348 bp of the 16S rRNA gene, this sequence is situated in the genus *Ehrlichia* in the *E. canis* group, and it is distinct from other known *Ehrlichia* spp. (Figures 7, 8) (*26*).

Detection of *Bartonella* spp. and *C. burnetii*

We tested 5 ticks by qPCR for the presence of *Bartonella* spp. and *C. burnetii*. Results were negative for these bacteria.

Co-infections

Of the 5 *A. vespertilionis* ticks analyzed by PCR, 4 were positive for >1 pathogen. Three of the 4 borreliae-positive ticks (nos. 62494, 62497, and 62498) were also infected with *Rickettsia* sp. AvBat. Two of the 3 *Ehrlichia* sp. AvBat–positive ticks (nos. 62495 and 62497) were also infected with *Borrelia* sp. CPB1, and 1 of those (no. 62497) was also infected with *Rickettsia* sp. AvBat.

Discussion

We showed that *A. vespertilionis* ticks collected from a bat-infested attic in southwestern France were infected with 3 bacteria: 1) *Rickettsia* sp. AvBat, a new species or subspecies of the SFG rickettsiae; 2) a novel *Ehrlichia* sp.

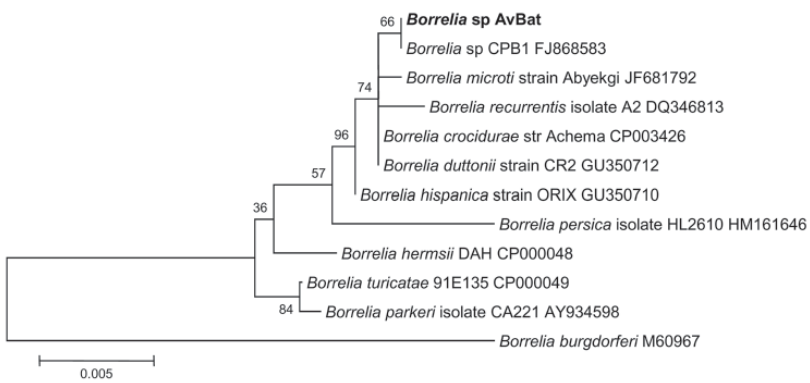

Figure 6. Phylogenetic tree drawn from an alignment of the 1206-bp 16S rRNA gene specific to *Borrelia* spp. by using the minimum evolution method. Bootstrap values are indicated at the nodes. Scale bar indicate the degree of divergence represented by a given length of branch. **Boldface** indicates the position of *Borrelia* sp. AvBat in the phylogenetic tree.

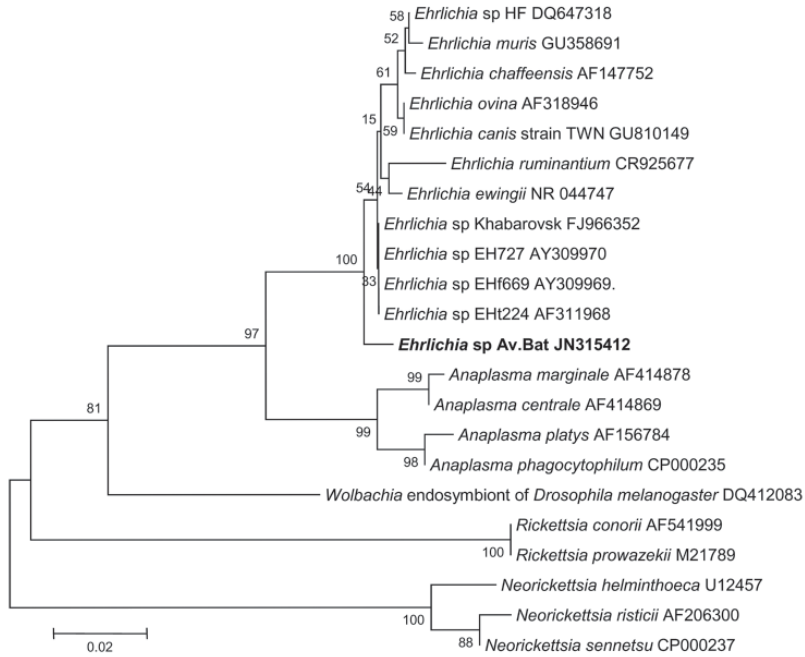

Figure 7. Phylogenetic trees drawn from an alignment of the 348-bp 16S rRNA gene specific to *Ehrlichia* spp. by using the minimum evolution method. Bootstrap values are indicated at the nodes. Scale bar indicate the degree of divergence represented by a given length of branch. **Boldface** indicates the taxonomic position of a new *Rickettsia* sp.

AvBat of the *E. canis* group of the genus *Ehrlichia*; and 3) *Borrelia* sp. from the relapsing fever group.

In 1956, Hoogstraal (*2*) reported that several *A. vespertilionis* ticks from Egypt were examined for rickettsiae, and all were negative. Through sequence analysis of the full-length *gltA* gene, we showed that the *Rickettsia* sp. detected in *A. vespertilionis* ticks in France can be classified within the SFG rickettsiae (*24*).

The association of *Rickettsia* spp. with soft ticks is poorly understood. First, intracellular rickettsia-like symbionts were described in laboratory-maintained *Argas (Persicargas) arboreus* ticks, but the organisms have not been shown to infect vertebrates or cause disease (*27*). *Ornithodoros papillipes* ticks that were sucking blood from guinea pigs infected with *Rickettsia sibirica,* the agent of North Asian tick typhus, were found to be infected with the bacterium (*28*). *O. papillipes* ticks can transmit bacteria vertically but cannot transmit it to vertebrate hosts (*28*). In addition, in a laboratory experiment, *O. parkeri* and *O. rostratus* ticks were infected with *R. rickettsii*, the agent of Rocky Mountain spotted fever, and transmitted the bacterium to a laboratory host (*29*). In 1974, Rehácek et al. (*30*) found that *Argas persicus* ticks collected in Armenia were massively infected with SFG group rickettsiae that were probably identical with *R. slovaca*, an emerging pathogen (*17*). Rickettsiae-infected *O. moubata* ticks (well-known vectors of *Borrelia duttonii,* an agent of tick-borne relapsing fever) were collected from human dwellings in central Tanzania and analyzed by PCR; phylogenetic analysis of the rickettsial species showed a unique cluster among the SFG rickettsiae (*31*). At the same time, *R. felis,* the agent of the so-called flea-borne spotted fever (*32*), was detected in 1 of 64 *C. capensis* ticks collected from a brown pelican rookery in South Carolina, USA (*33*). A rickettsial endosymbiont, later named *R. hoogstraalii* sp. nov., was detected by use of PCR and isolated from *C. capensis* ticks (*34*,*35*) collected in the United States and Japan (*33*,*36*) and, later, from *Carios kelleyi* bat ticks collected from residential buildings in Jackson County, Iowa, USA (*37*). Transstadial and transovarial transmission of this rickettsia have also been demonstrated (*33*). Our results support further investigation of the association of soft ticks and rickettsiae.

The sequences for the *Borrelia* sp. detected in this study share 100% similarity with sequences for species detected in the liver of a dead bat in the United Kingdom in 2008 (*25*). Phylogenetic analysis showed that the *Borrelia* sp. detected in the bat is close to, but distinct from, borreliae in a cluster containing *B. recurrentis, B. duttonii,* and *B. crocidurae* (*25*), which all cause relapsing fever in Africa (*13*). In the study in the United Kingdom, an *A. vespertilionis* larval tick (not tested for borreliae by PCR) was found attached to the infected bat, and the study authors suggested that the tick may have been the source of *Borrelia* infection (*25*). Usually, only the larvae of *A. vespertilionis* ticks are found on bats because they feed on their hosts from the time they are a few days old up to 2 weeks of age. Nymphs and adults become replete within 1 hour; thus, the probability is small for finding ticks in these growth stages on bats (*2*). In addition, adults tick may remain attached for as

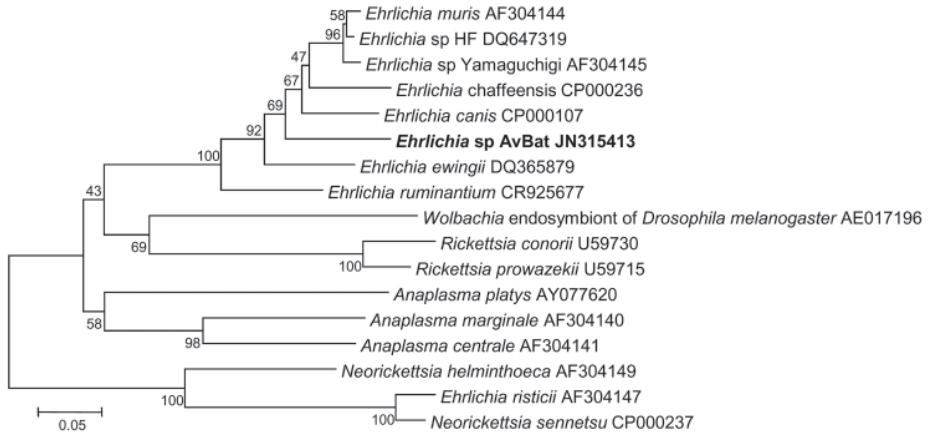

Figure 8. Phylogenetic trees drawn from an alignment of the 257-bp *gltA* gene specific to *Ehrlichia* spp. by using the minimum evolution method. Bootstrap values are indicated at the nodes. Scale bar indicate the degree of divergence represented by a given length of branch. **Boldface** indicates the taxonomic position of a new *Ehrlichia* sp.

long as 5 hours after completed engorgement. The results of our study confirm the association between *A. vespertilionis* ticks and this new *Borrelia* sp.

The presence of ehrlichiae was detected in 3 *A. vespertilionis* ticks in our study, but we did not culture the samples. The association between *Ehrlichia/Anaplasma* spp. and soft ticks is unknown. In 1990, Ewing et al. (*38*) reported that an *Otobius megnini* tick, which detached from the ear of a child who had serologic evidence of ehrlichiosis, was negative for ehrlichia by PCR. The authors of that study attempted to use laboratory-reared *O. megnini* ticks to transmit *E. canis,* the causative agent of canine ehrlichiosis; neither transstadial nor transovarial transmission occurred.

In our study, no residents of the bat-infested attic were bitten by ticks, although 2 persons were sleeping in the room the night before the ticks were collected. Adult and nymphal *A. vespertilionis* ticks occasionally bite humans, and they can be highly aggressive toward humans (*39*). Ticks of this species have been removed from persons in Iraq, the former Soviet Union, Japan, and Africa (*2*,*3*). In Huesca Province, Spain, 2 adult *A. vespertilionis* ticks were found feeding on the arm of a man inhabiting a country house with many bats living in the attic (*40*). In 1994, Jaenson et al. (*3*) reported that 2 persons living near Stockholm were bitten by ticks in their bedroom; bats had been breeding in a loft above the room during May–June 1993. Severe skin reactions, fever, ulceration, erythema, and edema developed in the bitten persons, and the ulcers did not begin to cicatrise until 10 days after penicillin treatment had been initiated. Thus, medical doctors should consider bacterial infections in sick person who may have been bitten by bat ticks.

The findings from our study have repercussions for public health in many parts of Europe, Asia, and Africa because *A. vespertilionis* ticks have a wide geographic range and may bite humans (*2*). Almost any bat, whether it lives in large colonies or in small groups, may be parasitized by *A. vespertilionis* ticks (*2*). We do not know whether the novel *Rickettsia* sp. AvBat and *Ehrlichia* sp. AvBat described in our work are pathogenic for vertebrate hosts; however, the *Borrelia* sp. detected in these ticks was shown to be a pathogen for bat hosts (*25*). In addition, the *Rickettsia* sp. AvBat that we cultivated in a cell line should be analyzed genomically to further define the taxonomic position of this *Rickettsia* sp. Future studies are needed to 1) assess the role of vector and/or reservoir for each of these pathogens in *A. vespertilionis*, including a more precise analysis of transstadial and transovarial transmission in ticks; 2) confirm tick transmission of the bacteria in animal models; and 3) detect tick transmission of these bacteria in humans.

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Dr Socolovschi is a medical doctor specializing in infectious diseases and tropical medicine at the Medical School of Marseille, France. Her research interests focus on vector-borne infectious tropical diseases and medical entomology.

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Address for correspondence: Philippe Parola, Unité de Recherche en Maladies Infectieuses et Tropicales Emergentes (URMITE), UMR 63, CNRS 7278, IRD 198, INSERM 1095, WHO Collaborative Center for Rickettsial Diseases and Other Arthropod-Borne Bacterial Diseases, 27 Bd Jean Moulin, 13385 Marseille Cedex 5, France; email: philippe.parola@univ-amu.fr

Nonprimate Hepaciviruses in Domestic Horses, United Kingdom

Sinéad Lyons, Amit Kapoor, Colin Sharp, Bradley S. Schneider, Nathan D. Wolfe, Geoff Culshaw, Brendan Corcoran, Bruce C. McGorum, and Peter Simmonds

Although the origin of hepatitis C virus infections in humans remains undetermined, a close homolog of this virus, termed canine hepacivirus (CHV) and found in respiratory secretions of dogs, provides evidence for a wider distribution of hepaciviruses in mammals. We determined frequencies of active infection among dogs and other mammals in the United Kingdom. Samples from dogs (46 respiratory, 99 plasma, 45 autopsy samples) were CHV negative by PCR. Screening of 362 samples from cats, horses, donkeys, rodents, and pigs identified 3 (2%) positive samples from 142 horses. These samples were genetically divergent from CHV and nonprimate hepaciviruses that horses were infected with during 2012 in New York state, USA. Investigation of infected horses demonstrated nonprimate hepacivirus persistence, high viral loads in plasma (10^5–10^7 RNA copies/mL), and liver function test results usually within reference ranges, although several values ranged from high normal to mildly elevated. Disease associations and host range of nonprimate hepaciviruses warrant further investigation.

Hepatitis C virus (HCV) is a positive-sense RNA virus, classified as the type member of the family *Flaviviridae* and the genus *Hepacivirus*. HCV is a major human pathogen, causing persistent infections that target and eventually destroy the liver in a substantial proportion of those infected (*1,2*). HCV infections are distributed worldwide and have spread epidemically within the past 40–60 years within western countries through blood-borne routes such as blood and blood product transfusion and injection drug use.

HCV shows considerable genetic diversity; 7 genotypes show >30% nt sequence divergence from each other (*3*). Several of these genotypes are associated with suspected endemic source areas in central and western sub-Saharan Africa (genotypes 1, 2, and 4) (*4–7*) and Southeast Asia (genotypes 3 and 6) (*8,9*). These regions harbor the greatest diversity of HCV subtypes, implying a long-term, endemic circulation of the virus for several hundred years. The spread of certain genotype variants from these populations, such as 1a and 1b to Western countries, 3a among injection drug users in Europe, and 4a to Egypt, where it was extensively transmitted by medical injections (*10–14*), show several parallels with the emergence and rapid spread of HIV-1 among new risk groups from a central African reservoir over a similar time frame (*15*).

Based on this model, it has been frequently speculated that HCV could have an ultimate animal origin in ≥1 nonhuman primate species, in much the same way as human HIV-1 originated from chimpanzees (*15*). Three of the *Pan troglodytes* chimpanzee subspecies are frequently infected in the wild with a lentivirus that ultimately was derived from simian immunodeficiency viruses infecting Old World monkey species (*16*). The hypothesis for an equivalent nonhuman primate origin for HCV fueled several published (*17,18*) and unpublished (S. Lyons et al., unpub. data) surveys for HCV or HCV-like viruses in chimpanzees, other apes, and a variety of Old World monkey species (*19*). These studies were encouraged by the serendipitous detection of a virus distantly related to HCV, termed GB virus B (GBV-B), in a laboratory-housed tamarin, a New World monkey (*20,21*). This detection was speculated to represent, in evolutionarily terms, the New World monkey homolog of HCV, a scenario that supports the possibility for the widespread distribution of further HCV-like viruses among Old World monkey species in Africa and Asia. Despite the plausibility of this hypothesis, we found that no survey to date has detected or obtained serologic evidence for infection with HCV or HCV-like viruses in any ape or Old World monkey species screened in published data (*17,18*) or in the aforementioned unpublished data of

Author affiliations: University of Edinburgh, Edinburgh, Scotland, UK (S. Lyons, C. Sharp, G. Culshaw, B. Corcoran, B.C. McGorum, P. Simmonds); Columbia University, New York, New York, USA (A. Kapoor); and Global Viral Forecasting Initiative, San Francisco, California, USA (B.S. Schneider, N.D. Wolfe)

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Lyons et al. As a further puzzling observation, GBV-B infection has not been reported in any other tamarin or other New World monkey species either in the wild or among captive animals. Like HCV, its origin remains unknown.

Considering this background and previous focus on primates for HCV origins, it came as a complete surprise that a virus, much more closely related to HCV than GBV-B, was recently described for domestic dogs (*22*). Its host and apparent tropism for the respiratory tract (and potential association with infectious respiratory disease) represent major differences from what might be expected for a close relative of HCV. It came as a further surprise that CHV RNA sequences were detected in plasma samples from 8 of 103 horses in New York state, USA (*23*). Results of a novel serologic test of samples from horses for antibodies to the conserved nonstructural protein 3 (NS3) showed an overall seropositivity of 35%, and 80 samples from dogs were negative by serologic testing and PCR. The wider host range of the virus implied by these findings led the authors to propose a new name, non-primate hepacivirus (NPHV) for CHV, and this new nomenclature is used in the current study.

To investigate the species distribution of NPHV or homologs in a range of mammalian species and to investigate clinical features of infection, we initiated large-scale PCR–based screening of plasma, respiratory, and postmortem liver, spleen, and lung samples from horses, dogs, cats, and other species originating in the United Kingdom. The PCR was specific for conserved sequences in the 5′ untranslated region (5′-UTR) and the NS3 regions of NPHV. NPHV was detected in 3 horses (horse 1, horse 2, and horse 3); initial characterization of the epidemiology and clinical features of NPHV infections were performed and compared with those of HCV.

Materials and Methods

Samples

A total of 552 samples were screened for the presence of NPHV RNA. Samples collected were from horses, dogs, cats, mice, pigs, and donkeys. All horse, cat, and donkey samples were sourced from either excess diagnostic samples or previously archived study samples from the Royal (Dick) School of Veterinary Studies, University of Edinburgh, where the laboratory investigation for this study was performed. Buccal swab samples were obtained from dogs (*Canis lupus familiaris*) undergoing veterinary examination at the Edinburgh Dog and Cat home. Bronchoalveolar lavage samples were collected from dogs undergoing investigation of respiratory disease.

Venus blood samples were collected from 353 nonprimates comprising 99 dogs, 158 horses and donkeys (142 *Equus ferus caballus, 16 Equus africanus asinus*), 56 cats (*Felis catus*), 63 rodents (47 *Apodemus sylvaticus*, 8 *Mus muscullus*, 5 *Myodes glareolus*, 3 *Microtus agrestis*), and 40 pigs (*Sus scrofa*). Plasma was separated by centrifugation and frozen at −80°C until testing. Lung, liver, and spleen samples were obtained from dogs during autopsy at the pathology department of the school of veterinary studies and placed in RNAlater (QIAGEN, Crawley, UK) before RNA extraction. Samples of mouse liver were collected from all rodents in East Lothian, Scotland except 2 *Mus muscullus* for which liver samples were unavailable.

All clinical sampling was undertaken with full owner consent and in line with Royal (Dick) School of Veterinary Studies institutional and UK ethical guidelines.

CHV and NPHV Screening

Screening for CHV and NPHV infections was performed by using PCR; serologic screening for antibodies against CHV/NPHV was precluded by the non-availability of NS3 antigen used in a previous study (*23*). To validate the PCR, RNA transcripts were generated from a plasmid containing partial CHV NS3 cDNA by using the Ambion T7 transcription kit (Promega Corp., Southampton, UK). Transcripts were purified with the RNeasy kit (QIAGEN), and concentrations were determined by using the NanoDrop 2000 (NanoDrop Products, Wilmington, DE, USA). RNA extractions were performed on 140 μL of plasma or respiratory sample by using the QIAmp viral extraction kit (QIAGEN) according to the manufacturer›s instructions and eluted in a final volume of 60 μL. All tissue samples were homogenized in lysis buffer; RNA was extracted by using the RNeasy Mini Kit (QIAGEN) according to instructions and eluted in a final volume of 60 μL. Peripheral blood mononuclear cells were separated from whole blood immediately after collection by centrifugation on a Ficoll-Hypaque density gradient by using Histopaque 1077 according to manufacturer's instructions (Sigma Aldrich, St. Louis, MO, USA), and RNA was extracted by using QIAmp RNA blood mini kit as instructed (QIAGEN) and eluted in final volume of 100 μL.

RNA was converted to cDNA by using random hexamers with the Reverse Transcription System A3500 (Promega) and then used in nested PCR with previously published CHV NS3 primers (*22*) Chv-0F1,Chv-0R1S1, Chv-0F2, and Chv-0R2 and new equine-based NS3 primers (Table 1) and amplified by using 2 rounds of 30 cycles at 94°C for 18 s, 50°C for 21 s, and 72°C for 1.5 min; and 1 cycle of 72°C for 5 min, with 2 μL of first-round amplicon added to the second round. Degenerate equine- and canine-based NS3 primers were designed on the basis of the sequence variability observed in the published NPHV sequences and used to additionally screen all samples from dogs and equids. The CHV NS3 transcript was tested by using both NS3 primer sets and used as a control in screening, with sensitivity of 0.5–5 RNA copies in a reaction (Tables 1,2).

Table 1. Transcript titration of nonprimate hepaciviruses with NS3 primers in samples from domestic horses, United Kingdom

Transcript RNA copies/mL	Published NS3	New NS3
5×10^6	2/2	2/2
5×10^5	2/2	2/2
5×10^4	2/2	2/2
5,000	2/2	2/2
500	2/2	2/2
50	2/2	2/2
5	2/2	2/2
0.5	0/2	0/2
0	0/2	0/2

*NS, nonstructural protein.

To confirm positive results of screening, we designed degenerate primers derived from the NPHV sequences for the 5′-UTR and NS5B (Table 1). For all detected positive results, we used SuperScript III One-Step RT-PCR (Life Technologies, Paisley, UK) with 6 μL of RNA and cycling conditions as published (*23*) with 1 of the following first-round primer sets: EQ5→UTROS and EQ5→UTROAS or EQNS5BIS and EQNS5BIAS. From the first round, 2 uL was added to the second-round PCR with respective forward and reverse primer sets: EQ5→UTRIS and EQ5→UTRIAS or EQNSBIS2 and NS5BIAS2, with the following cycling conditions; 30 cycles at 94°C for 18 s, 50°C for 21 s, and 72°C for 1.5 min; and 1 cycle of 72°C for 5 min.

Positive second-round PCR amplicons were sequenced in both directions by using the inner sense and inner antisense primers used in the second round of amplification. Sequencing was executed by using Big Dye Terminator version 3.1 (Applied Biosystems, Paisley, UK) according to the manufacturer's instructions. Sequences were analyzed by using SSE version 1.0 software (*24*). Sequences obtained in this study have been assigned the GenBank accession nos. JX948116–JX948121.

Viral loads of positive samples were determined by real-time quantitative PCR and a standard calibration curve generated from a dilution series of the NS3 transcript. Dilutions were prepared from the CHV NS3 transcript from concentrations of 10^6 to 1 copy/μL; 5 μL of transcript RNA was used to generate cDNA by using random hexamers and reverse transcription. Five-microliter aliquots of cDNA were assayed in triplicate for the 3 positive samples in the same way. To quantify viral loads of positive samples, EQNS3IS and EQNS3IAS primers were used with 4 μL of cDNA in the SensiFAST SYBR Hi-ROX Kit (Bioline, London, UK) per manufacturer's instructions (Table 1) with the exception that the annealing temperature was reduced to 50°C and the extension time extended to 15 s. Samples were analyzed in triplicate and fluorescence measured by using the Rotor-Gene Q system (QIAGEN). Viral loads were read from the standard curve generated and converted to RNA copies/mL for sample volume used in extraction and elution of the RNA.

Results

Sample Screening

To investigate the frequency of NPHV infection in dogs, 46 respiratory samples collected from dogs over a 6-month period in the Edinburgh area were screened by published PCR-based screening methods (*22,23*) by using primers from the 5′-UTR and NS3 regions. An RNA transcript from the NS3 region (*23*) verified the sensitivity of the NS3-based assay to single RNA copies per amplification reaction (Table 2). All samples were negative in both genome regions (Table 3). Ninety-nine plasma samples from dogs that had a variety of clinical conditions and had been referred for virology screening, along with 15 autopsy lung, liver, and spleen samples from dogs, were additionally screened; results were uniformly negative in both regions.

Since publication of the NS3- and 5′-UTR–based PCRs (*22*), comparative sequence data from several NPHV-infected horses have become available (*23*). These data revealed sequence variability in the primer binding regions of both primer sets. We therefore redesigned the screening primers in both regions (Table 1) to accommodate this. In the 5′-UTR region, it was additionally possible to ensure that primers matched homologous regions of HCV genotypes 1–7. The new nested NS3 primers showed similar sensitivity for the NS3 transcript (Table 2). These new

Table 2. Nonprimate hepaciviruses primer sequences for 5′UTR, NS3, and NS5B in samples from horses, United Kingdom*

Primer	Position	Sequence, 5′ →3′
EQ5→UTROS	Forward outer sense	ACA YYA CCA TGT GTC ACT CCC CCT
EQ5→UTROAS	Reverse outer antisense	CYC ATG TCC TAT GGT CTA CGA GA
EQ5→UTRIS	Forward inner sense	ACA CGG AAA YGG GTT AAC CAY ACY C
EQ5→UTRIAS	Reverse inner antisense	GCC CTC GCA AGC ATC CTA TCA G
EQNS3OS	Forward outer sense	ATW TGT GAT GAR TGC CAY AGY AC
EQNS3OAS	Reverse outer antisense	TAG TAG GTB ACA GCR TTA GCY CC
EQNS3IS	Forward inner sense	TCY AAR GGT GTD AAG CTT GTT GT
EQNS3IAS	Reverse inner antisense	TGG CAG AAG YTA AGR TGY CTY CC
EQNS5BIS	Forward outer sense	AAR TGY TTT GAC TCY ACB GTC ACT C
EQNS5BOIAS	Reverse outer antisense	ACT RTG ACT RAT YGT YTC CCA ACT CG
EQNS5BIS2	Forward inner sense	CAY GAT ATA GAH ACT GAG AGR GA
EQNS5BIAS2	Reverse inner antisense	TCR TCT TCC TCR ACG CCY TTR CTG G

*UTR, untranslated region; NS, nonstructural protein.

Table 3.Nonprimate hepaciviruses sequences detected by using PCR on mammal samples, United Kingdom*

Animal/sample type	No.	NS3	5′-UTR	Published NS3	New primers, 5′-UTR
Dog					
Respiratory	53	0	0	0	0
Plasma	99	0	0	0	0
Lung	15	0	0	0	0
Liver	15	0	0	0	0
Spleen	15	0	0	0	0
Horse					
Respiratory	40	0	0	0	0
Plasma	142	3	3	3	3
Donkey					
Plasma	16	NA	NA	0	0
Cat					
Plasma	56	NA	NA	0	0
Pig					
Serum	40	NA	NA	0	0
Mouse					
Liver	61	NA	NA	0	0

*Data from *23*. NS, nonstructural protein; UTR, untranslated region; NA, not applicable.

primers were used to repeat screening of all canine respiratory, plasma, and autopsy samples that produced uniformly negative results (Table 3).

To investigate the possible infection of non-canine mammalian species, we screened available plasma/serum, respiratory, and liver samples from horses (n = 175), donkeys (n = 16), domestic cats (n = 56), pigs (n = 40), and wild mice (n = 61) by using both sets of conserved primers (Table 3). From this extended survey, 3 plasma samples from horses were positive on initial screening and confirmed positive on reextraction and reamplification in 5′-UTR and NS3 regions. PCR of samples of all types from all other studied mammalian species showed negative results.

To confirm the presence of NPHV sequences in the 3 screen-positive horses, we further amplified each sample using conserved primers in the NS5B region and comparing amplified sequences from each region with homologous regions of previously identified positive horses (Figure). Although this method does not represent a comprehensive genetic analysis, these sequence comparisons demonstrated that each of the positive horses was infected with NPHV variants distinct from the transcript-positive control and from each of the 8 previously identified infected horses in the USA. All 3 variants showed similar branching orders in each genome region, consistent with the observed lack of recombination in previous analyses (*23*).

Virologic and Clinical Examination of NPHV-positive Horses

The 3 infected horses originated in Scotland and comprised 2 geldings and 1 mare, 12–20 years of age (Table 4). Clinical records of each horse from the time of sample collection failed to identify evidence of hepatitis or systemic disease. Liver function tests provided no evidence for hepatic inflammation: γ-glutamyl transferase (GGT) and glutamate dehydrogenase were within reference range, except a mildly elevated GGT level in horse 2 (Table 5). Testing also ruled out hepatic insufficiency: bile acid lev-

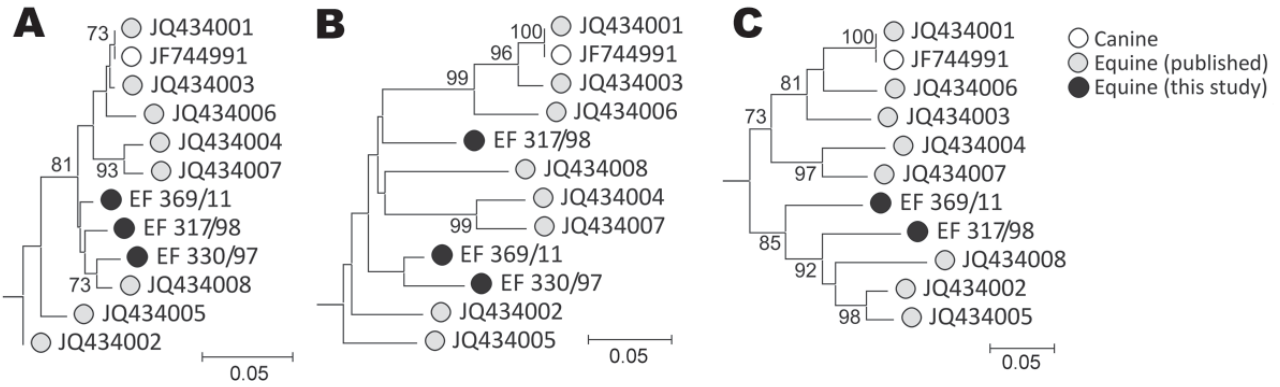

Figure. Phylogenetic analysis of A) 5′ untranslated region, B) nonstructural protein 3, and C) nonstructural protein 5B regions of nonprimate hepatitis virus sequence amplified from screen-positive study animals. Neighbor-joining trees of nucleotide sequences from each genome region were constructed from Jukes-Cantor corrected pairwise distances calculated by using the program MEGA version 5 (*25*; datasets were bootstrap re-sampled 500× to indicate robustness of branching (values ≥70% shown on branches). The hepacivirus genotype 1a sequence, M62321, was used to root the tree (not shown). Scale bars indicate nucleotide substitutions per site.

Table 4. Clinical features of domestic horses infected by nonprimate hepaciviruses, United Kingdom*

Horse	Sample	Collection date	Area	Age, y/sex	Presenting disease
1	EF_317/98	1998	Caithness	8/F	Lameness
2	EF_330/97	1997	Perthshire	12/M	Inflammatory airway disease
3	EF_369/11	2011 Dec 2	Lothian	20/M	Lameness; no lung disease
	EF_374/12	2012 Mar 1			Lameness; no lung disease
	EF_523/12	2012 Mar 23			Lameness; no lung disease

els were within reference range. Viral loads, measured by using real-time PCR against the NS3 transcript standard, ranged from 7×10^4 to 5×10^7 RNA copies/mL among the 3 horses.

To clinically characterize NPHV infection, we further examined 1 of the positive horses (horse 3) and collected samples at 4 and 5 months after the original sample collection. The horse remained clinically unremarkable and had no specific signs indicative of systemic disease. The horse regularly competed in equestrian events and had traveled extensively worldwide during the 10 years preceding detection of infection with NPHV. No specific risk factors, such as operations, exposure to unsterilized needles, or history of systemic illness were elicited by interviewing the owner. During the 5-month follow-up period, the horse remained viremic, but samples showed lower viral loads (7×10^4 to2×10^5 RNA copies/mL) than found in the initial sample (5×10^7 copies/mL) (Table 5). Although liver indices were within the reference range, liver enzymes and bile acids were frequently at the upper end of the reference range. Although of questionable clinical significance, these observations and the elevated GGT level in horse 2 are potentially consistent with low-grade hepatitis. UK veterinary rules precluded taking a liver biopsy sample from horse 3 to further investigate this.

Nasal and mouth swab samples and peripheral blood mononuclear cells collected at month 5 were NPHV negative by PCR when both sets of primers were tested. Screening plasma samples from 6 horses stabled with horse 3 were uniformly NPHV negative in 5′-UTR and NS3 by PCR.

Discussion

Although the study was designed as an investigation of the frequency of CHV infection in dogs, initial findings of uniformly negative results from a large number of respiratory, plasma, and autopsy samples prompted us to widen our sampling to other mammalian species. Consistent with a then-recent report (*23*), we found viruses similar to CHV and now termed NPHV in »3% of horse plasma samples but in no samples from other species (cats, pigs, or mice), irrespective of sample type (Table 3). The detection frequency in samples from horses 1, 2, and 3 was lower (but not significantly so by the Fisher exact test; $p = 0.06$) than the previous 8 from 103 viremia frequency among horses in New York state (*23*).

The restriction of NPHV infection to horses was consistent with serology-based screening (*23*) that showed 35% of samples from horses to contain antibodies against a recombinant NPHV NS3 peptide (of which »25% were additionally viremic) but an absence of NS3 antibodies in other species. These included dogs, deer and rabbits, although 1 intermediately seroreactive (but NPHV-negative by PCR) sample was found in 84 samples screened from cows. Whether these findings represent rare infection in another ruminant species or assay non-specificity requires further investigation. Although these initial surveys provide preliminary evidence that horses could be the natural host of NPHV, its previous detection in dogs with respiratory disease (*22*) provides evidence for its potential spread to humans. In this respect, NPHV specificity would differ from the narrow specificity observed in other hepaciviruses; e.g., HCV can infect humans and chimpanzees (although it does not naturally circulate in the latter species) and cannot infect Old World monkey species, such as macaques (*26*). The host range of GBV-B appears similarly restricted to New World monkeys (*27*). Further studies are needed to determine whether additional equally or more divergent hepaciviruses are distributed in other mammalian species; the 5′-UTR primers developed for the current study used with primers selective for regions conserved between NPHV and HCV might provide a useful assay for this purpose.

The detection of NPHV RNA sequences in samples obtained 5 months apart from horse 3 provides evidence for an ability of NPHV to establish persistent infections. Although longer term sampling is required to confirm this possibility, the observation is consistent with the high viremia frequencies among seropositive horses in a previous study (8/37) (*23*). This proportion would probably not be

Table 5. Laboratory indices for domestic horses infected with nonprimate hepaciviruses, United Kingdom

Horse	Sample	GGT (<40 U/mL)	GLDH (<10 U/mL)	Bile acids (<10 U/mL)	Viral load, copies/mL
1	EF_317/98	15	2	1	1.3×10^5
2	EF_330/97	59	2	3	4.4×10^5
3	EF_369/11	15	2	6.4	4.8×10^7
	EF_374/12	36	1	7.4	2.1×10^5
	EF_523/12	24	4	6.3	7.1×10^4

*GGT, γ-glutamyl transferase; GLDH, glutamate dehydrogenase.

observed on random sampling if infections had rapidly resolved. In this respect, NPHV may at least partly reproduce the documented high rates of HCV persistence, in which >50% of those exposed showed decades- or lifelong viremia and active liver disease in the absence of treatment. GBV-B, although clearly hepatotropic, does not establish persistent infections in tamarins or owl monkeys (*20,27,28*). However, more recent studies have demonstrated long-term persistence among experimentally infected marmosets (*29,30*).

Neither the clinical signs nor the liver function tests of the 3 NPHV-infected horses provided a clear indication of the organism's association with hepatic or other systemic disease (Tables 4, 5). GGT and glutamate dehydrogenase are sensitive markers of liver inflammatory processes in the horse but with 1 exception were in the reference range. Reference levels of bile acids similarly demonstrated adequate liver function. Although the sample size was small, these relatively normal liver indices contrast with the frequent GGT and ALT elevations associated with chronic HCV infection and found in New World monkeys experimentally infected with GBV-B. Although in the current study, UK veterinary regulations did not permit liver biopsies to be performed on horses without evidence of liver disease, the current findings do not rule out a lower grade infection or potential replication in the liver without the associated immunologic response to HCV that is primarily responsible for liver damage (*31,32*). Future studies, perhaps refocused on NPHV screening of horses with idiopathic liver disease that have undergone biopsy sampling and have been clinically characterized, are needed to investigate further the potential for hepatotropic NPHV and manifest its clinical effects. In the longer term, and acknowledging that the horse is not the ideal experimental animal, inoculation of horses with NPHV and subsequent monitoring for viremia development, liver function abnormalities, and B- and T-cell immune responses would provide further insights into the nature of NPHV infections and associated immune response and similarity of these developments to current observations for HCV and GBV-B.

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Ms Lyons is completing a PhD degree in virology in the Royal (Dick) School of Veterinary Studies at University of Edinburgh. Her research interests are focused on the evolutionary history, cross-species transmission, and host adaptation of human viruses and their primate/nonprimate homologs; including hepatitis B, GB, and hepatitis C viruses.

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Address for correspondence: Peter Simmonds, Infection and Immunity Division, Roslin Institute, University of Edinburgh, Easter Bush, Edinburgh, EH25 9RG, UK; email: peter.simmonds@ed.ac.uk

Transmission Routes for Nipah Virus from Malaysia and Bangladesh

Bronwyn A. Clayton, Deborah Middleton, Jemma Bergfeld, Jessica Haining, Rachel Arkinstall, Linfa Wang, and Glenn A. Marsh

Human infections with Nipah virus in Malaysia and Bangladesh are associated with markedly different patterns of transmission and pathogenicity. To compare the 2 strains, we conducted an in vivo study in which 2 groups of ferrets were oronasally exposed to either the Malaysia or Bangladesh strain of Nipah virus. Viral shedding and tissue tropism were compared between the 2 groups. Over the course of infection, significantly higher levels of viral RNA were recovered from oral secretions of ferrets infected with the Bangladesh strain. Higher levels of oral shedding of the Bangladesh strain of Nipah virus might be a key factor in onward transmission in outbreaks among humans.

Nipah virus (NiV), a bat-borne paramyxovirus, has caused outbreaks of human disease with high mortality rates in Malaysia, Singapore, India, and Bangladesh. Two divergent NiV strains (NiV-Malaysia and NiV-Bangladesh) share 91.8% nt sequence identity (*1*).

NiV-Malaysia emerged in 1998 during an outbreak of infectious respiratory and neurologic disease in commercially farmed pigs, presumably after virus spillover from Malaysian flying foxes (*2*). Pigs were the source of infection for farm and abattoir workers, resulting in a widespread outbreak of severe febrile encephalitic disease among humans (*3*–*5*); >250 cases were reported in Malaysia and Singapore, and the case-fatality rate approached 40% (*2*,*5*,*6*). No cases of human-to-human transmission were reported during the outbreak (*7*,*8*). However, rare instances of human-to-human transmission have been suggested by asymptomatic seroconversion against NiV-Malaysia in a health care worker, which was recognized after the outbreak (*9*), and by a recently reported case of late-onset NiV encephalitis attributed to transmission from infected family members (*10*).

NiV-Bangladesh emerged in 2001 in Bangladesh (*11*,*12*), and subsequent outbreaks of disease have occurred almost annually (*12*–*20*). Since 2001, >200 cases in humans have been identified in Bangladesh; the overall case-fatality rate is >70% (*21*). In contrast to the rare instances of human-to-human transmission of NiV-Malaysia, human-to-human transmission of NiV-Bangladesh is a major pathway for human infection (*13*).

The different transmission characteristics of NiV-Malaysia and NiV-Bangladesh might be attributable to differences in infectivity and pathogenicity of virus strains and in tissue tropism, reflected by higher incidence of respiratory disease in NiV-Bangladesh–infected patients (*14*,*21*). We assessed the role that tissue tropism and shedding characteristics of NiV-Malaysia and NiV-Bangladesh might play in clinical outcomes and increasing transmission risk. For this purpose, we used a mammalian infection model, the ferret, in which NiV causes fulminating systemic disease, with fever and neurologic and/or respiratory signs, similar to those in humans (*15*). Here we describe a ferret model for NiV-Bangladesh infection and our comparison of the characteristics of infections caused by NiV-Malaysia and NiV-Bangladesh in the ferret.

Author affiliations: Commonwealth Scientific and Industrial Research Organisation Livestock Industries, Geelong, Victoria, Australia (B.A. Clayton, D. Middleton, J. Bergfeld, J. Haining, R. Arkinstall, L. Wang, G.A. Marsh); University of Melbourne, Parkville, Victoria, Australia (B.A. Clayton); and Duke–National University of Singapore Graduate Medical School, Singapore (L. Wang)

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Materials and Methods

Animal Infection, Handling, and Housing

A total of 15 male ferrets, 12–18 months of age, were oronasally exposed to 5,000 50% tissue culture infective doses of low-passage isolates of Nipah virus from hu-

mans. Animals were randomly assigned to receive NiV-Bangladesh (n = 8, ferrets B1–B8) or NiV-Malaysia (n = 7, ferrets M9–M15). The 2 groups were housed under separate biosafety level 4 conditions as described (*17*). The specific NiV-Bangladesh isolate was Nipah Bangladesh/human/2004/Rajbari, R1, which came from the oropharynx of 1 of 12 patients infected during an outbreak of NiV encephalitis in Rajbari district, Bangladesh (*1*,*16*). The patient was a 10-year-old boy with neurologic disease and respiratory involvement characterized by coughing, wheezing, and difficulty breathing (S. Luby, pers. comm.). The specific NiV-Malaysia isolate was Nipah virus/Malaysia/human/99, which came from the cerebrospinal fluid of a patient with encephalitis. We selected a challenge dose that was expected to infect all exposed animals with NiV-Malaysia (*15*) and NiV-Bangladesh (D. Middleton, unpub. data). Ferrets were anesthetized as described before viral challenge and for subsequent sample collection (*17*). Procedures involving live animals were approved by the Commonwealth Scientific and Industrial Research Organisation, Australian Animal Health Laboratory, Animal Ethics Committee.

Animal Monitoring and Sampling

After receiving the challenge dose, animals were assessed daily for clinical signs of disease. Every 48 hours, nasal wash samples, oral and rectal swab samples, and blood (axillary vein) were collected and temperature and weight were recorded. Sampling days were staggered so that sampling of ferrets B1–B4 and M9–M11 started on 1 day postinfection (dpi) and sampling of ferrets B5–B8 and M12–M15 started on 2 dpi. Environmental samples of urine and feces were obtained daily from cage floors.

Ferrets were euthanized at predetermined humane end points as described (*15*). Clinical samples and various tissues were collected immediately before euthanasia or during postmortem examination.

Sample Collection, Processing, and Analysis

Nasal wash, swab, urine, whole blood (EDTA treated), tissue, and fecal samples were collected and processed in the same manner as tissue samples and then used for virus isolation, RNA extraction, and TaqMan reverse transcription PCR (RT-PCR; selective for the NiV N gene) as described (*17*–*19*). Samples with a mean NiV N gene cycle threshold value ≤39.1 were defined as positive for NiV RNA. For tissue samples, NiV N gene values were normalized to host cell 18S rRNA by multiplex RT-PCR as described (*22*) but by using probe (5′-VIC-TGCTGGCACCAGACTTGCCCTC-TAMRA-3′). Tissues were also processed for histopathologic and immunohistochemical examination with rabbit a-NiV N protein antiserum (*20*).

Statistical Analysis

To compare trends in virus shedding over time, we analyzed transformed RT-PCR data from nasal, oral, and rectal swab samples by using a residual maximum-likelihood (REML) model in GenStat statistical software, version 3 (VSN International, Hemel Hempstead, UK). Data were collapsed into 48-hour periods, thereby generating 4 time points for comparison: dpi 1–2, 3–4, 5–6, and 7–8. We omitted dpi 9–10 from analysis because few animals in either group survived this long. To analyze the trend of shedding over time for each virus, we fitted the interaction of virus and days to the model.

On the basis of REML analysis outcomes, we also estimated the amount of NiV-Bangladesh and NiV-Malaysia shed by individual animals over the course of infection by calculating the area under the curve (AUC) for viral RNA (by using the trapezoidal rule) for nasal wash and oral and rectal swab samples. Blood samples were similarly assessed. Estimates were transformed to the $\log_{10}$ scale, and mean AUCs for NiV-Bangladesh (n = 8) and NiV-Malaysia (n = 7) samples were compared by using an independent-samples *t* test.

At the time of euthanasia, we compared levels of viral RNA in nasal, oral, rectal, urine, and blood samples between the 2 groups by using independent-sample *t* tests of transformed data. Also at the time of euthanasia, we similarly assessed levels of viral RNA in tissue. Analysis by *t* test did not assume equal differences. All tests used were 2-sided, and $p<0.05$ was defined as statistically significant.

Results

Clinical Features of Infection

Clinical signs were those of lower respiratory tract and neurologic system infection. Clinical signs were similar for all 8 ferrets challenged with NiV-Bangladesh and for 6 of 7 challenged with NiV-Malaysia (Table 1). In 1 ferret challenged with NiV-Malaysia (ferret M11), localized bacterial lymphadenitis (confirmed by histopathologic examination) developed, and the ferret was euthanized on humane grounds at 5 dpi, at which time no clinical signs of NiV infection had been observed. The first sign of disease was pyrexia (rectal temperature ≥40°) for most animals; disease progressed rapidly to its humane end point within 72 hours of pyrexia onset.

Hemorrhage was another clinical sign, but it differed between the 2 groups. At the terminal stage of disease, NiV-Malaysia–infected animals experienced cutaneous petechial hemorrhage, accompanied by bleeding from oral, nasal, and rectal mucosa; whereas, only 1 NiV-Bangladesh–infected animal experienced bleeding (of the oral mucosa at the time of euthanasia).

Virus Loads

Viral RNA and virus isolation results are presented in Tables 2 and 3. Viral RNA was recovered from clinical samples from all animals with clinical disease, and virus was isolated from some samples. For ferret M11, viral RNA was detected in clinical samples as early as 3 dpi and in blood, indicating a productive infection after experimental challenge; data for ferret M11 were therefore included in analysis of shedding. RNA was detected in nasal, oral, or rectal samples from similar numbers of animals exposed to

Table 1. Clinical disease in ferrets after experimental infection with NiV from Bangladesh or Malaysia*

NiV type and ferret no.	Euthanasia, dpi	Resp†	Neuro	Hemorr‡	Criteria for euthanasia	Clinical signs
Bangladesh						
B1	7	–	–	–	Obtundation	Severely obtunded; hunched posture
B2	7	+	+/–	–	Respiratory +/– mild neurologic signs	Hunched posture; possible mild neurologic disease (agitation); sneezing; >10% reduction in body weight§
B3	7	+	+/–	–	Respiratory +/– neurologic signs	Possible mild neurologic disease (continuous licking, smacking lips); dehydration;¶ vomiting; rapid deterioration in clinical condition after sampling at 7 dpi
B4	7	+	+	–	Respiratory signs, neurologic signs, and obtundation	Fine tremors/myoclonus of forelimbs; nasal discharge
B5	8	–	+	–	Neurologic signs and obtundation	Hind limb myoclonus/paresis, ataxia; dehydration; periorbital/facial/ventral neck edema
B6	9	+	+	+	Respiratory signs, neurologic signs, hemorrhage, and obtundation	Forelimb myoclonus; sneezing; mucoid nasal discharge; reduced feces production; periorbital/facial edema; hemorrhage of oral mucosa at euthanasia time point; >10% reduction in body weight
B7	8	–	+	–	Neurologic signs	Myoclonus and muscular spasm affecting the tail, ataxia; ventral neck edema
B8	8	+	+	–	Respiratory signs, neurologic signs, and obtundation	Myoclonus of the flanks, ataxia, hind limb paralysis; vomiting; ventral neck edema
Malaysia						
M9	7	+	+	+	Respiratory signs, neurologic signs, hemorrhage, and obtundation	Severe ataxia, facial and hind limb tremors, head tilt and torticollis (left); sneezing; nasal discharge; facial edema; hemorrhage of rectal mucosa at euthanasia
M10	7	+	+	+	Respiratory signs, neurologic signs, hemorrhage, and obtundation	Dyspnea with prolonged expiration phase; mild ataxia; reduced feces production; facial and ventral neck edema; hemorrhage from nose and mouth at euthanasia
M11	5	NA	NA	NA	NA	Euthanasia at 5 dpi for humane reasons; no evidence of clinical disease associated with NiV infection
M12	8	–	+	+	Neurologic signs and severe hemorrhage	Spastic paralysis of right forelimb, rhythmic myoclonus of right trunk, ataxia; sneezing; nasal discharge; facial edema; extensive cutaneous petechial hemorrhages and facial bruising
M13	9	+	+/–	+	Respiratory signs, hemorrhage, and obtundation	Mild neurologic disease (hind limb paresis) at 6 dpi but not apparent at euthanasia; nasal discharge; facial edema; inappetence; >10% reduction in body weight
M14	8	–	+	+	Neurologic signs, hemorrhage, and obtundation	Hunched posture; spastic paralysis of hind limbs, fine muscular fasciculations over flanks, ataxia; hunched posture; dehydration; nasal discharge; cutaneous petechial hemorrhages; >10% reduction in body weight
M15	10	–	+	+	Neurologic signs, hemorrhage, and obtundation	Sporadic hind limb myoclonus; recumbency; nasal discharge; cutaneous petechial hemorrhages and hemorrhage from mouth; >10% reduction in body weight

*NiV, Nipah virus; dpi, days postinfection; resp, respiratory involvement; neuro, neurologic involvement; hemorr, hemorrhage; +/–, vague clinical signs that might indicate neurologic involvement; NA, not applicable.
†Increased respiratory effort and/or rate unless otherwise stated under clinical disease.
‡Cutaneous hemorrhages or frank hemorrhage from oral, nasal, and rectal mucosa.
§Based on weight data collected before experimental infection.
¶Based on the observation of reduced skin turgor at physical examination.

NiV-Malaysia or NiV-Bangladesh (by linear mixed-model analysis; data not shown). Mean levels of viral RNA in clinical samples increased throughout the course of infection and were highest at 7–8 dpi, the time of onset of severe clinical disease for most animals (Figure 1). In 4 NiV-Bangladesh–infected animals and all NiV-Malaysia–infected animals, viral RNA was detected in nasal secretions at least 24 hours before it was detected in blood and/or before onset of pyrexia.

Levels of viral RNA were significantly higher in oral secretions from NiV-Bangladesh–infected than from NiV-Malaysia–infected animals; predicted mean viral RNA levels were at least 10-fold higher in the NiV-Bangladesh–infected group at 5–6 and 7–8 dpi (Figure 1; REML analysis; $p = 0.038$ for virus by days), and mean AUC was >30-fold higher (Figure 2; $p = 0.001$; $t_{12} = 4.3$). However, the rate of virus isolation from oral swab samples did not differ significantly between infection groups (Z test of 2 proportions; data not shown). NiV-Bangladesh was isolated from oral swab samples from ferrets B5 (4 dpi and at euthanasia), B6 (6 dpi), and B7 (4 and 6 dpi). Oral swabs from ferrets infected with NiV-Malaysia yielded isolates in ferret M9 at 3 dpi and in ferrets M10, M13, M14, and M15 at euthanasia (Table 3).

Table 2. Viral RNA and virus isolation results from ferrets experimentally infected with Nipah virus, Bangladesh strain*

Ferret no., sample	Virus in shedding samples and blood over time, RNA/virus isolation†									
	Dpi 1	Dpi 2	Dpi 3	Dpi 4	Dpi 5	Dpi 6	Dpi 7	Dpi 8	Dpi 9	Dpi 10
B1										
NW	–		Indet		+/–‡		+/+			
OS	–		–		–‡		+/–			
RS	–		Indet		–‡		+/–			
Blood	–		+/‡		–‡		+/§			
B2										
NW	–		+/–		+/–		+/+			
OS	–		–		+/–		+/–			
RS	–		–		–		+/–			
Blood	–		–		+/+		+/§			
B3										
NW	–		+/–		–‡		+/–			
OS	–		–		–‡		+/–			
RS	–		–		–‡		+/+			
Blood	–		Indet		–‡		+/§			
B4										
NW	–		+/–		+/–		+/+‡			
OS	–		–		+/–		+/–‡			
RS	–		–		–		–‡			
Blood	–		–		–		+/§			
B5										
NW		–		+/–		+/+		+/+		
OS		–		+/+		+/–		+/+		
RS		Indet		–		–		+/–		
Blood		Indet		–		+/+		+/+		
B6										
NW		–		–		+/–‡		NS	+/+	
OS		–		–		+/+‡		NS	+/–	
RS		–		–		–‡		NS	+/–	
Blood		–		–		+/+‡		NS	+/–	
B7										
NW		–		+/–		+/+		+/+‡		
OS		–		+/+		+/+		+/–‡		
RS		–		–		–		+/+‡		
Blood		+/+		–		+/–		+/+‡		
B8										
NW		–		–		–‡		+/+		
OS		–		–		+/–‡		+/–		
RS		–		–		–‡		+/+		
Blood		–		–		+/+‡		+/+		

*Dpi, days postinfection; NW, nasal wash; –, Nipah virus not detected by reverse transcription PCR (RT-PCR) for N gene; therefore, virus isolation was not attempted for this sample; empty cells represent days on which sampling was not scheduled for that animal; Indet, mean cycle threshold value >39.1 (the defined cutoff value for positive samples); +/–, virus detected by RT-PCR but negative by virus isolation; +/+, sample positive by RT-PCR and virus isolation; OS, oral sample; RS, rectal sample; NS, not sampled.
†RNA detection by RT-PCR.
‡Pyrexia first detected.
§Virus isolation not attempted for this sample.

Table 3. Viral RNA and virus isolation results from ferrets experimentally infected with Nipah virus, Malaysia strain*

Ferret no., sample	Virus in shedding samples and blood over time, RNA/virus isolation†									
	Dpi 1	Dpi 2	Dpi 3	Dpi 4	Dpi 5	Dpi 6	Dpi 7	Dpi 8	Dpi 9	Dpi 10
M9										
NW	–		+/–		+/–‡		+/+			
OS	–		+/+		–‡		+/–			
RS	–		–		+/+‡		+/–			
Blood	–		–		+/–‡		+/+			
M10										
NW	–		+/–		+/+‡		+/+			
OS	–		–		–‡		+/+			
RS	–		–		+/–‡		+/–			
Blood	–		–		–‡		+/+			
M11										
NW	–		+/–		+/+					
OS	–		–		+/–					
RS	–		–		–					
Blood	–		–		+/+					
M12										
NW		+/+		+/+‡		+/–		+/+		
OS		–		–‡		Indet		+/–		
RS		–		–‡		+/–		+/+		
Blood		–		–‡		+/+		+/+		
M13										
NW		–		+/–		+/+‡		NS	+/+	
OS		–		–		–‡		NS	+/+	
RS		–		–		+/+‡		NS	+/–	
Blood		–		–		–‡		NS	+/+	
M14										
NW		–		+/–		+/+‡		+/+		
OS		–		–		+/–‡		+/+		
RS		–		–		–‡		+/–		
Blood		–		–		+/+‡		+/+		
M15										
NW		–		+/–		+/+		+/–‡		+/+
OS		–		–		+/–		–‡		+/+
RS		–		–		+/–		+/§		+/–
Blood		–		–		+/+		+/+‡		+/+

*Dpi, days postinfection; NW, nasal wash; –, Nipah virus not detected by reverse transcription PCR (RT-PCR) for N gene; therefore, virus isolation was not attempted for this sample; empty cells represent days on which sampling was not scheduled for that animal; +/–, virus detected by RT-PCR but negative by virus isolation; +/+, sample positive by RT-PCR and virus isolation; OS, oral sample; RS, rectal sample; Indet, mean cycle threshold value >39.1 (the defined cutoff value for positive samples); NS ,not sampled
†RNA detection by RT-PCR.
‡Pyrexia first detected.
§Virus isolation not attempted for this sample.

Nasal shedding of viral RNA for both viruses over time was higher than oral and rectal shedding (Figure 1). However, the shedding trend over time was similar between the 2 groups, and no significant difference was found between the mean AUCs (data not shown).

Rectal shedding of viral RNA was observed for most animals with clinical disease in both groups. Predicted means for rectal shedding were higher for the NiV-Malaysia–infected group at 5–6 and 7–8 dpi; for the NiV-Bangladesh–infected group, detection of viral RNA in rectal swab samples was delayed until 7–8 dpi (Figure 1; REML analysis $p = 0.006$ for virus by days). However, the total amount of viral RNA shed in rectal swab samples over the course of infection did not differ significantly between the 2 viruses (by AUC analysis; data not shown).

Viral RNA was generally detected in blood from 5 dpi on (Tables 2, 3). AUC analysis did not demonstrate a difference between the groups in total viral RNA in blood over the course of infection (data not shown).

Urine collection was rarely achieved by manual bladder expression during the course of infection. However, at the time of euthanasia, small volumes of urine were collected from all animals in the NiV-Bangladesh–infected group and from 5 of 6 animals in the NiV-Malaysia–infected group. The rate of detection of viral RNA and isolation of virus in urine was similar for each group (data not shown).

Environmental urine and fecal samples from both groups were NiV positive by RT-PCR and by virus isolation over the course of infection from as early as 3 dpi for 1 cage in the NiV-Malaysia–infected group (Table 4). The rate of detection and isolation of NiV from environmental samples was highest at 7 dpi, coinciding with onset of severe clinical disease in most animals.

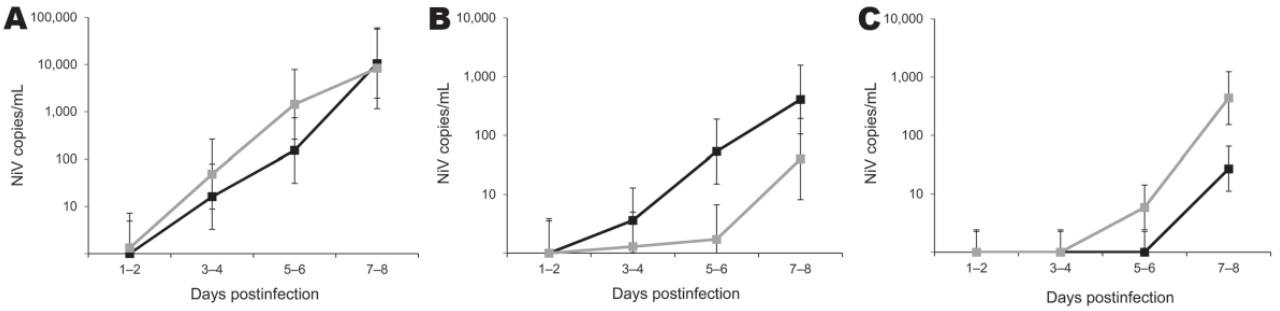

Figure 1. Predicted means for detection of Nipah virus (NiV) RNA in nasal wash samples (A), oral swab samples (B,) and rectal swab samples (C) from experimentally infected ferrets over time, based on residual maximum-likelihood analysis. Black line, NiV-Bangladesh; gray line, NiV-Malaysia. NiV N gene copies per milliliter of sample were calculated from reverse transcription data, then the transformation $\log_{10}(x_1 + 780)$ was calculated, where x_1 = NiV gene copies per milliliter. Values were fitted in the residual maximum-likelihood model by using transformed data and are plotted as values relative to the original scale (y-axis; logarithmic scale). Error bars represent approximate upper and lower limits for 95% CIs for the mean (calculated as mean ± 2 SE relative to the transformed scale).

Histopathologic and Immunohistochemical Findings

NiV-Bangladesh

The main histopathologic findings for ferrets from both groups are summarized in Table 5. In ferrets exposed to NiV-Bangladesh, multisystemic inflammatory lesions developed, most consistently affecting the upper and lower respiratory tract, lymphoid tissue, kidneys, and liver. Lesions comprised mild to severe acute necrotizing rhinitis affecting olfactory and respiratory epithelium, focal necrotizing bronchoalveolitis, and marked lymphadenitis (most notably involving the submandibular and retropharyngeal lymph nodes, caudal cervical lymph nodes, and associated peritracheal and periesophageal lymph vessels). Tonsillitis and nasopharyngitis were also noted. In some animals, lymph node lesions were confined to subcapsular and cortical regions; in others, the entire nodal architecture was effaced. There was also glomerular necrosis with hyaline tubular casts; focal proximal renal tubular necrosis and interstitial nephritis; focal adrenal, splenic, and hepatic necrosis; and mild esophagitis and tracheitis. Vasculitis was detected in nasal submucosae, lungs, lymph nodes, spleen, and testes; syncytial cells of epithelial (bronchiole, renal tubule), endothelial (lymph node, testis), and unknown derivation (spleen, lymph node) were also identified. Viral antigen was found in tissues from each animal, including tonsillar (Figure 3, panel A) and nasopharyngeal (Figure 3, panel B) epithelium; vascular endothelium; syncytia; foci of inflammation in lung, bronchial, and bronchiolar epithelium; necrotic areas within lymphoid tissues and adrenal glands; necrotic glomeruli and renal tubular cells; necrotic hepatic acinar tissue; and the esophageal exudate from 1 animal. Within the nasal cavity, viral antigen was identified not only in respiratory and olfactory epithelium but also adjacent to submucosal nerve fibers in 2 animals (Figure 4). Mild testicular degeneration was observed in all animals and was attributed to fever.

Although encephalitis was not detected in any animal, viral antigen was found in endothelial cells within brain parenchyma of 7 and within meninges of 4 of these. Antigen was occasionally detected in neurons and glial cells adjacent to affected capillaries (Figure 5), consistent with hematogenous spread.

NiV-Malaysia

In ferrets exposed to NiV-Malaysia, multisystemic inflammatory disease developed as described (*15*), which was generally similar to that observed in ferrets exposed to

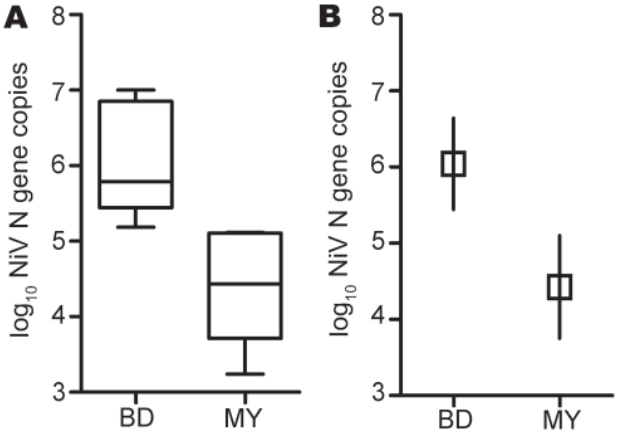

Figure 2. Oral shedding of Nipah virus (NiV) in experimentally infected ferrets. A) Results of viral RNA area under the curve (AUC) calculation. Lower margin, inner line, and upper margin of the boxes represent 25th percentiles, medians, and 75th percentiles, respectively. Whiskers show maximum and minimum values for each group. B) Comparison of mean AUC between NiV-Bangladesh and NiV-Malaysia. Mean AUC for the NiV-Bangladesh infection group was significantly higher than that for the NiV-Malaysia–infected group; p = 0.001. Interval bars represent 95% CIs for the means. BD, Bangladesh; MY, Malaysia.

Table 4. NiV in environmental samples after experimental infection of 15 ferrets*

Virus strain, cage no. (ferret no.)	log_{10} NiV copies/mL†																			
	Dpi 1		Dpi 2		Dpi 3		Dpi 4		Dpi 5		Dpi 6		Dpi 7		Dpi 8		Dpi 9		Dpi 10	
	U	F	U	F	U	F	U	F	U	F	U	F	U	F	U	F	U	F	U	F
Bangladesh																				
1 (B1, B2)	–	–	–	–	–	–	–	–	–	–	–	–	5.1‡	–	NA	NA	NA	NA	NA	NA
2 (B3, B4)	–	–	–	–	–	–	–	–	NS	NS	–	–	5.2‡	4.1	NA	NA	NA	NA	NA	NA
3 (B5, B6)	–	–	–	–	–	–	–	–	–	–	–	–	4.1	3.4	4.6‡	–	5.3‡	4.6	NA	NA
4 (B7, B8)	–	–	–	–	–	–	–	–	–	–	–	–	3.6	4.3	–	4.9‡	NS	–	NA	NA
Malaysia	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	NA	NA
5 (M9)	–	–	–	–	–	–	–	–	–	–	–	–	4.9‡	–	NA	NA	NA	NA	NA	NA
6 (M10, 11)	–	–	–	–	–	3.6‡	–	–	–	–	–	–	4.9‡	–	NA	NA	NA	NA	NA	NA
7 (M12, 13)	–	–	–	–	–	–	–	–	–	–	5.1‡	–	–	–	4.9‡	–	–	–	NA	NA
8 (M14, 15)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4.6‡	–	3.6	–	–	–

*NiV, Nipah virus; dpi, days postinfection; U, urine; F, feces; –, negative; NA, not applicable because cage was empty after euthanasia of ferrets; NS, no sample available.
†Calculated from standard curve generated for NiV N gene copies by reverse transcription PCR. Samples with mean cycle threshold ≤39.1 (based on duplicate reactions) were defined as NiV positive.
‡Sample was also NiV positive by virus isolation.

NiV-Bangladesh. Mild myocarditis was found in 1 animal and cholecystitis was found in another. Unlike findings in NiV-Bangladesh–infected ferrets, focal hepatic necrosis was found in only 1 NiV-Malaysia–infected ferret, a difference that was statistically significant (p = 0.001, Fisher exact test) but of uncertain pathogenic relevance. Mild nonsuppurative meningitis was found in 2 of 7 animals, and vasculitis in the choroid plexus was found in 1 of these. NiV antigen was identified in meningeal endothelial cells of 5 ferrets; in 3 of these 5 ferrets, it was found in the choroid plexus (Figure 6, panel A), ependyma (Figure 6, panel B), parenchymal vascular endothelium, and adjacent neurons and glia, consistent with hematogenous spread.

Virus in Clinical Samples and Tissues at Euthanasia

Because ferret M11 did not fulfill the defined criteria for reaching humane end point attributable to NiV infection, euthanasia data for this animal were omitted from statistical analysis. Viral RNA levels in oral swab, nasal wash, and rectal swab samples and urine collected at euthanasia were comparable between ferrets infected with NiV-Bangladesh or NiV-Malaysia (by *t* test; data not shown). All animals with clinical disease had detectable viral RNA in blood at euthanasia. Mean viral load in blood at euthanasia was 10-fold higher in the NiV-Malaysia–infected group (p = 0.008; t_{12} = 3.2; difference between means: $10^{1.1}$ [95% CI $10^{0.3}$–$10^{1.8}$]). Viral RNA was detected in virtually all tissues examined from all animals with clinical disease, and levels were generally higher in tissues from the NiV-Malaysia–infected group; this difference was significant for tissue from the olfactory pole of the brain, the nasal turbinates, pharynx, retropharyngeal lymph nodes, spleen, and bladder (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0875-Techapp.pdf) and was attributed to the higher RNA levels in blood in this group at euthanasia. Although rectal shedding was detected in most animals over the course of clinical disease, virus reisolation from the jejunoileum was not successful. Virus was reisolated from rectal tissues from 1 of 6 and from 3 of 5 animals positive for viral RNA in the NiV-Bangladesh and NiV-Malaysia infection groups, respectively.

Discussion

We compared the characteristics of viral shedding and tissue tropism between NiV-Malaysia and NiV-Bangladesh in the ferret model to examine whether these characteristics might contribute to observed differences in the clinical outcome and transmission of disease during outbreaks among humans. We found that viral shedding by nasal, oral, rectal, and urinary routes occurred in ferrets infected with both strains, as has been reported for outbreaks among humans (*23*), and we found that levels of viral genome over time were significantly higher in oral secretions from ferrets infected with NiV-Bangladesh than with NiV-Malaysia.

Table 5. Histopathologic and immunohistochemical findings in major systems of ferrets infected with NiV from Bangladesh or Malaysia*

System, predominant lesion	No. animals with lesion/antigen/vasculitis†	
	NiV-Bangladesh, n = 8	NiV-Malaysia, n = 7
Respiratory		
Acute rhinitis	7/5/1	6/7/0
Acute bronchoalveolitis	8/8/1	7/7/4
Lymphoid		
Lymphadenitis	8/8/1	7/7/0
Splenic necrosis	7/7/1	6/7/1
Renal, glomerular necrosis	8/6/0	6/7/1
Hepatic, focal hepatic necrosis	8/7/0	1‡/2/0
Central nervous		
Meningitis	0/4/0	2/5/1
Encephalitis	0/7/0	0/3/0

*NiV, Nipah virus.
†No. animals in which the predominant lesion was observed/antigen staining was observed in that organ or tissue/vasculitis was present in that organ or tissue. For all animals, vasculitis was associated with antigen staining in blood vessel endothelium or tunica media.
‡This animal also had cholecystitis with associated viral antigen.

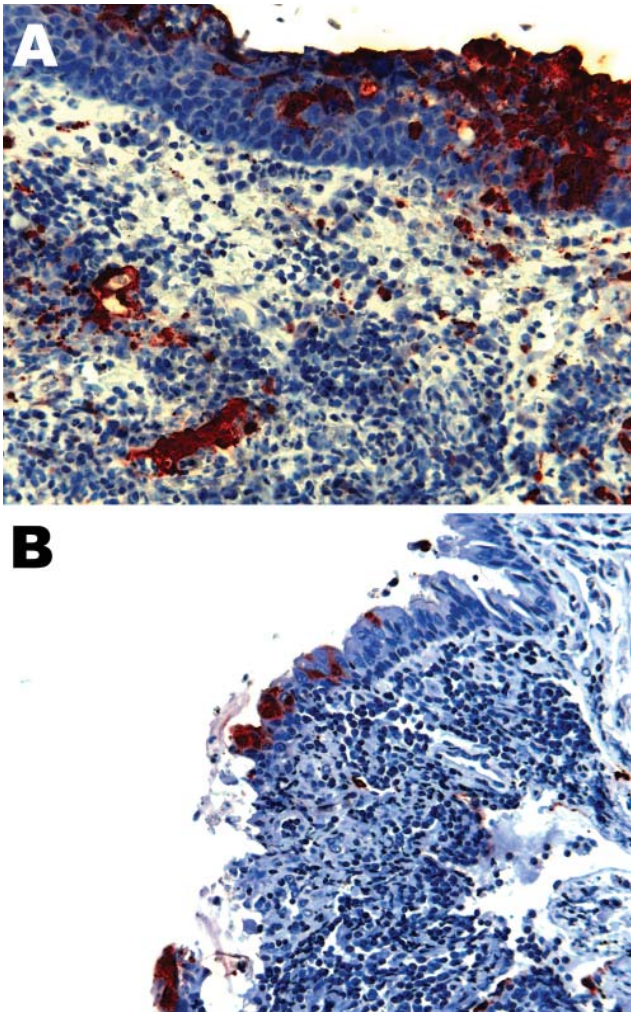

Figure 3. Nipah virus (NiV) antigen in acutely inflamed tonsillar tissue and overlying epithelium (A) and nasopharyngeal epithelium (B) in 2 ferrets infected with NiV-Bangladesh. Rabbit a-NiV N protein antiserum. Original magnification ×200. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/12/12-0875-F3.htm).

Although this finding was not reflected by a higher rate of virus isolation in that group, an observation that was attributed at least in part to the relative insensitivity of virus isolation assay compared with RT-PCR (*24*), increasing viral RNA over time was consistent with active virus replication in the oropharynx of these animals. Accordingly, this finding in oral secretions suggests that strain differences in replication at sites relevant to transmission might occur.

Although, to our knowledge, data on viral shedding in humans over the course of infection have not been reported, epidemiologic studies identified exposure to infectious saliva or respiratory secretions from patients as a major risk factor for human-to-human transmission of NiV-Bangladesh (*13*,*25*,*26*). Lower respiratory tract involvement and associated signs, including coughing, are more commonly reported for humans infected with NiV-Bangladesh than with NiV-Malaysia and have been suggested as a contributing factor in the higher likelihood of transmission from patients so affected (*21*). We did not observe differences between the 2 strains in the form of viral antigen distribution, lesion distribution and severity, or levels of viral RNA in the oropharynx or lower respiratory tract at the time of advanced clinical disease that would offer an immediate explanation for the increased oral shedding of NiV-Bangladesh. It might be that a higher level of oral shedding of NiV-Bangladesh reflects additional, more extensive, or more efficient viral replication in the oropharynx or lower respiratory tract earlier in the infection process that is later masked by fulminant NiV infection. In addition, our criteria for euthanasia might not have reflected a consistent biological time point in the infection process with each strain. More in vivo studies of viral infection of the oropharynx and lower respiratory tract, particularly soon after exposure, are warranted to explore these points further. Differences in infection and replication efficiency between virus strains might also be elucidated by in vitro comparisons of NiV-Bangladesh and NiV-Malaysia replication kinetics in respiratory cell lines.

It is noteworthy that with both NiV strains, shedding was observed in nasal wash and oral swab samples before the onset of pyrexia, as has been reported for hamsters infected with NiV-Malaysia (*27*). This finding suggests risk for transmission during the incubation period and before hematogenous virus spread.

Isolation of virus and detection of viral RNA from rectal swab samples from animals in both infection groups

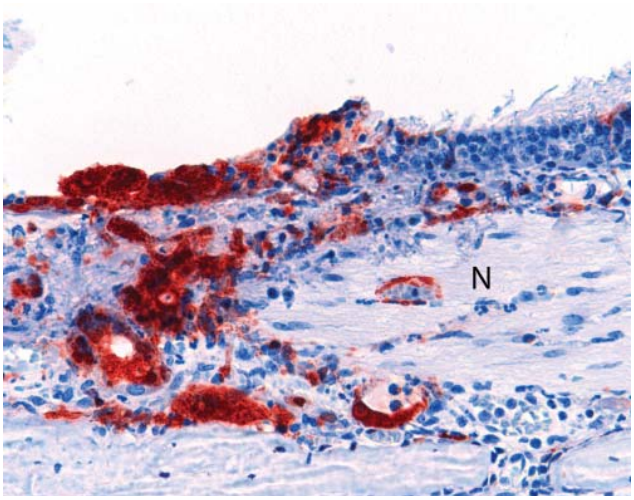

Figure 4. Olfactory epithelium of a ferret infected with Nipah virus (NiV)-Bangladesh. NiV antigen was observed in close association with submucosal nerve fibers (N). Rabbit a-NiV N protein antiserum. Original magnification ×200. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/12/12-0875-F4.htm).

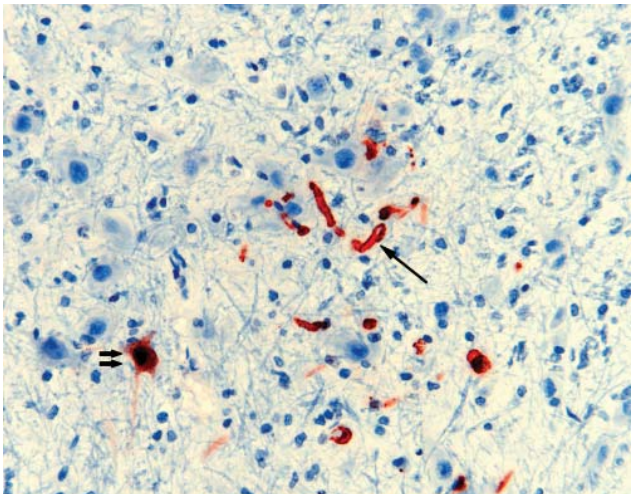

Figure 5. Nipah virus (NiV) antigen in neuron (double arrows) and capillary endothelia (single arrow) of a ferret experimentally infected with NiV-Bangladesh. Rabbit a-NiV N protein antiserum. Original magnification ×200. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/12/12-0875-F5.htm).

supports the potential for oral–fecal NiV transmission with a comparatively higher risk for NiV-Malaysia during terminal disease. Because rectal shedding typically occurred later in the course of infection and was not associated with viral localization in the gastrointestinal tract at euthanasia, it was attributed to effusion of blood-borne virus from compromised gastrointestinal tract vasculature. The higher mean levels of viral RNA in blood at euthanasia in the group infected with NiV-Malaysia are of uncertain pathogenic significance, but it is noteworthy that increased severity of hemorrhagic diathesis was observed in this group. Thrombocytopenia and gastrointestinal bleeding have been reported for some humans with advanced NiV-Malaysia infection (*28–30*), and a hemorrhagic syndrome has been observed in green monkeys (*24*) and ferrets (*18*) after infection with NiV-Malaysia. In our study, animals with hemorrhagic disease tended to be those that had reached their end points for euthanasia late (9 dpi in the NiV-Bangladesh–infected group and ≥8 dpi for 4 of the 6 animals in the NiV-Malaysia–infected group), and they might have had more prolonged endothelial infection.

We were unable to assess shedding in urine over time, but virus was reisolated from urine collected at euthanasia from ferrets infected with NiV-Bangladesh and NiV-Malaysia. This finding is consistent with findings for NiV-Malaysia–infected human patients (*23*). Virus reisolation from urine and feces collected from cages containing infected animals in both groups also suggests that environmental contamination might pose an infection risk.

Viral antigen was observed in neurons and glia and in meningeal and parenchymal vascular endothelium of animals in both groups. This finding is consistent with the dual mechanism proposed for the pathogenesis of NiV neurologic disease in humans, namely, direct cellular dysfunction resulting from neuronal infection and vasculitis-associated ischemic injury to the brain (*2*,*31–33*).

Our observation that oropharyngeal shedding occurred at higher levels in NiV-Bangladesh–infected ferrets suggests a mechanism for the higher risk for human-to-human transmission that is observed for this NiV strain in the field, although the mechanism for enhanced shedding of NiV-Bangladesh has not yet been elucidated. However, it is recognized that heterogeneity of NiV-Bangladesh isolates (*1*,*34*) is more substantial than has been observed for NiV-Malaysia isolates. It would be of value to compare the shedding characteristics of the NiV-Bangladesh isolate

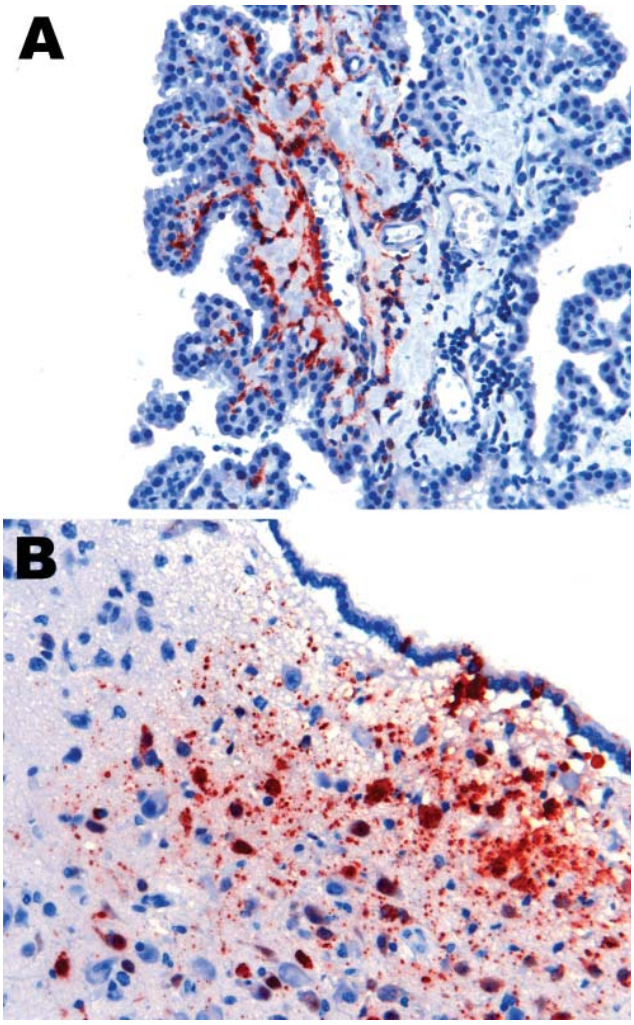

Figure 6. Nipah virus (NiV) antigen in ferret infected with NiV-Malaysia. A) Choroid plexus endothelium. B) ependymal epithelium and subependymal tissue, including neurons. Rabbit a-NiV N protein antiserum. Original magnification ×200. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/12/12-0875-F6.htm).

studied here (from an outbreak in which human-to-human transmission was not observed) (*16*) with characteristics of isolates obtained during outbreaks with differing epidemiologic features. Transmission of NiV-Malaysia has been recently described in the hamster model (*35*), but the application of this observation to enhancing infection control with human NiV-Bangladesh is unclear, and natural transmission of NiV-Malaysia among humans occurs at low frequency (*9*,*10*). Our observations for ferrets support the view that although transmission of NiV-Malaysia between humans is possible, an increased propensity for oral shedding of NiV-Bangladesh (of pharyngeal or lower respiratory tract origin) within the context of social environmental factors in play during outbreaks of human disease leads to a higher incidence of human-to-human transmission of NiV-Bangladesh. Whether increased oral shedding of NiV-Bangladesh is predictive for increased transmissibility under controlled conditions in an animal model remains to be seen. In addition to time-course studies, in vivo studies that simulate various levels of interaction between infected and in-contact animals are warranted. On the basis of the virus shedding reported here for the ferret model, we propose that the ferret is a suitable human surrogate for further investigation of NiV transmission.

Acknowledgments

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Dr Clayton is a postgraduate student at the Australian Animal Health Laboratory, Commonwealth Scientific and Industrial Research Organisation, Livestock Industries, enrolled in a PhD program through the University of Melbourne. Her research interests include viral pathogenesis and emerging viral diseases.

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Address for correspondence: Glenn A. Marsh, CSIRO Livestock Industries, Australian Animal Health Laboratory, 5 Portarlington Rd East, Geelong, Victoria 3219, Australia; email: glenn.marsh@csiro.au

Virulent Avian Infectious Bronchitis Virus, People's Republic of China

Jinling Feng, Yanxin Hu, Zhijun Ma, Qi Yu, Jixun Zhao, Xiaodong Liu, and Guozhong Zhang

A virulent avian infectious bronchitis virus (IBV) was isolated from 30-day-old broiler chickens that exhibited respiratory symptoms, nephropathologic lesions, and a high proportion of deaths in the People's Republic of China during 2005. The strain, designated YN, was genetically and pathologically characterized. Phylogenetic analysis showed that YN and most of the previously characterized IBV isolates found in China were phylogenetically classified into 2 main genetic clusters. The YN isolate caused severe lesions and resulted in deaths of 65% in experimental infections of 30-day-old specific-pathogen–free chickens. Tracheal and severe kidney lesions developed in all infected birds, confirming the ability of YN strain to induce both respiratory and renal disease. IBV antigens were detected by immunohistochemical analysis in the trachea, lung, kidney, and bursa, consistent with histopathologic observations, virus isolation, and reverse transcription PCR detection. We showed that YN IBV exhibits severe pathogenicity in chickens, and that similar viruses are prevalent in China.

Avian infectious bronchitis virus (IBV), a member of family *Coronaviridae,* order Nidovirales, causes a highly contagious respiratory and sometimes urogenital disease of chickens that is characterized by respiratory signs, nephritis, or reduced egg production and quality in layer chickens. IBV is a major poultry pathogen that is endemic worldwide and leads to serious economic losses.

The main method of protecting poultry from infectious bronchitis (IB) is the administration of live or killed vaccines. However, IB continues to cause economic losses in the poultry industry despite intensive vaccination programs in many countries (*1*–*5*). Outbreaks of IB often are due to infections with strains serologically different from those used for vaccination (*2*,*6*). Since IBV was first described in 1931, a large number of serotypes or variants have emerged, and some have become endemic worldwide (*4*,*7*–*12*). That little or no cross-protection occurs between different serotypes of IBV is well known (*13*). Therefore, continuous determination of the epidemic serotype and production of new generations of vaccines are crucial for controlling IB in each geographic region or country.

IBV was first isolated in the People's Republic of China in the early 1980s; since then, the Massachusetts-type (e.g., H120, H52, Ma5, W93, and 28/86) or Connecticut-type live attenuated IB vaccines and the inactivated killed oil-emulsion vaccine have been used to prevent and control the disease. However, the serotypes of the vaccines used have been epidemiologically determined to differ from those of the predominant IBV isolates in China that form 2 large groups of unique strains, named A2-like and QXIBV-like strains (*5*,*6*,*14*–*18*). Therefore, because of the lack of a vaccine against endemic strains of IBV in China, IBV infection has remained a problem in the Chinese poultry industry.

We isolated a virulent IBV strain from 30-day-old broiler chickens in the Yunnan Province and characterized it using sequence alignment, phylogenetic analysis, pathogenicity studies, histopathologic observation, and immunohistochemical (IHC) examination. The results indicate that the isolate is genetically similar to most of the prevalent strains of IBV found in China. We also showed that IBV YN isolate displays more severe pathogenicity than previously characterized strains in China.

Methods

Virus and Animals

The YN strain was isolated in 2005 from 30-day-old broilers that had been inoculated oculonasally with H120

Author affiliations: The Key Laboratory of Animal Epidemiology and Zoonosis, Ministry of Agriculture, China Agricultural University, Beijing, People's Republic of China (J.L. Feng, Y.X. Hu, J.X. Zhao, G.Z. Zhang) and Animal Husbandry and Veterinary Station of Beijing, Beijing (Z.J. Ma, Q. Yu, X.D. Liu)

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vaccines of IBV on days 1, 7, and 21, respectively. The sick birds had respiratory signs, severe renal disease, and a death rate of 30%. The virus was propagated in 10-day-old specific-pathogen–free (SPF) embryonated chicken eggs at 37°C for 40 h. Allantoic fluid was recovered from infected eggs and stored at −80°C. All animal research was approved by the Beijing Administration Committee of Laboratory Animals under the leadership of the Beijing Association for Science and Technology (approval ID is SYXK [Beijing] 2007–0023).

Viral RNA Extraction, Reverse Transcription PCR, and DNA Sequencing

On the basis of IBV nucleotide sequences (M41, A2, BJ, ZJ971, and SC021202; GenBank accession nos. DQ834384, AY043312, AY319651, AF352313, and AY237817, respectively), 22 pairs of specific primers (Table 1) were designed to amplify the complete genome (excluding the 5′- and 3′-terminal segments) of the YN strain. Viral RNA was extracted from virus-infected allantoic fluid by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription (RT) was performed at 37°C for 1 h by using 3 μg total RNA, 1 μL random primers (500 μg/mL, random hexadeoxynucleotides; Promega, Madison, WI, USA) and 0.5 μL M-MLV RT (200 U/μL) (Promega). For PCR, 1 U Taq DNA Polymerase (Promega) and 10 pmol of each primer were added to 100 ng cDNA as template in a total of 20 μL reaction volume. PCR was performed at 95°C for 5 min, followed by 35 cycles of denaturation (95°C, 45 s), annealing (53°C or 55°C, 45 s), and polymerization (72°C, 2 min), and the postpolymerization step was performed at 72°C for 10 min. Amplified sequences were analyzed by 1.2% agarose gel electrophoresis.

The amplified DNA products were purified by using an AxyPrep DNA Gel Extraction Kit (AxyGEN, Union City, CA, USA), and the purified products were ligated to the pMD18-T Easy Vector system (Promega). Recombinant plasmids were extracted from positive clones by using the E.Z.N.A.R. Plasmid Miniprep Kit (Omega, Norcross, GA, USA) and identified by *Eco*RI restriction digestion (Promega). Nucleotide sequencing reactions were performed by Sunbio Biotech (Bejjing, China).

Sequence and Phylogenetic Analysis

Complete genome or S1 gene sequences of IBV were obtained from GenBank, and these IBV sequences and the complete coding sequence of the IBV YN isolate were aligned and analyzed by using the ClustalW multiple alignment algorithm in the MegAlign program of the DNASTAR software suite (version 3.1; DNAstar, Madison, WI, USA).

The phylogenetic tree of S1 gene or complete genomic sequences was constructed by using MEGA4.0 software (www.megasoftware.net) by the neighbor-joining method (1,000 bootstrap replicates). Evolutionary distances were computed by the pairwise distance method using the maximum composite likelihood model (*19*).

Clinicopathologic Assessment in Chickens

Forty 30-day-old SPF white leghorn chickens were randomly divided into 2 groups of 20 birds each. All birds in 1 group were challenged intranasally with at least 10^5 50% egg infectious dose per 0.2 mL of IBV YN strain. Birds

Table 1. Primers used to amplify the complete genomic sequence of avian infectious bronchitis virus YN strain, People's Republic of China

Primer*	Location, bp	Upstream primer	Downstream primer	Length, bp
1	22–1,475	ATATCATACATACTAGCCTTG	AGTTAAGTCGTTTCGCATG	1,454
2	1,302–2,411	CCAACTGGTTTTATGGGTGC	TGGAAGTGTCACTGCCTCG	1,110
3	2,240–3,784	ACTTGGTAGAGTTTCTGGGG	TTGACATACGAAGGTGTGACA	1,545
4	3,635–5,161	TGTAAACGCCGCAAATGAG	GGCAACTTGGAATCCTCTT	1,527
5	5,040–6,636	GATCTTACTGACTTTGAAC	CACTGAAACACTTAACTG	1,597
6	6,424–7,936	GGAATTGTGACGAGTATGG	TGAAAGTATACCCTATGAGGA	1,513
7	7,746–9,246	GAAATTGTCGGTTACACTCAG	TAGAACGCATAGTAACAGGG	1,501
8	9,130–9,985	CTAACGCTGAAACTCCA	GTTGGCAATAGGAAAGTA	856
9	9,082–10,645	TCAGTAGGCGGATAAAAGG	ATAGGCAACACACGGTCG	1,564
10	10,516–12,092	ATTTCAAAGTTTCGGTTGACC	CACAGAAGCTCCTCCATAG	1,577
11	11,861–13,471	GTTTTACAATCTAAAGGTCAT	AAGAGCGGGATCTCCTAC	1,611
12	13,270–14,908	CAACATTCTTTTCTCTACAC	GATATAACGCTCCATAACT	1,639
13	14,712–16,138	CTGATTCTAAGTGTTGGGTTG	TCCTTTGAGGTACTATGCGA	1,427
14	16,027–17,539	TGCTCGTGTTGTTTTTACTGC	CACTTGCTCCTTGCCTATTTT	1,513
15	17,362–18,993	CCACTTGAGGGCTTTGT	TAAACATACAGGTTCGCT	1,632
16	18,945–20,442	AAGCGGTATYCNTATGTAGAA	ATAGTRCAVACAAAAKRGTCA	1,498
17	20,336–21,920	ACTGAACAAAAGACMGACTT	CCACCAGAAACTACAACT	1,585
18	21,873–22,919	AAGGTTAATCCCTGTGAAG	AGTYTCVGTAAGAATAGCA	1,047
19	22,723–23,837	CTTTTGCHACTCAGATDCA	AGATTTCTTACCACACTTACT	1,115
20	23,435–24,898	ATTTGTAGAAGATGACGAT	CATTGTTGACCATTAGTTA	1,464
21	24,747–26,141	GCAGCGATAATACTTACCGTG	GCTTGGCGTCTCCAGTATC	1,395
22	25,965–27,650	TATCAAACTAGGAGGACCA	GCTCTAACTCTAAACTAGCCT	1,686

*Primer locations are listed according to strain SC021202 (GenBank accession no. EU714029).

in another group were inoculated with 0.2 mL phosphate-buffered saline (PBS) as noninfected controls. Birds were housed in isolators and provided feed and water ad libitum.

All birds were observed daily for signs of disease (e.g., disheveled feathers, depression, respiratory signs, or diarrhea) and death for 21 days. Gross pathologic changes were observed, and tissues (trachea, lung, kidney, bursa, liver, and brain) were collected for RT-PCR from 10 randomly selected chickens that died within 4–10 days after inoculation, virus isolation, and histopathologic and IHC analyses. Serum samples collected from birds that survived were tested for IBV antibodies by a commercial ELISA kit (IDEXX Laboratories, Westbrook, ME, USA). The endpoint titers were calculated according to the manufacturer's instructions, and titers >396 were considered positive for IBV antibody.

RT-PCR of Tissue Samples

Tissue samples from deceased birds were collected and used for RT-PCR to determine tissue distribution of the virus. Total RNA was obtained by using Trizol Reagent (Invitrogen) as recommended for tissue samples. RT-PCR was performed as described above by using a pair of primers (forward: 5′-TTTTGGTGATGACAAGATGAA-3′; reverse: 5′-CG CATTGTTCCTCTCCTC-3′), which amplify and detect a 403-bp fragment of the S1 gene of IBV. PCR products were analyzed on 1.5% agarose gels.

Virus Re-isolation from Infected Tissue Samples

Tissue samples collected after challenge were used for virus isolation. Briefly, at least 6 SPF embryos were inoculated through the allantoic cavity, with each sample, containing 10,000 U/mL penicillin and 10,000 μg/mL streptomycin (0.2 mL/egg). The eggs were candled daily, and allantoic fluids from 3 inoculated embryos were collected 48 h after inoculation for RT-PCR amplification. The remaining embryos were examined 6 days after inoculation for characteristic IBV lesions, such as dwarfing, stunting, or curling of embryos.

Histopathology

Tissues (trachea, lung, kidney, bursa, liver, and brain) were collected and fixed by immersion in 10% neutral formalin at room temperature for 48 h. Tissue was then routinely processed, embedded in paraffin wax, and cut into 5-μm sections. The sections were stained with hematoxylin and eosin and examined by light microscopy for lesions resulting from IBV infection.

Immunohistochemistry

All sampled tissues were examined by IHC analysis to detect viral antigen. Briefly, 5-μm tissue sections were subjected to antigen retrieval (*20*) and were then incubated in 10% normal goat serum in PBS for 30 min to block nonspecific binding sites. Slides were further incubated with chicken anti-IBV hyperimmune serum at 1:500 dilution in PBS for 2 h, followed by incubation with a horseradish peroxidase–conjugated rabbit chicken IgG for 1 h. The reaction was visualized by the addition of 3,3-diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) for 15 min. After IHC staining, sections were counterstained with hematoxylin, air dried, and examined by light microscopy.

Results

Genome Sequencing

The complete genome sequence (excluding the 5′- and 3′-terminal segments) of YN strain was obtained by assembling 22 overlapping sequences that ranged from 856 bp to 1,686 bp by the DNAstar software. The nucleotide sequences were submitted to GenBank under accession no. JF893452. The complete genomic sequences (excluding the 5′- and 3′-terminal segments) of YN isolate comprised 27,635 nt, which putatively contains 6 different genes, each containing single or multiple open reading frames.

Phylogenetic Analysis

Phylogenetic trees for the genome and each gene of IBV reinforced the viral nucleotide sequencing results, suggesting that the YN isolate shares an immediate ancestor with the SC021202 strain (Figure 1). To elucidate the phylogenetic relationships of China IBV strains, we further analyzed S1 genes of 70 IBVs, including 51 China isolates and 19 standard strains or vaccine strains (Figure 1, panel B). The data indicated that China isolates could be differentiated into 3 distinct genetic groups or genotypes. Group I included 41 of the 51 field isolates, which were tentatively named A2-like viruses. Four China field isolates were included in group II, which were tentatively named LSD-like viruses. China group III comprised 6 isolates, which were grouped with the Massachusetts serotype. These results showed that at least 3 distinct groups existed in chicken flocks in China, and A2-like and M41-like viruses were mainly responsible for the IB panzootic, consistent with findngs of our previous studies (*5*,*16*).

Sequence Comparisons

Ten virus sequences were selected for pairwise comparisons with the YN strain according to the phylogenetic analysis for S1 (Figure 1, panel B), which included 2 common vaccine strains in China (M41 and H120) and 8 China isolates from 3 different gene clusters (DY07, LX4, A2, and SC021202 from cluster I; CK/CH/LSD/05I and CK/CH/LHB/100801 from cluster II; CK/

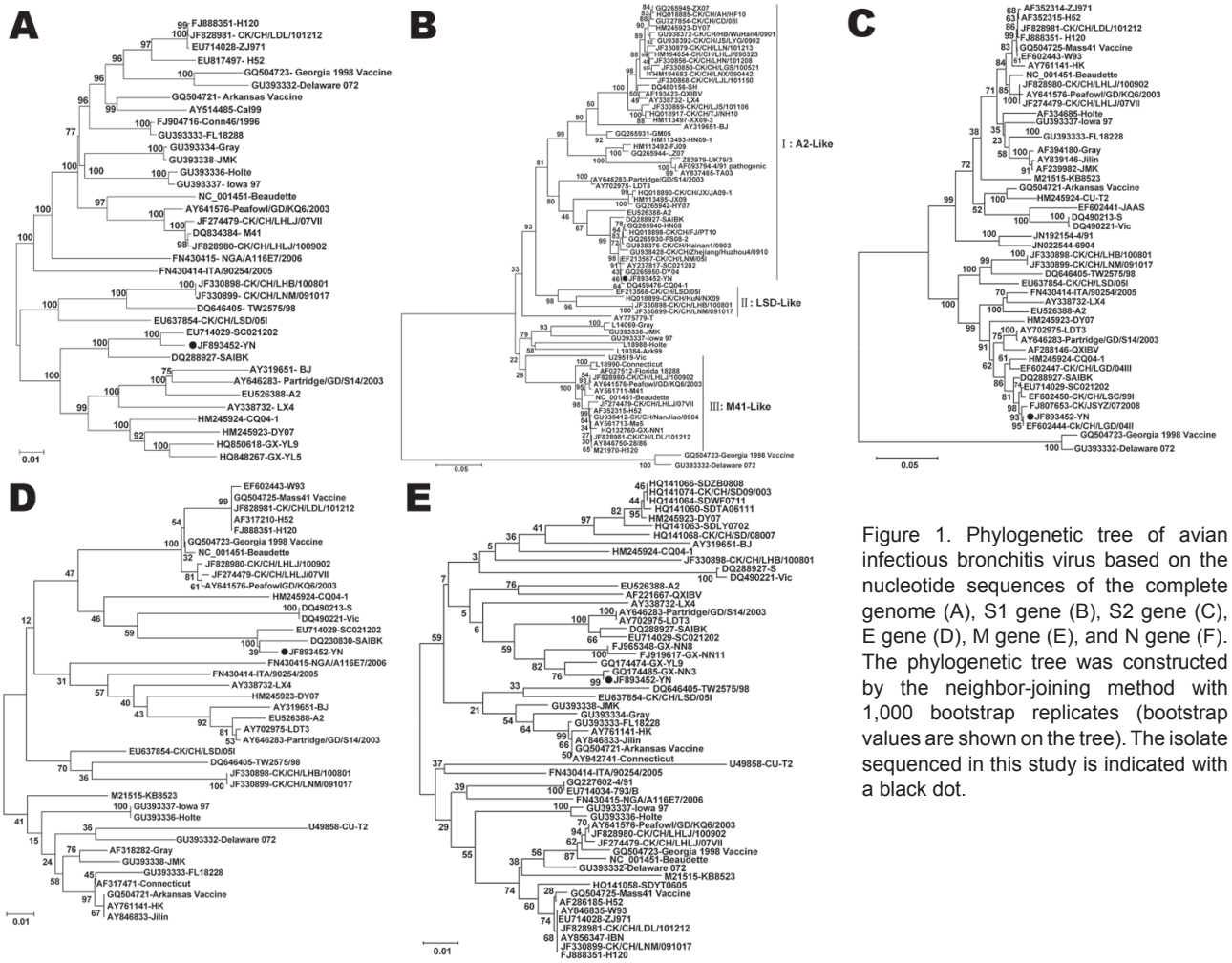

Figure 1. Phylogenetic tree of avian infectious bronchitis virus based on the nucleotide sequences of the complete genome (A), S1 gene (B), S2 gene (C), E gene (D), M gene (E), and N gene (F). The phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates (bootstrap values are shown on the tree). The isolate sequenced in this study is indicated with a black dot.

CH/LHLJ/07VII and CK/CH/LDL/101212 from cluster III) (Table 2, Appendix, wwwnc.cdc.gov/EID/article/18/12/12-0552-T2.htm). Overall, the complete genomic sequences (excluding the 5′- and 3′-terminal segments) were 87.1%–98.0% identical among the isolates, and the YN strain had the highest nucleotide identity (98.0%) to the SC021202 strain isolated in southern China (GenBank accession no. EU714029). For each gene, the identity hanged from 93.7% to 100% between YN and SC021202 strains (Table 2).

The S glycoprotein is the major functional protein for IBV; thus, the putative differences between the YN strain and other IBVs were investigated. Analyses showed that a single amino acid insertion was present at position 22 (Figure 2, panel A) in addition to a 7-aa insertion at positions 74—81 (Figure 2, panel B) in the S1 gene of the YN isolate, in which a putative N-glycosylation site was found. Among available sequences in GenBank, strains A2, BJ, CQ04–1, SC021202, and SAIBK had the same insertion in the S1 gene. Compared with other available strains, 9-aa deletions were located at the N terminal of the S2 gene (Figure 2, panel C) from a base transition (G×T) at position 1,849 nt, resulting in premature stop codon in the open reading frame.

Clinicopathologic Assessment in Chickens

Clinically, some birds appeared depressed with ruffled feathers; these birds had increased water intake and huddled together in YN-inoculation group at 2 days postinfection (dpi). At 4 dpi, IB-like signs became obvious in all infected chickens, and some birds were euthanized because of severe disease. Deaths occurred until 21 dpi; the death rate reached 65% (Figure 3, panel A). No deaths or clinical signs were observed in the control group.

Slight hemorrhage with serous catarrhal exudate could be seen in the trachea of all euthanized birds. Air sac lesions also were present, characterized by marked thickening of the air sac wall and a yellow caseous exudate. All euthanized chickens showed typical kidney lesions. The affected kidneys were substantially enlarged, and deposits of pale urate were frequently observed in the tubules and

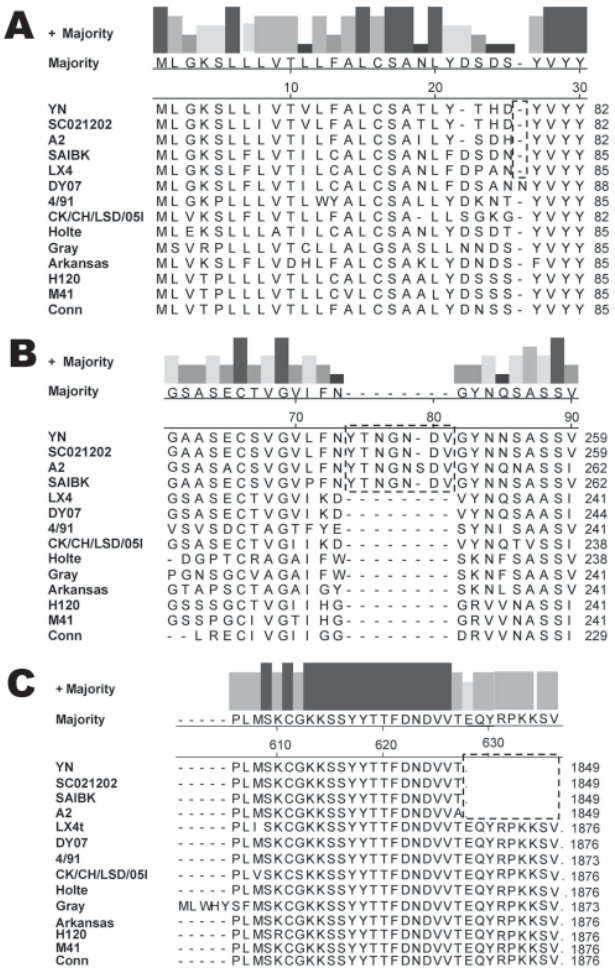

Figure 2. Amino acid sequence alignment for the S1 gene (A, B) and S2 gene (C) of 14 strains of avian infectious bronchitis virus. Strains YN, SC021202, A2, and SAIBK have a 7-aa insertion in the S1 gene and a 9-aa deletion in the S2 gene, compared with most strains.

ureters (Figure 4). No gross lesions were observed in any birds in the control group.

Antibody responses in birds that survived infection were measured by a commercial ELISA kit (IDEXX Laboratories). All but 1 chicken showed a positive reaction (Figure 3, panel B) by ELISA, and the mean titer induced by the YN strain was 1,250.35 at 21 dpi.

RT-PCR and Virus Isolation on Tissues

To further examine the pathogenicity of IBV YN, the tissue tropism of the strain was investigated by RT-PCR and virus isolation by using tissue suspensions from dead birds. YN strain replicated well in the various organs, including the kidneys, trachea, lungs, and bursa (Table 3; online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0552-Techapp.pdf).

Histopathologic and IIHC Analyses

Microscopic examination of the tracheal tissues revealed extensive degeneration and necrosis of the ciliated epithelial cells, sometimes with pseudoacinar structures resulting from dropout of dead cells (Figure 5, panel B). Viral antigen was detected at high levels in the epithelial cells of the tracheal mucosa (Figure 6, panel B). Lung lesions were characterized by hemorrhage, congestion, and lymphocytic infiltration in the alveolar lumen (Figure 5, panel D), and viral antigen was detected in alveolar cells (Figure 6, panel D). Severe renal lesions, including degeneration and necrosis of renal tubular epithelial cells, lymphocytic infiltration in the interstitium, exfoliated renal tubular epithelial cells, and erythrocytes were frequently observed (Figure 5, panel F). Viral antigens were detected extensively in the renal tubular epithelial cells (Figure 6, panel F). Serious atrophy of lymphoid follicles and widening of the interstitium were observed in the bursa of Fabricius (Figure 5, panel H), and viral antigens were detected widely in the mucosal epithelium of the bursa of Fabricius (Figure 6, panel H). Sporadic congestion also was observed in the liver and cerebrum.

Discussion

IBV infection leads to severe economic losses in the poultry industry because of poor weight gain and feeding

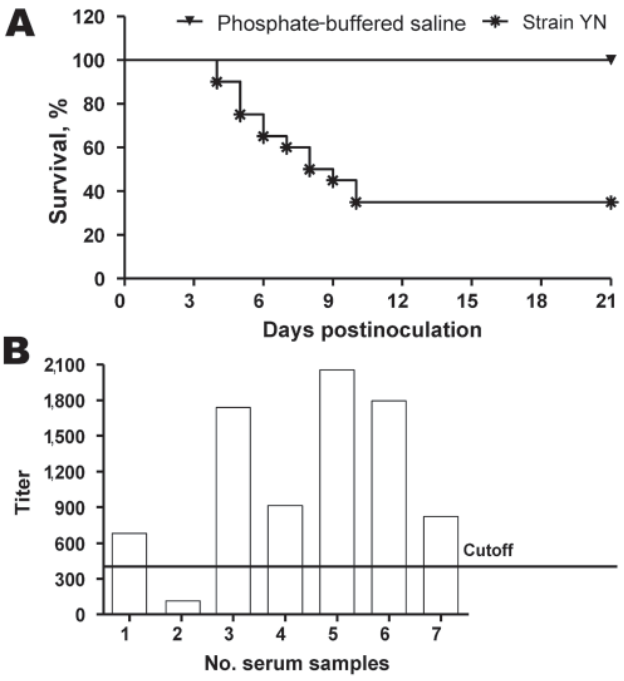

Figure 3. Seroconversion and percentage survival of chickens experimentally infected with infectious bronchitis virus (IBV), People's Republic of China. A) Survival of chickens after inoculation with IBV YN strain. B) Detection of IBV antibodies by ELISA at 21 days postinoculation. Cutoff titer = 396.

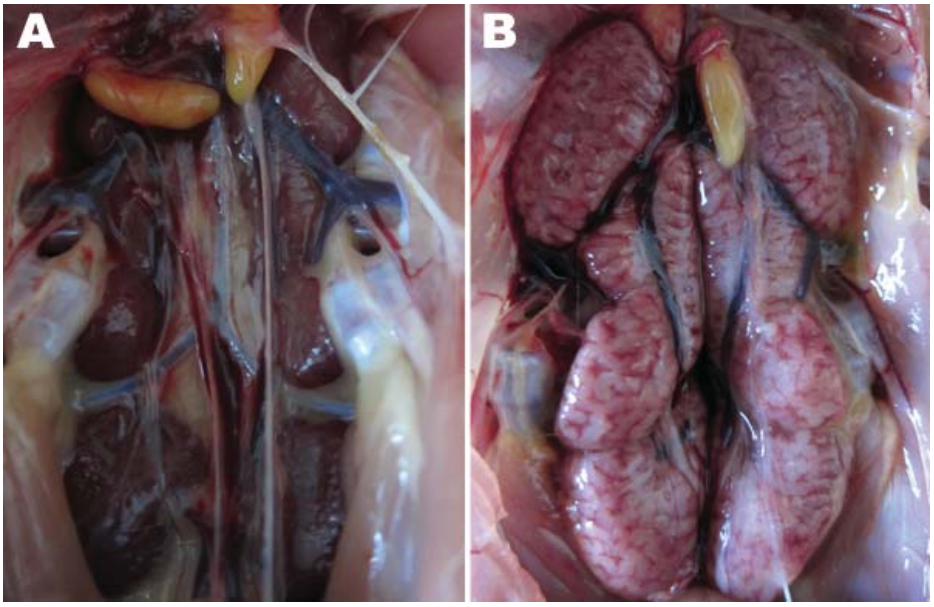

Figure 4. Gross lesions from kidney tissues from chickens experimentally infected with infectious bronchitis virus (IBV). A) Kidney tissue of an uninfected control chicken. B) Obvious enlargement and urate deposition in the kidney of a chick infected with the IBV YN strain at 7 days postinfection.

efficiency in broilers or reduced egg production and egg quality in laying birds. Death rates are often low (<30%) unless secondary bacterial infections cause increased deaths of birds (*21*,*22*). Evaluation of the pathogenicity of IBV was based on several criteria, including: clinical signs, gross pathologic lesions, histopathologic changes, and tissue tropism. In this study, a China IBV strain isolated from a 30-day-old vaccinated broiler flock that had a history of respiratory signs, severe renal disease, and higher than expected mortality rate was analyzed by clinical observation, histopathologic examination, RT-PCR, virus isolation, and immunohistochemistry. Analyses revealed that the YN strain induced severe pathogenicity in 30-day-old SPF chickens with a 65% death rate and substantial kidney lesions.

Sequence analysis of the S1 gene of YN isolate showed high nucleotide similarities to CQ04–1 (99.5% nt and 98.8% aa identities) and SC021202 (99.3% nt and 98.6% aa identities). Phylogenetic analysis further indicated that at least 3 distinct genetic clusters of IBV were present in chicken flocks in China, named A2-like, LSD-like, and M41-like strains. Most China IBV field isolates belonged to the same genetic cluster (A2-like), but the serotypes of these prevalent IBV strains differed from those of the currently used vaccine strains in China (M41-like, e.g., H120, Ma5, 28/86, and W93), which are considered the main cause of disease outbreaks (*5*,*15–17*). An attenuated or inactivated vaccine that matches with pandemic strains is urgently needed in China to control IBV infection.

IBV has been shown to replicate in many respiratory tissues (including trachea, lungs, and air sac), causing respiratory disease; in some urogenital tissues (including kidney), causing minor or major nephritis; and in many parts of the alimentary tract (including esophagus, proventriculus, and intestine) (*23–25*). As expected, YN was most often detected in the trachea, lung, kidney, and bursa by RT-PCR, virus isolation, and IHC analysis. In addition, we found YN antigens partly from liver (1/10) and brain (1/10).

Among 4 major structural proteins, the S glycoprotein is known to contain regions that induce neutralizing, serotype-specific, membrane fusion, attachment, and hemagglutination-inhibiting antibodies (*5*,*26–28*). In addition, the S protein is a determinant of cell tropism (*29*,*30*). Our data showed a 7-aa insertion between positions 72 and 78 (Figure 2, panel A) in the S1 gene of the YN strain and a 9-aa deletion in the N terminal of the S2, generating a stop codon. These changes are possibly related to the increased virulence observed for YN IBV, and reverse genetic analyses are needed to confirm these findings.

In conclusion, we showed that YN-like viruses are a predominant strain in China and that YN IBV exhibits

Table 3. Tissue tropism of YN strain of avian IBV, People's Republic of China*

Method	Tissue examined†					
	Trachea	Lung	Kidney	Bursa	Brain	Liver
RT-PCR‡	10/10	10/10	8/10	7/10	1/10	1/10
Virus isolation§	7/10	8/10	10/10	9/10	ND	ND

*Thirty-day-old SPF chickens were inoculated intranasally with YN IBV (10^5 50% egg infectious dose per bird). IBV, infectious bronchitis virus; SPF, specific-pathogen free; RT-PCR, reverse transcription PCR; ND, not detected.
†Ten tissue samples from euthanized birds were randomly collected and examined.
‡RT-PCR was directly performed on collected tissues.
§Tissue samples were collected for re-isolation of the challenge virus using SPF embryos. Data are the number of tissues from which the virus was isolated/number of tissues examined.

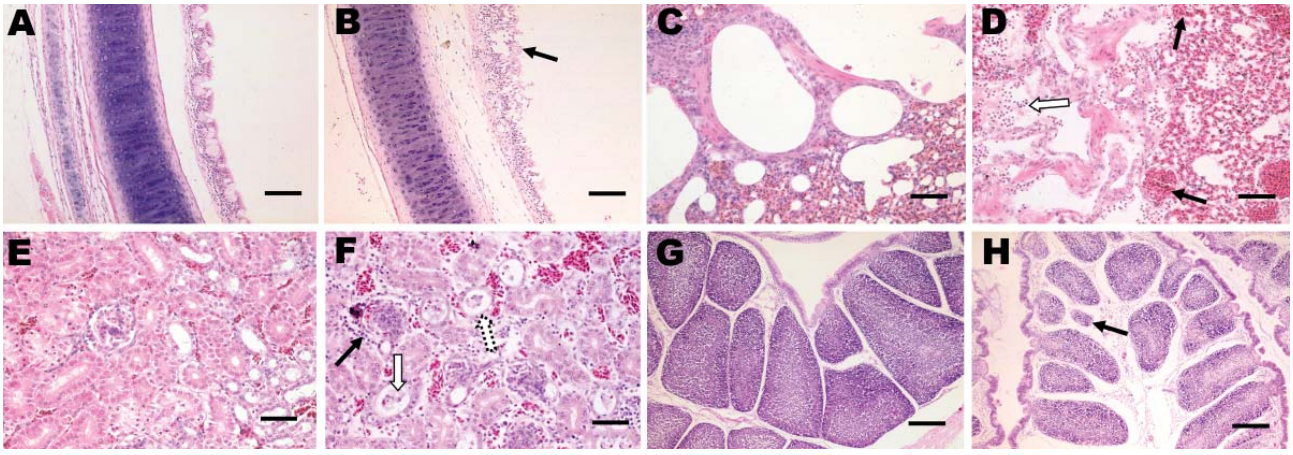

Figure 5. Histopathologic analysis (hematoxylin and eosin stain) of tissues from 30-day-old chickens infected with infectious bronchitis virus YN strain. Panels A, C, E, and G correspond to control tissues. B) Trachea, extensive dropout, degeneration, and necrosis of the ciliated epithelial cells (black arrow). Scale bar = 100 μm. D) Lung tissue with hemorrhage (black arrow), congestion, and lymphocytic infiltration in alveolar lumen (white arrow). Scale bar = 50 μm. F) Kidney tissue with severe renal lesions, including degeneration (white arrow), and necrosis of renal tubular epithelial cells, lymphocytic infiltration in the interstitium (black arrow), exfoliated renal tubular epithelial cells and erythrocytes were observed extensively. Scale bar = 50 μm. H) Bursa tissue with serous atrophy of lymphoid follicles and widening of the interstitium were observed in bursa of Fabricius (black arrow). Scale bar = 200 μm.

broad tissue tropism and severe pathogenicity in chickens. To better control IBV in China, more detailed analysis of the biologic and antigenic characteristics of the predominant IBV isolates is warranted, and assessments of the efficacy of current vaccines against these isolates are needed.

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Ms Feng is a postgraduate student at the College of Veterinary Medicine, People's Republic of China Agricultural University, in Beijing. Her scientific interests include the epidemiology and pathogenicity of avian infectious bronchitis viruses.

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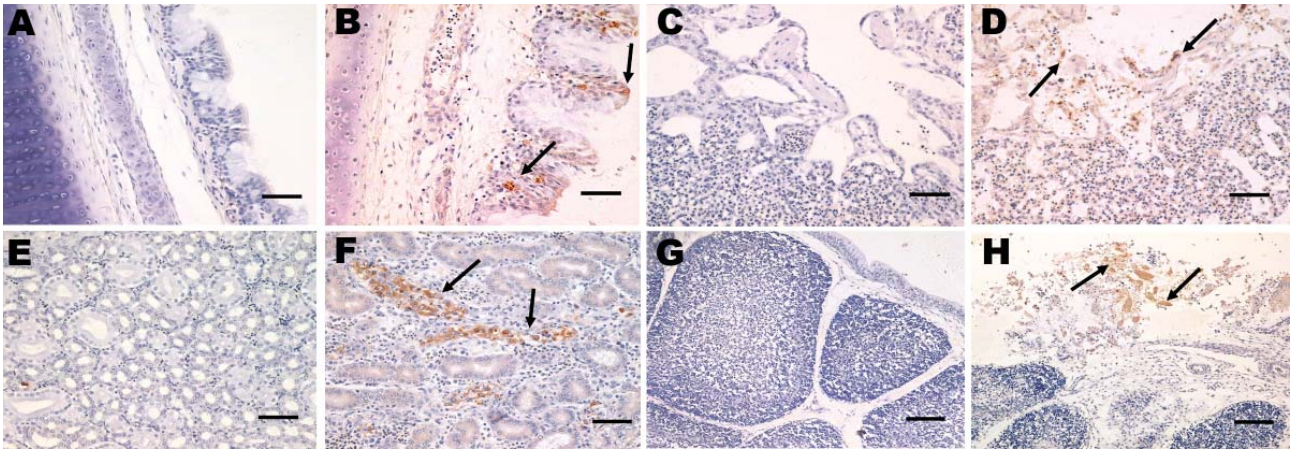

Figure 6. Immunohistochemical detection of avian infectious bronchitis virus (IBV) antigens in tissues after experimental infection with IBV YN strain. Scale bar = 100 μm. Panels A, C, E, and G correspond to control tissues. B) Tracheal tissue with viral antigen detected extensively in the epithelial cells of the tracheal mucosa (black arrow). Scale bar = 50 μm. D) Lung tissue with viral antigen detected in alveolar cells (black arrow). Scale bar = 50 μm. F) Kidney tissue with viral antigens detected widely in the renal tubular epithelial cells (black arrow). Scale bar = 50 μm. H) Bursa tissue with viral antigens detected at high levels in mucosal epithelium in the bursa of Fabricius (black arrow).

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Address for correspondence: Guozhong Zhang, College of Veterinary Medicine, People's Republic of China Agricultural University, Beijing 100193, No. 2 Yuanmingyuan West Rd, Haidian District, People's Republic of China; email: zhanggz@cau.edu.cn

Enterovirus 71–associated Hand, Foot, and Mouth Disease, Southern Vietnam, 2011

Truong Huu Khanh, Saraswathy Sabanathan, Tran Tan Thanh, Le Phan Kim Thoa, Tang Chi Thuong, Vu thi Ty Hang, Jeremy Farrar, Tran Tinh Hien, Nguyen van Vinh Chau, and H. Rogier van Doorn

We prospectively studied 3,791 children hospitalized during 2011 during a large outbreak of enterovirus 71–associated hand, foot, and mouth disease in Vietnam. Formal assessment of public health interventions, use of intravenous immunoglobulin and other therapies, and factors predisposing for progression of disease is needed to improve clinical management.

In Southeast Asia, human enterovirus 71 (EV71) is a frequent cause of hand, foot, and mouth disease (HFMD) in Southeast Asia and resulting neurologic and cardiopulmonary complications. Children <5 years of age are at risk for symptomatic and severe disease, but the factors predisposing for severity are largely unknown.

In Vietnam, EV71 was first isolated in 2003. In 2005, an outbreak of HFMD was caused by an early peak of coxsackievirus A16 (CVA16), followed by a peak of EV71, associated with severe disease (*1*).

HFMD outbreaks occurring every 3 years have been reported from countries in the region to which it is endemic (*2*,*3*), but Vietnam had a high number of cases during February 2011–July 2012: a total of 174,677 cases (110,897 during 2011; 63,780 during the first 6 months of 2012) and 200 deaths were reported from Vietnam during this period. The outbreak peaked in week 38 (September 18–24, 2011, with ≈2,500 reported hospital admissions countrywide. Reported case-patients were mainly from southern Vietnam in 2011; in 2012, the outbreak spread to the northern provinces of Vietnam (*4*).

Author affiliations: Children's Hospital 1, Ho Chi Minh City, Vietnam (T.H. Khanh, L.P.K. Thoa, T.C. Thuong); Oxford University Clinical Research Unit, Oxford, UK (S. Sabanathan, T.T. Thanh, V.t.T. Hang, J. Farrar, T.T. Hien, H.R. van Doorn); Oxford University, Oxford (S. Sabanathan, J. Farrar, T.T. Hien, H.R. van Doorn); and Hospital for Tropical Diseases, Ho Chi Minh City (N.v.V. Chau)

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Learning Objectives

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

- Distinguish the virus type associated with the outbreak of hand, foot, and mouth disease (HFMD) in Vietnam in 2011
- Analyze how to grade the clinical severity of HFMD
- Evaluate the clinical presentation of HFMD in the current report
- Assess the management of HFMD

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CME Author

Charles P. Vega, MD, Health Sciences Clinical Professor; Residency Director, Department of Family Medicine, University of California, Irvine. *Disclosure: Charles P. Vega, MD, has disclosed no relevant financial relationships.*

Authors

Disclosures: ***Truong Huu Khanh, MD; Saraswathy Sabanathan, MD; Tran Tan Thanh, PhD; Le Phan Kim Thoa, MD; Tang Chi Thuong, MD, PhD; Vu thi Ty Hang, PhD; Jeremy Farrar, BSc, MBBS, FRCP, DPhil; Tran Tinh Hien, MD, PhD; Nguyen van Vinh Chau, MD, PhD;*** *and* ***H. Rogier van Doorn, MD, Phd,*** *have disclosed no relevant financial relationships.*

The Vietnamese Ministry of Health has implemented a clinical grading system to guide disease management. It is based on a grading system from Taiwan (*5*) and is a consensus of experienced physicians; it describes 4 grades of disease. Grade 1 is uncomplicated disease with fever and vesicles or papules on hands, feet, buttocks, and oral mucosa. In grade 2 disease, the central nervous

system is involved, usually as myoclonus starting in the fingers. Grade 2 is further divided into grade 2a disease, when myoclonus is reported by the caregiver, and grade 2b disease, when myoclonus is observed by a physician. In grade 3 disease, autonomic dysfunction occurs with fever that is unresponsive to antipyretics and with hypertension and persistent tachycardia. Patients with grade 4 disease exhibit cardiopulmonary compromise with pulmonary edema or hemorrhage. Grades 2b, 3, and 4 describe severe disease and are indicators for hospital admission and treatment. Patients with grade 2a disease (reported myoclonus) also usually are admitted, and a small proportion of patients seeking care in the outpatient clinic with grade 1 disease are admitted for observation, on the basis of clinical judgment.

Myoclonus and more severe neurologic complications are treated with oral or intravenous phenobarbital. Heart rate, respiratory rate, blood pressure, and saturations are recorded to monitor progress of disease. When persistent tachycardia, fever unresponsive to antipyretics, irregular breathing, or persistent hypertension occur, intravenous immunoglobulin (IVIg) is administered. Children have arterial lines inserted for close observation of blood pressure. Hypertension is treated with milrinone, a phosphodiesterase inhibitor. When a child's condition does not improve, hemofiltration is used in conjunction with full intensive care support as needed.

The Study

Data were collected prospectively from 3,791 patients with HFMD in Children's Hospital 1, Ho Chi Minh City, Vietnam, during September 18–November 30, 2011, of whom 2,364 (62%) were male. Patients' median age was 20 months; 95% of children were 1–4 years of age (Figure 1). On admission, 647 (17%) patients were given the following grade 1; 2,750 (73%), grade 2a; 338 (9%), grade 2b; 42 (1%), grade 3; and 14 (0.4%), grade 4. Of all admissions, 159 (4%) had a maximum grade of 3 or 4 during their hospital course. Six 6 (0.2%) children died, of whom 2 had grade 4 disease, one had grade 3, two had grade 2b, and one had grade 2a. Median time to maximum grade was <24 hours; the median duration of hospitalization was 3 days (interquatrile range [IQR] 2–4) for all patients and 5 days (IQR 4–8) for patients who were admitted with grade 3 or 4 disease or whose illness progressed to that degree of severity.

A total of 2,750 (73%) of the 3,791 patients were admitted with grade 2a HMFD; however, disease in only 121 (4%) progressed to grade 2b (94 children) or further (27 children), whereas disease in 75 (22%) of 338 children admitted with grade 2b progressed to grade 3 or 4. Disease in only 1 (0.2%) of 647 children admitted with grade 1 progressed to grade 3 or 4 (Table 1). A total of 443 (12%) children with severe cases of grade 2b or higher, were treated with IVIg, of which 22 (0.6%) were treated with hemofiltration.

Samples were analyzed with generic enterovirus reverse transcription PCR (RT-PCR) as described (*6*) and with EV71-specific real-time RT-PCR and CVA16-specific RT-PCR (Table 2) by using SuperScript One-Step RT-PCR reagents (Invitrogen, Carlsbad, CA, USA) under the following conditions: 50°C for 30 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 20 s. Sequencing of viral protein (VP) 1 of EV71 was performed by using ABI Dye Terminator sequencing (Applied Biosystems, Foster City, CA, USA).

Virologic analysis was done on nose/throat or rectal swab specimens from 174 (33%) of 522 children with grade 2b disease or higher. A total of 132 (76%) of these were positive for by RT-PCR. CVA16 was not detected among these 174 children, and other enteroviruses were detected only sporadically.

In contrast to results from previous outbreaks in Vietnam, all of 11 sequences from this and other studies (*7*) showed that viruses belonged to subgenogroup C4, replacing C5 (Figure 2). Subgenogroup C4 has been found in the region since 1998 (*8*), and viruses from our study cluster with recent C4 viruses from the People's Republic of China. Emergence of novel subgenotypes, often related to recombination events in noncoding regions (*9*), and switches of dominant subgenotypes are common and may be associated with large outbreaks. Most outbreaks

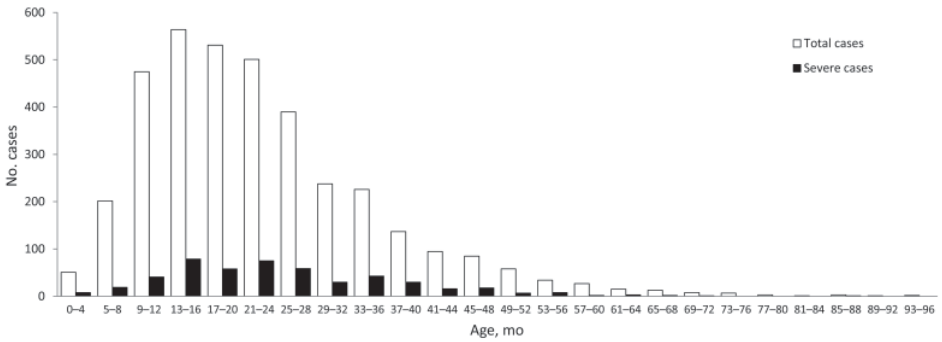

Figure 1. Age distribution by 4-month interval of 3,667 children admitted to Children's Hospital 1, Ho Chi Minh City, Vietnam, who had clinical diagnoses of hand, foot, and mouth disease, September 18–November 30, 2011. White bars indicate total number of cases; black bars indicate severe cases (grade 2b or worse). Severe cases are defined as grade 2b, 3, or 4 disease.

Table 1. Hand, foot, and mouth disease grades at admission and grades to which disease progressed during hospitalization for 3,791 children, Children's Hospital 1, Ho Chi Minh City, Vietnam, September 18–November 30, 2011

Grade at admission	Grade to which illness progressed, no. (%) cases					Total
	1	2a	2b	3	4	
1	640 (98.9)	0	6 (0.9)	1 (0.2)	0	647
2a		2,629 (95.6)	94 (3.4)	25 (0.9)	2 (0.1)	2,750
2b			263 (77.8)	71 (21.0)	4 (1.2)	338
3				38 (90.5)	4 (9.5)	42
4					14 (100.0)	14

reported in the Asia-Pacific region during the last decade were caused by previously undefined EV71 subgenogroups (*10*), but there is no evidence of differences in virulence. Data suggest cross-antigenicity among the different subgenotypes (*11*), i.e., EV71 constitutes 1 serotype.

Conclusions

EV71 has emerged as a frequent cause of clinically severe HFMD and affects a large number of countries in the region. Although spreading locally, large epidemics with severe disease are confined to Southeast Asia. The potential for pandemic spread is unknown.

In Vietnam and surrounding countries, EV71 has become endemic, and seroprevalence studies show a high force of infection with a seroconversion rate of up to 14% during the second year of life in southern Vietnam (*12*). The case-fatality rate in this and other outbreaks is generally low (<0.5%) (*13*), but the large number of cases and relative absence of prognostic factors for progression to more severe disease considerably affect the health care system, requiring monitoring and observation of large numbers of patients.

This study included all patients hospitalized during September 18–November 30, 2011, in the largest children's referral hospital in southern Vietnam. The study's limitations are as follows: because observations are only from hospitalized patients in 1 hospital, the study did not include all cases of severe disease in southern Vietnam or any outpatients. In addition, virologic testing was available only for patients with severe disease and only for one third of those.

Until a vaccine becomes available, control of EV71 is limited to promotion of public health interventions, such as hand washing, exclusion of ill children from school settings, and improved clinical management of EV71-associated HFMD. During the EV71 outbreak in southern Vietnam during 2011–2012, most children (90% in this study) with HFMD were hospitalized with mild disease (grade 2a or below), and more severe disease (grade 2b or higher) developed in only a small fraction (4% in this study) of these patients. To improve clinical management and reduce the strain on the health care system, formal assessment of public health interventions and use of IVIg and other therapeutic options and of factors predisposing the patient for progression of disease is needed to improve clinical management and reduce the strain on the health care system.

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Dr Khanh has been head of the infectious diseases department of Children's Hospital 1 in Ho Chi Minh City, Vietnam, since 1996. He was involved in writing the guidelines for HFMD management for the WHO Regional Office for the Western Pacific and has longstanding experience in treating patients with moderate and severe HFMD.

Table 2. Primers and probes used in study of EV71-associated hand, foot, and mouth disease, Children's Hospital 1, Ho Chi Minh City, Vietnam, September 18–November 30, 2011*

Assay	Primer and probe	Sequence, 5′ → 3′	GenBank accession no.	PCR product size, bp
EV71 real-time RT-PCR	EV71-VP1-634F	GGAGAACACAARCARGAGAAAGA	AM490160.1	Real-time analysis
	EV71-VP1-743R	ACTAAAGGGTACTTGGACTTVGA		
	EV71-VP1-TaqMan	FAM-TGATGGGCACGTTCTCAGTGCG-BHQ1		
CVA16 RT-PCR	CoxA-VP1-526F	AACCCATCTGTGTTTGTGAAAA	JF317969.1	110
	CoxA-VP1-635R	CCGAAGGTGGGATAACCAT		
EV71 VP1 sequencing	EV71-VP1-3F	AGAYAGGGTGGCRGATGT	AM490160.1	701
	EV71-VP1-703R	CTGAGAACGTGCCCATCA		

*EV71, enterovirus 71; RT-PCR, reverse transcription PCR; VP, viral protein; FAM, carboxyfluorescein; BHQ1, black hole quencher 1; CVA16, coxsackievirus A16; CoxA, coxsackievirus.

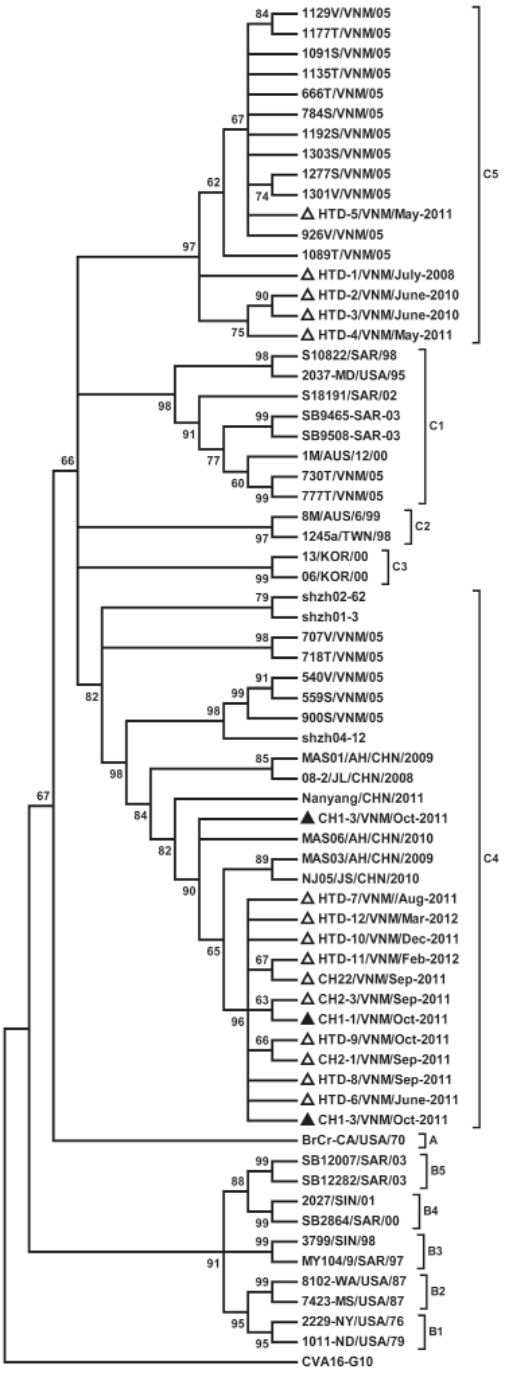

Figure 2. Phylogenetic tree of enterovirus 71 viral protein 1 constructed by MEGA4 (www.megasoftware.net) with neighbor-joining method showing the relationship of 18 local sequences from 2010 and 2011 (triangles). Sequence names consist of the following information: the hospital at which the sample was obtained (HTD, Hospital for Tropical Diseases; CH1, Children's Hospital 1; CH2, Children's Hospital 2 (all from Ho Chi Minh City, Vietnam]); number in chronologic order/VNM for Vietnam/date (month-year). Reference genotypic sequences used in tree construction were obtained from GenBank. Detailed information about these sequences is available in reference (*1*).

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Address for correspondence: H. Rogier van Doorn, Oxford University Clinical Research Unit, 764 Vo Van Kiet, Ward 1, District 5, Ho Chi Minh City, Vietnam; email: hrogier@gmail.com

Epizootic Spread of Schmallenberg Virus among Wild Cervids, Belgium, Fall 2011

Annick Linden,[1] Daniel Desmecht,[1] Rosario Volpe, Marc Wirtgen, Fabien Gregoire, Jessica Pirson, Julien Paternostre, Deborah Kleijnen, Horst Schirrmeier, Martin Beer, and Mutien-Marie Garigliany

Schmallenberg virus was detected in cattle and sheep in northwestern Europe in 2011. To determine whether wild ruminants are also susceptible, we measured antibody seroprevalence in cervids (roe deer and red deer) in Belgium in 2010 and 2011. Findings indicated rapid spread among these deer since virus emergence ≈250 km away.

During summer and fall of 2011, a nonspecific febrile syndrome among adult dairy cows in northwestern Europe was reported. During November 2011, an enzootic outbreak causing fetal death or neurologic signs in newborn lambs, kids, and calves emerged throughout several countries in Europe. Both syndromes were associated with the genome of a new Shamonda/Sathuperi-like orthobunyavirus named Schmallenberg virus (SBV) in the blood (adults) or central nervous system (newborns) (*1*,*2*). Susceptibility of wild ruminants can be expected on the basis of the behavior of related viruses of the Simbu serogroup. Therefore, we measured seroprevalence of antibodies against SBV in wild red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) and looked for the viral genome in fetuses from pregnant deer found dead.

The Study

Blood samples were collected during postmortem examination of 313 red deer and 211 roe deer shot during the 2010 and 2011 hunting seasons. The 524 samples were randomly collected during October–December from 35 hunting estates in 4 of the 5 provinces in southern Belgium (Figure 1). The animals' sex; age; body condition; and macroscopic aspects of hooves, mucosae, and internal organs were recorded. IgG against the recombinant nucleoprotein of the emerging SBV was detected by using an ELISA kit (ID Screen Schmallenberg Virus Indirect, version 1; ID.vet Innovative Diagnostics, Montpellier, France). Results are expressed as percentages of the reference signal yielded by the positive control serum; serologic status is defined as negative (<60%), doubtful (60%–70%), or positive (>70%). Neutralizing antibodies against SBV were sought as described (*3*) in subsets of roe deer serum (IgG-negative and IgG-positive according to ELISA), and a linear relationship between percentages and reciprocal neutralizing titers was found. In addition, necropsies were performed on 22 fetuses and 5 newborn red deer fawns; brain samples were tested for SBV genomic RNA and cellular β-actin transcripts by reverse transcription quantitative PCR (*3*). Contingency tables were analyzed by using χ^2 analysis to detect associations between seroconversion and species, sex, age, sampling location, and sampling date. Significance level was $p<0.05$.

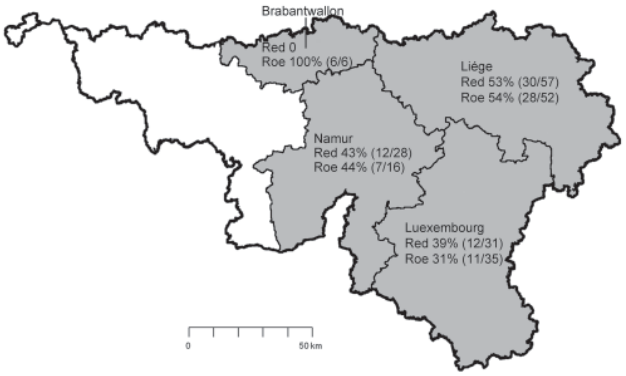

Figure 1. Location of 4 provinces in southeast Belgium (shaded) where 524 wild cervids (313 red deer and 211 roe deer) were killed during hunting seasons 2010 and 2011 and sampled. Seroprevelance for Schmallenberg virus is shown for each of the 225 deer killed in 2011. Source: Institut Géographique National, Brussels, Belgium, 2001.

No gross lesions compatible with any disease were found in any deer. All 299 serum samples collected during the fall of 2010 were negative for IgG against SBV. However, among the 225 samples from deer killed in 2011, seroprevalence was 43.1% (95% CI 36.6%–49.6%). No significant association was found between species and seroconversion: 40.5% (95% CI 31.6%–49.5%) among red deer and 45.9% (95% CI 36.5%–55.2%) among roe deer ($p = 0.42$). Acquired immunity against SBV was thus already high, suggesting that SBV had quickly spread since its emergence ≈250 km northeast during late summer 2011.

A significant association between month of sampling and seroconversion was detected for both deer species

Author affiliations: University of Liège, Liège, Belgium (A. Linden, D. Desmecht, R. Volpe, M. Wirtgen, F. Gregoire, J. Pirson, J. Paternostre, D. Kleijnen, M.-M. Garigliany); and Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany (H. Schirrmeier, M. Beer)

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[1]These authors contributed equally to this article.

($p = 0.0016$ and 0.0083 for red and roe deer, respectively). Seroprevalence increased during weeks 40–50 of 2011: for red deer, 20.0% (95% CI 8.3%–31.7%) in October, 52.6% (95% CI 36.8%–68.5%) in November, and 54.6% (95% CI 37.6%–71.5%) in December and for roe deer, 34.0% (95% CI 20.5%–47.6%) in October, 49.1% (95% CI 35.6%–62.5%) in November, and 88.9% (95% CI 68.4%–100%) in December, thus suggesting that the virus had circulated in the areas sampled until at least mid-November (Figure 2).

This late circulation of virus might be surprising because biting midges of the genus *Culicoides*, which reportedly transmit SBV (*4*), are not usually active during cold months. However, during fall 2011, temperatures in the region were substantially higher than normal (*5*) and thus compatible with persistent wild-ruminant exposure to biting midges until mid-December. No association was found between seroconversion and sex of the deer ($p = 0.71$ and 0.85 for red and roe deer, respectively), age ($p = 0.99$ and 0.24), and location of sampling ($p = 0.47$ and 0.23). These results suggest a similar level of exposure to infected vectors and a similar degree of susceptibility to infection among all animals in the study area (13,058 km^2).

In most animals that had been found dead, gross lesions were consistent with trauma (e.g., fractures, hematomas, hemoperitoneum/thorax, ruptured spleen) suggestive of impact against a vehicle. No fetus or newborn showed morphologic alterations of the neck, trunk, or limbs suggestive of arthrogryposis. No macroscopic abnormalities were seen in the cerebral cortex, cerebellum, and spinal cord. All β-actin–positive samples of these 27 fetuses and newborns remained negative for SBV RNA. Unfortunately, postmortem decay rendered fetal serum not suitable for analysis.

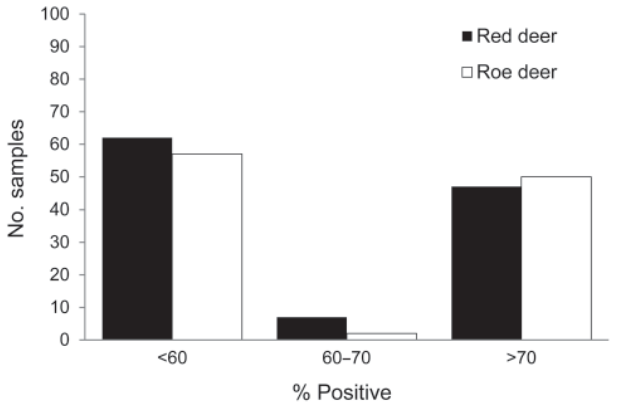

Figure 2. Frequency distribution of the results yielded by indirect ELISA for detecting IgG targeting recombinant nucleoprotein of emerging Schmallenberg virus in serum samples collected from 116 red deer and 109 roe deer in southeast Belgium during the fall of 2011. Results are expressed as percentages of the reference signal yielded by the positive control serum. Serologic status is defined as negative (<60%), doubtful (60%–70%), or positive (>70%).

Conclusions

SBV infects wild cervid populations, and infected insect vectors were homogeneously distributed over southern Belgium in the fall of 2011. Emergence probably took place in 2011. However, because seroprevalence was already 20% in red deer and 34% in roe deer during October and because our results show that the proportion of the infected population increased exponentially during October–December, we suggest that the virus began circulating months earlier than the currently believed August/September (*3*). We recently showed that among the fetuses of pregnant cows that were infected after the establishment of the first placentome, 28% were infected and that an arthrogryposis/hydranencephaly syndrome follows if transplacental virus transmission occurs before fetuses are immunocompetent (*6*). For this study, no feedback from forest rangers, no macroscopic observations, and no PCR results suggested transplacental contamination. However, aborted fetuses and stillborn and distorted nonviable newborn fawns are almost impossible to collect in the wild (quickly eaten by scavengers), and the absence of SBV-specific genetic material or morphologic alterations at necropsy are not evidence of noninfection. Therefore, no objective facts confirm or refute transplacental transfer.

Because the virus can infect the fetus only after the first placentome has developed and because roe deer embryos remain in diapause until January (*7*), it is unlikely that SBV has contaminated many roe deer fetuses. Because 90% of roe deer were already SBV positive in mid-December and because circulating antibodies prevent transplacental passage of the closest phylogenetic relatives of the virus (*8*), we suggest that roe deer fetuses were probably not infected. On the contrary, red deer mate in September, and the first functional placentome is established by the end of October (*9*); thus, 80% of pregnant red deer were exposed to the emerging virus when placental transfer was possible. Furthermore, 35% of pregnant red deer were infected in November and December, i.e., after establishment of the first placentome and before the fetus was immunocompetent. By extrapolating the rate of transplacental infection among cattle (*6*), we determined that 28% of these pregnancies resulted in contamination of the fetus, i.e., 10%, of expected pregnancies. Because unrestricted replication of Simbu-like viruses occurs in the central nervous system of immunologically incompetent ruminant fetuses (*1*), which can lead to a typical arthrogryposis/hydranencephaly syndrome, a 10% loss among fawns can be expected in 2012.

In the same geographic area, 5 years apart, 2 arboviruses have emerged: bluetongue virus serotype 8 (BTV-8) during the summer of 2006 and SBV during the summer of 2011. For each virus, *Culicoides* spp. midges function

as vectors and infect sheep, goats, cattle, and red deer. Although most (>50%) red deer seroconverted against BTV-8, only a few (<3%) roe deer sampled in the same places and at the same time were BTV-8-positive (*10*), which sharply contrasts with the SBV seroconversion rates reported here. This finding invalidates the assumption that less exposure of roe deer to infected midge bites explains the almost complete absence of seroconversion against BTV-8 in this species. The emergence of SBV thus reveals the existence of roe deer–specific anti–BTV-8 host factors, posing a fascinating question.

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Dr Linden is a professor in the Department of Infectious Diseases in the Veterinary Faculty of the University of Liège. Her research interests focus on wildlife diseases.

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Address for correspondence: Daniel Desmecht, Department of Pathology, Faculty of Veterinary Medicine, University of Liège, B-4000 Liège, Belgium; email: daniel.desmecht@ulg.ac.be

Variant Rabbit Hemorrhagic Disease Virus in Young Rabbits, Spain

Kevin P. Dalton, Inés Nicieza, Ana Balseiro, María A. Muguerza, Joan M. Rosell, Rosa Casais, Ángel L. Álvarez, and Francisco Parra

Outbreaks of rabbit hemorrhagic disease have occurred recently in young rabbits on farms on the Iberian Peninsula where rabbits were previously vaccinated. Investigation identified a rabbit hemorrhagic disease virus variant genetically related to apathogenic rabbit caliciviruses. Improved antivirus strategies are needed to slow the spread of this pathogen.

Rabbit hemorrhagic disease (RHD) is rapidly fatal, with mortality rates of 70%–100% in adult rabbits (*1*); young rabbits (kits) are unaffected or subclinically infected (*1*,*2*). This difference in disease susceptibility is poorly understood, but it may be due to changes in tissue-specific receptors that occur as young rabbits develop to adulthood (*3*).

RHD is caused by *Rabbit hemorrhagic disease virus* (RHDV; genus *Lagovirus*, family *Caliciviridae*) (*4*), a virus with a positive-sense, single-stranded RNA genome of 7.4 kb. The single serotype of RHDV is divided into 2 subtypes, classic RHDV and RHDVa. Effective inactivated vaccines prepared from liver extracts of rabbits experimentally infected with classic RHDV strains are used as a prophylactic and postoutbreak strategy to combat disease (*1*).

RHDV is not cultivatable in cell culture; therefore, detection of virus genome, virions, and anti-RHDV antibodies and experimental infection of rabbits are required for diagnosis and virus characterization (*1*). Sequence regions of the major capsid protein viral protein (VP) 1 are used to type and classify strains.

The identification of rabbit caliciviruses (RCVs) (*5*,*6*), nonpathogenic viruses antigenetically similar to RHDV, and recent descriptions of a pathogenic RCV (*7*), an RHDV variant grouping with RCV viruses in phylogenetic analysis (*8*), and nonpathogenic RHDV (*9*) raise questions about the origins, classification, and nomenclature of these viruses. On the Iberian Peninsula, RHDVa or pathogenic or nonpathogenic RCV isolates had not been reported (*10*). We report the results of an investigation of outbreaks of RHD among young rabbits on farms on the Iberian Peninsula where rabbits were previously vaccinated for RHDV.

Author affiliations: Universidad de Oviedo Instituto Universitario de Biotecnología de Asturias, Oviedo, Spain (K.P. Dalton, I. Nicieza, A.L. Álvarez, F. Parra); Centro de Biotecnología Animal (SERIDA), Gijón, Spain (A. Balseiro, R. Casais); INTIA División ITG, Navarra, Spain (M.A. Muguerza); and Cunivet Service, Tarragona, Spain (J.M. Rosell)

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The Study

During September 2011–February 2012, our laboratory received liver samples from 9 rabbitries from 3 areas of northeastern Spain where acute outbreaks of RHD were occurring in adult rabbits and kits. We analyzed 35 tissue samples from 20 kits (age 14–35 days), 9 growers (age 36–57 days), and 6 adults. Macroscopic lesions in infected kits were consistent with RHDV infection usually observed only in adult rabbits (*4*). The lesions in young rabbits consisted of hemorrhages in heart, trachea, thymus, lungs, liver, kidneys, and gut; jaundice was also seen. Mortality rates of up to 20% and 50% in adult and young rabbits, respectively, were observed. Infected samples came from vaccinated (n = 23) and unvaccinated young and adult rabbits.

Reverse transcription PCR was performed by using RNA extracted from 20 mg of liver samples using the mini RNAeasy RNA extraction kit (QIAGEN Iberia, Madrid, Spain), Superscript III reverse transcription (Invitrogen Corp., Carlsbad, CA, USA), LA-Taq polymerase (Takara Bio, Otsu, Japan), and forward and reverse primers annealing at nt 6056–6075 and 6775–6794, respectively (positions refer to the genomic sequence of RHDV Ast/89; GenBank accession no. Z49271). A band of the expected size (738 bp) was purified after gel electrophoresis and sequenced; this isolate was named RHDV-N11 and deposited into GenBank (accession no. JX133161). The sequenced region consisted of nt 6108–6716, corresponding to domains CDE and partial B and F domains of the VP1 capsid protein (*11*). Sequence analysis showed that samples from each farm contained the same virus (96.6% identity) and that the virus detected had 81% identity with both RHDV and RHDVa sequences (data not shown).

Multiple sequence alignment and phylogenetic analysis were performed by using the RHDV-N11 VP1 sequence and 37 other sequences (18 classic RHDV, 12 RHDVa, and 6 RCV-like, with European brown hare syndrome virus as an outlier). The RHDV-N11 sequence was shown to form a branch falling between RCV and RCV-A1, separated from RHDV and RHDVa (Figure 1). We suggest the term RHDVb for this new isolate type.

To confirm the presence of virions in the infected livers, liver homogenates (10% in sterile phosphate-buffered

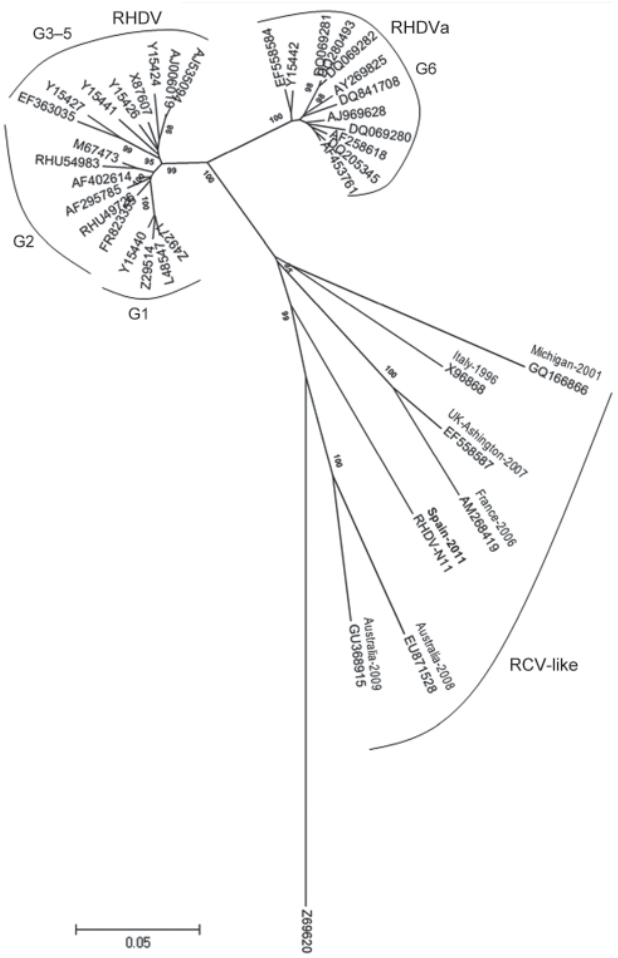

Figure 1. Evolutionary relationships of rabbit hemorrhagic disease virus (RHDV) and related viruses. A total of 38 nt sequences were analyzed: the isolate from this study, designated RHDV-N11 (GenBank accession no. JX133161); 18 classical RHDV and 12 RHDVa isolates; 6 rabbit calicivirus (RCV)–like isolates; and European brown hare syndrome virus (GenBank accession no. Z69620) as an outlier. Evolutionary history was inferred by using the neighbor-joining method; optimal tree with the sum of branch length = 1.33075964 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed by using the p-distance method (*14*) and are in the units of the number of base differences per site. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair; the final dataset consisted of 646 positions. Evolutionary analyses were conducted in MEGA5 (*15*). RHDV genetic groups are indicated. GenBank accession numbers of the sequences were: RCV-like strains: EU871528, GU368915, X96868, EF558587, AM268419, GQ166866; RHDV strains; AJ006019, AJ535094, Y15424, Y15426, Y15441, EF363035, Y15427, AF402614, RHU54983, AF295785, Z49271, L48547, Y15440, RHU49726, Z29514, M67473, X87607, FR823355; RHDVa strains: DQ069280, DQ280493, DQ069282, AY269825, DQ841708, DQ205345, AF258618, DQ069281, AJ969628, AF453761, EF558584, Y15442.

saline) were clarified by low-speed centrifugation followed by ultracentrifugation through a 30% sucrose cushion. Pellets were suspended in phosphate-buffered saline for further study. A major band of ≈60 kDa was observed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and detected by Western blot by using a rabbit polyclonal antibody against RHDV Ast/89 (*4*). Dot-blot analyses using monoclonal antibodies 1H8 and 6G2 (*12*) revealed that, although RHDV Ast/89 reacted with both monoclonal antibodies, the new RHDV-N11 isolate reacted with 6G2 only (data not shown). This type of reactivity (negative 1H8, positive 6G2) was found (L. Capucci, pers. comm.) for a recent French variant (*8*).

Agglutination studies showed that RHDV Ast/89 agglutinated human blood of all group types (O, A, B, and AB), as described for RHDV genetic group 1 members (*13*). RHDV-N11 showed no agglutination of blood groups O or A, but did agglutinate blood groups B and AB; this pattern is similar that of G4 and G6 groups (*13*) (Table 1).

To prove that the virus isolated was the etiologic agent of RHDV in these animals, we conducted a small-scale experiment. Six New Zealand white rabbits, 3 kits (30 days of age) and 3 adult rabbits were experimentally infected in a biosecurity level 2 laboratory with 15,000 hemagglutination units of purified RHDV-N11 virions (using B-type human erythrocytes). One additional adult and kit were used as uninoculated controls. Two routes of infection were used: 2 adult and 2 kits were infected subcutaneously, and 1 adult and 1 kit were infected intranasally. Forty-eight hours postinfection, 2 rabbits (1 adult and 1 kit) died. Postmortem analysis revealed discoloration of the liver and extensive hemorrhaging in the lungs. Ninety-six hours postinfection, the kit control died, also showing macroscopic lesions consistent with RHDV.

Immunohistochemical staining of tissues using the anti-RHDV mouse monoclonal 6G2 (1:700) and the Avidin-Biotin complex (Vectastain ABC Kit; Vector Laboratories Ltd., Peterborough, UK) confirmed the presence of RHDV-N11 VP1 in the liver, heart, kidney, spleen, lung, and intestine of infected rabbits. Data for RHDV-N11 antigen detection in the experimentally infected rabbits are shown in Table 2. Immunohistochemical analyses of tissues from the subcutaneously inoculated kit that died 48 h postinfection show hepatocytes with intense

Table 1. Agglutination titers for RHDV (Ast/89) and RHDV-N11, by human blood type, Spain

Virus	Human blood type†			
	O	A	B	AB
RHDV (Ast/89)	32,768	16,384	131,072	131,072
RHDV-N11	<2	<2	2,048	2,048

*RHDV, rabbit hemorrhagic disease virus.
†0.5% in phosphate-buffered saline at 4°C.

Table 2. Immunohistochemical detection of RHDV-N11 in tissues from experimentally infected and control rabbits by using monoclonal antibody 6G2*

Rabbit no. and age	Infection method	Tissue					
		Liver	Lung	Kidney	Spleen	Intestine	Heart
1. Adult (control)	SC	–	–	–	–	+/–	–
2. Young (control)†	SC	+	++	++	+	++	+/–
3. Young	SC	–	–	–	–	–	–
4. Adult‡	SC	+	+	++	+++	+	+/–
5. Young‡	SC	+++	+	++	+++	++	+/–
6. Adult	SC	–	–	–	–	–	–
7. Adult	IN	–	–	–	–	+	–
8. Young	IN	–	–	–	–	–	–

*Young rabbits were 30 d of age on the day of experimental infection. Control rabbits (1 and 2) were each injected with 500 µL sterile PBS. RHDV, rabbit hemorrhagic disease virus; PBS, phosphate-buffered saline; SC, subcutaneous injection; IN, intranasal administration; –, not detected; +, ++, +++, increasing levels of antigen.
†Died 96 h after experimental infection of animals 3–8.
‡Died 48 h after experimental infection.

RHDV-specific immunolabeling (Figure 2, panel A). Areas of focal necrosis and epithelial cells showing strong immunolabeling were also observed in the intestinal villi in the small intestine (Figure 2, panel B).

RNA extracted from the livers of the rabbits that died after experimental RHDV infection was analyzed by RT-PCR. These samples were confirmed to contain virus sequences corresponding to those of the new variant RHDV.

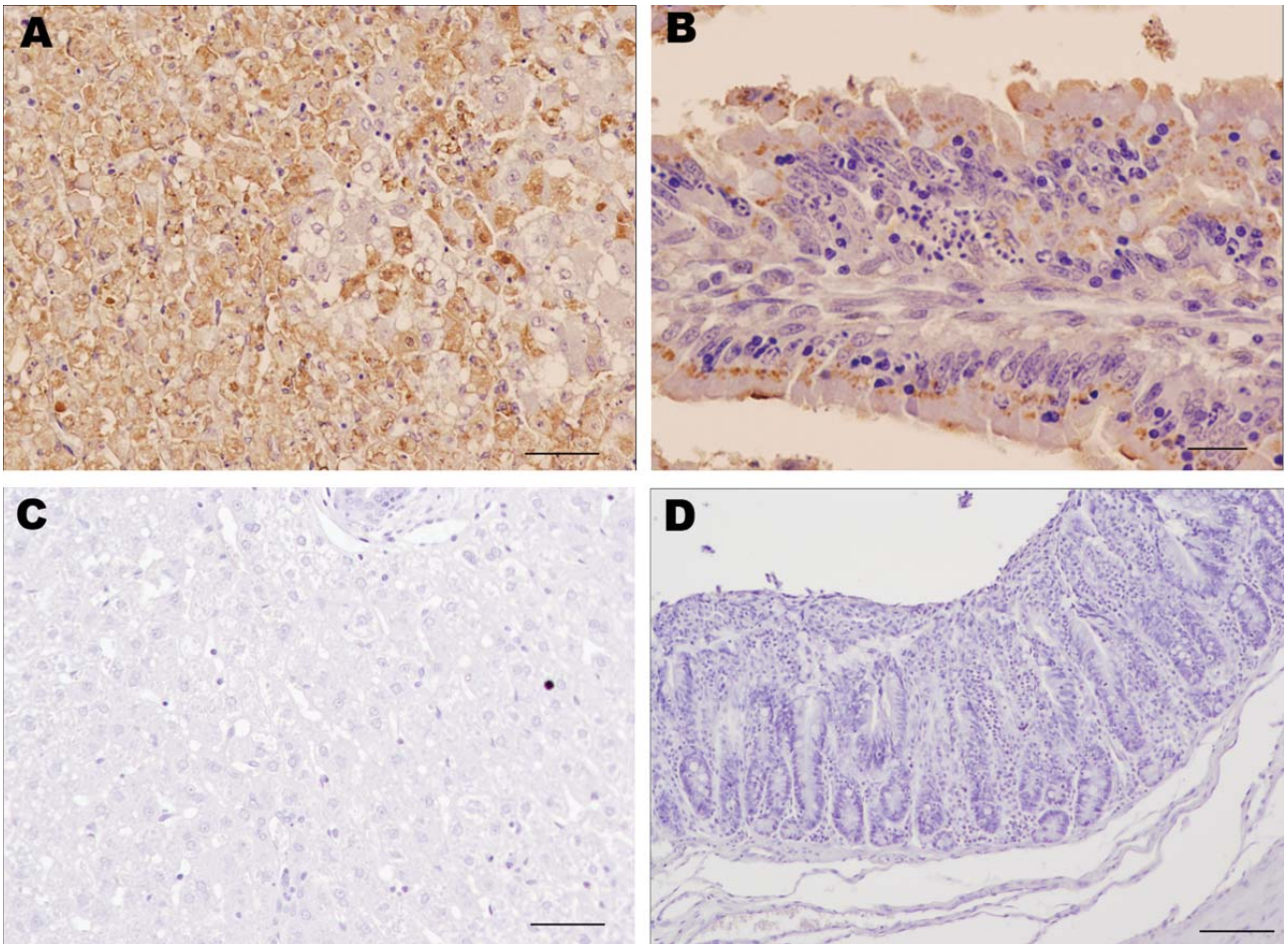

Figure 2. Results of immunohistochemical staining using monoclonal antibody 6G2 and the ABC complex technique of liver and intestine samples from young rabbits infected with rabbit hemorrhagic disease virus (RHDV) isolate RHDV-N11 and control rabbits. A) Liver of RHDV-N11–infected rabbit. Hepatocytes show intense 6G2-specific immunolabeling. Scale bar = 50 µm. B) Intestinal villi in small intestine of RHDV-N11–infected rabbit. Areas of focal necrosis and epithelial cells show strong immunolabeling. Scale bar = 20 µm. C) Liver of control rabbit. Hepatocytes do not show positive immunolabeling. Scale bar = 50 µm. D) Epithelial cells of intestinal villi of control rabbit do not show positive results on staining. Scale bar = 100 µm.

Conclusions

A variant of RHDV has been detected on the Iberian Peninsula in Spain and found to be responsible for causing disease and death in kits <30 days of age, even though rabbits of this age were not known to be susceptible to illness caused by RHDV infection. The RHDV-N11 variant differs antigenically from classic RHDV, as determined by dot-blot analyses using monoclonal antibodies and hemagglutination analysis. Phylogenetic analysis suggests that this virus isolate is genetically distant from RHDV and RHDVa and is more closely related to the so-called RCV apathogenic viruses. On the basis of these data, we suggest the use of the term RHDVb for this variant.

The occurrences of these outbreaks on farms where rabbits were previously vaccinated against RHDV raises serious concerns about the efficacy of the vaccines. Mutations of critical amino acids that result in antigenic differences, as suggested by the different monoclonal antibody recognition patterns, could explain why the immune response in vaccinated rabbits did not protect against RHDV-N11. Changes in receptor usage, as suggested by the modified agglutination patterns, may explain the different pathogenicity of this variant RHDV. These findings suggest the need to improve prophylactic reagents and reinforce hygienic measures to avoid the spread of this reemerging pathogen.

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Dr Dalton is a postdoctoral researcher at the Instituto Universitario de Biotecnología de Asturias, University of Oviedo, Spain. His research interests include molecular diagnosis and characterization of rabbit viruses.

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Address for correspondence: Francisco Parra, Departamento de Bioquímica y Biología Molecular, Edificio Santiago Gascón, Campus El Cristo, Universidad de Oviedo, 33006 Oviedo, Spain; email: fparra@uniovi.es

Reservoir Competence of Vertebrate Hosts for *Anaplasma phagocytophilum*

Felicia Keesing, Michelle H. Hersh, Michael Tibbetts, Diana J. McHenry, Shannon Duerr, Jesse Brunner, Mary Killilea, Kathleen LoGiudice, Kenneth A. Schmidt, and Richard S. Ostfeld

Fourteen vertebrate species (10 mammals and 4 birds) were assessed for their ability to transmit *Anaplasma phagocytophilum*, the bacterium that causes human granulocytic anaplasmosis, to uninfected feeding ixodid ticks. Small mammals were most likely to infect ticks but all species assessed were capable of transmitting the bacterium, in contrast to previous findings.

Human granulocytic anaplasmosis (HGA), formerly known as human granulocytic ehrlichiosis, is an emerging infectious disease in the United States, Europe, and Asia (*1*,*2*). In the United States, most reported cases are concentrated in north-central and northeastern states. Patients with HGA typically have nonspecific febrile symptoms, including fever, chills, headache, and myalgia (*1*). Most patients with HGA respond well to antimicrobial drug treatment, but complications are not uncommon and some cases are fatal (*2*). Because of difficulties in diagnosis and lack of awareness of HGA by physicians and the public, many cases are misdiagnosed, and national statistics likely dramatically underreport this disease (*1*).

HGA is caused by a rickettsial bacterium, *Anaplasma phagocytophilum* (*1*), groups of which form dense aggregations in granulocytes (*3*). The bacterium is passed from host to host through the bite of an infected ixodid tick: *Ixodes scapularis* in the eastern and central United States and *Ix. pacificus* in the western United States (*4*–*6*). Serosurveys and molecular diagnostics within disease-endemic zones show that many ground-dwelling vertebrate species are exposed to or infected with *A. phagocytophilum* (*2*). These data indicate that tick-to-host transmission rates are high and that infection is widespread in host communities.

However, few studies have examined rates of transmission from infected hosts to uninfected ticks, a trait known as the reservoir competence of these hosts. Quantification of host species–specific reservoir competence can identify animals most responsible for producing infected ticks and therefore increasing risk for human exposure. Overall, robust quantitative information on reservoir competence is scarce and key hosts remain unstudied. We determined the reservoir competence for *A. phagocytophilum* of 14 species (10 mammals and 4 birds) in a disease-endemic region of the eastern United States.

The Study

All procedures were conducted after approval from the Cary Institute of Ecosystem Studies Institutional Animal Care and Use Committee. We conducted our research in Dutchess County, New York, a region where human cases of anaplasmosis are rapidly increasing. We trapped hosts on the property of the Cary Institute of Ecosystem Studies (Millbrook, NY, USA) during the peak abundance of larval blacklegged ticks (*Ix. scapularis*) during July–September in 2008, 2009, and 2010. Detailed methods have been reported (*7*).

We held members of 10 mammal and 4 bird species (Table 1) for 3 days in cages with wire mesh floors suspended over pans lined with wet paper towels. Ticks feeding on hosts were allowed to feed to repletion and drop from hosts into the pans, from which they were collected. In some cases, if hosts did not drop >10 ticks within 3 days, we infested them with unfed larval ticks following methods described (*8*). Because no evidence has been found for transovarial transmission of *A. phagocytophilum* (*9*) or of infection in larval ticks, these infestations likely did not affect host exposure to the pathogen. Hosts that had been infested were held for an additional 4 days, and engorged ticks were collected each day. All engorged larval ticks were held in moistened glass vials at constant temperature and humidity until they molted into the nymphal stage. Newly molted nymphs were flash-frozen in liquid nitrogen and stored at −80°C.

DNA extraction was conducted as described (*7*). To amplify extracted DNA, we used protocols reported by Courtney et al. (*10*). Briefly, we used primers ApMSP2f and ApMSP2r and probe ApMSP2p, which are specific for the *msp2* gene of *A. phagocytophilum* and generate a 77-bp fragment. Real-time PCR was performed by using a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) . We used extracted DNA from unfed larval ticks and ultrapure water as negative controls to account for potential

Bard College, Annandale-on-Hudson, New York, USA (F. Keesing, M.H. Hersh, M. Tibbetts, D.J. McHenry); Cary Institute of Ecosystem Studies, Millbrook, NY, USA (F. Keesing, M.H. Hersh, S. Duerr, R.S. Ostfeld); Washington State University, Pullman, Washington, USA (J. Brunner); New York University, New York, New York, USA (M. Killilea); Union College, Schenectedy, NY, USA (K. LoGiudice); and Texas Tech University, Lubbock, TX, USA (K.A. Schmidt)

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Table 1. Host species tested for *Anaplasma phagocytophiluum* reservoir competence, southeastern New York, USA, 2008–2010*

Host species	Common name	No. hosts tested	No. ticks tested	Mean no. ticks sampled per host (range)
Mammals				
Blarina brevicauda	Northern short-tailed shrew	28	529	18.9 (11–25)
Didelphis virginiana	Virginia opossum	25	501	20.0 (11–25)
Glaucomys volans	Southern flying squirrel	4	59	14.8 (6–25)
Mephitis mephitis	Striped skunk	1	21	21.0 (21–21)
Peromyscus leucopus	White-footed mouse	30	571	19.0 (10–25)
Procyon lotor	Raccoon	25	484	19.4 (10–25)
Sciurus carolinensis	Eastern gray squirrel	20	358	17.9 (10–25)
Sorex cinereus	Masked shrew	6	41	6.8 (4–10)
Tamias striatus	Eastern chipmunk	19	300	15.8 (9–25)
Tamiasciurus hudsonicus	Eastern red squirrel	15	297	19.8 (11–25)
Birds				
Catharus fuscescens	Veery	21	427	20.3 (10–25)
Dumetella carolinensis	Gray catbird	14	235	16.8 (9–24)
Hylocichla mustelina	Wood thrush	28	496	17.7 (10–24)
Turdus migratorius	American robin	18	321	17.8 (8–24)

*Number of ticks tested per host can include samples from either natural body loads or experimental infestations, as described in the text, and is not representative of mean total body loads.

contamination during the extraction and PCR processes, respectively. The cloned 77-bp fragment was used as a positive control. Barrier pipette tips were used throughout the process to prevent contamination. We conducted 3 replicate PCRs per tick.

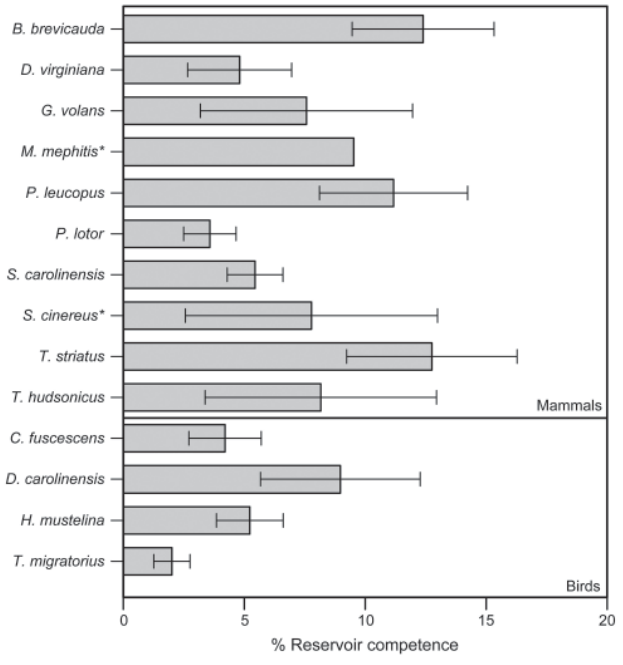

Figure 1. Mean reservoir competence of 14 host species (10 mammals and 4 birds) for *Anaplasma phagocytophilum*, southeastern New York, USA, 2008–2010. Error bars indicate SE. Reservoir competence is defined as the mean percentage of ticks infected by any individual host of a given species. Host species with <10 individual hosts sampled are indicated by an asterisk. See Table 1 for sample sizes. Single-letter abbreviations for genera along the left indicate *Blarina*, *Didelphis*, *Glaucomys*, *Mephitis*, *Peromyscus*, *Procyon*, *Sciurus*, *Sorex*, *Tamias*, *Tamiasciurus*, *Catharus*, *Dumetella*, *Hylocichla*, and *Turdus*, respectively.

Ticks were considered positive for *A. phagocytophilum* if any 1 of 3 replicate samples showed amplified DNA for *A. phagocytophilum* relative to negative controls. Ticks with marginal results (i.e., moderate fluorescence) were tested a second time with the same primers and SYBR green dye. For these confirmatory tests, we included a melt curve analysis in which we determined the temperature at which half of the PCR products had denatured. PCR products were heated from 70°C through 85°C, raising the temperature by 0.5°C every 10 s. Positive controls consistently had melting point maxima of 80.5°C. Using a TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), we cloned and sequenced 140 fragments that had a melting point of 80.5°C. Identity of sequences was confirmed by conducting BLAST searches (National Center for Biotechnology Information, Bethesda, MD, USA) of GenBank using the blastn algorithm (*11*). One hundred thirty-one of 140 fragments were identified as *A. phagocytophilum*; the remaining 9 fragments either had poor-quality sequences or did not have the cloning vector inserted. If any replicate was positive in the confirmatory test, ticks were considered positive for *A. phagocytophilum*. If all 3 replicates in the confirmatory test showed marginal or negative results, the ticks were considered negative. Reservoir competence for each host species was calculated as the average percentage of ticks infected per individual host.

Using data for 4,640 ticks collected from 254 animals over 3 years, we assessed levels of reservoir competence for 14 host species (10 mammals and 4 birds) (Table 1). Short-tailed shrews, white-footed mice, and eastern chipmunks had mean levels of reservoir competence >10% (Figure 1). All other hosts, including opossums, gray and red squirrels, and all 4 species of birds, had mean levels of reservoir competence ranging from 2% to 10%. Reservoir competence differed significantly among these 11 species (F = 2.294, df = 10,232, p = 0.014, by 2-way analysis of

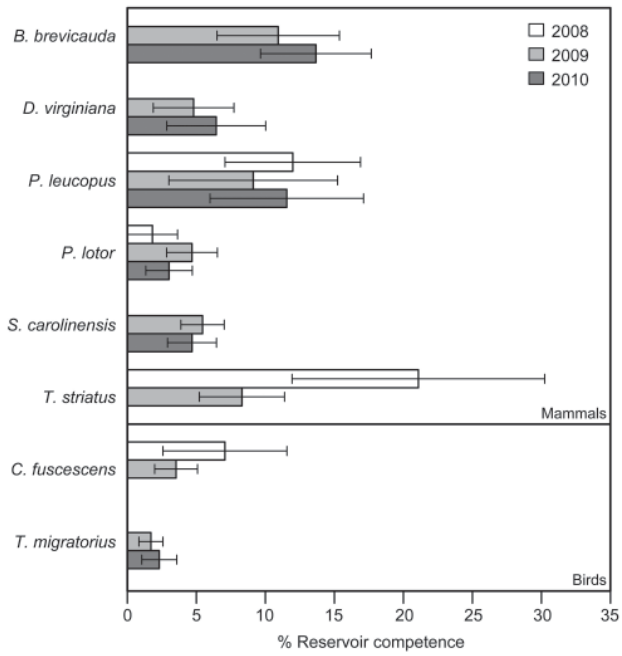

Figure 2. Mean reservoir competence of 14 host species (10 mammals and 4 birds) for *Anaplasma phagocytophilum*, southeastern New York, USA, 2008–2010. Error bars indicate SE. Reservoir competence is defined as the mean percentage of ticks infected by any individual host of a given species. For inclusion, sample sizes for a species had to be ≥4 in ≥2 years. No species showed significant variation in reservoir competence across years (p>0.10, by 2-way analysis of variance or Kruskal-Wallis test as appropriate, for all species tested). Single-letter abbreviations for genera along the left indicate *Blarina*, *Didelphis*, *Peromyscus*, *Procyon*, *Sciurus*, *Tamias*, *Catharus*, and *Turdus*, respectively.

variance). Southern flying squirrels, striped skunks, and masked shrews all transmitted *A. phagocytophilum* to ticks, but our sample sizes were too small to draw strong conclusions about reservoir competence. For species that we collected in abundant numbers in multiple years (≥4 animals in ≥2 years), reservoir competence of each species did not vary significantly from year to year (p>0.10 for all species tested, by analysis of variance or Kruskal-Wallis tests as appropriate) (Figure 2).

Conclusions

Our data contradict several assumptions about the role of hosts in infecting ticks with *A. phagocytophilum*. First, the role of the white-footed mouse in infecting ticks has been controversial (*2*). Our data suggest that although the mouse is a major reservoir, short-tailed shrews and eastern chipmunks have comparable levels of reservoir competence. In addition, previous work has suggested that chipmunks, skunks, and opossums do not infect feeding ticks (*12*). At our sites, all of these species infected feeding ticks (Table 2). Thus, the potential for these hosts to contribute to human risk for HGA should not be ignored.

Because hosts are capable of clearing *A. phagocytophilum* infections (*13*), surveys of host exposure might not represent species-specific probabilities of transmitting the pathogen to uninfected ticks. Instead, the role of particular species in contributing to the pool of infected ticks is best assessed by determining host reservoir competence using field-captured animals that usually carry ticks. On the basis of the community of hosts we sampled, small mammals are most responsible for infecting uninfected larval ticks in nature, and this result is consistent across years.

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Table 2. Host species infected with *Anaplasma phagocytophilum* southeastern New York, USA, 2008–2010*

Host species	No. hosts infected/no. tested (%)	No. (%) ticks infected	Mean % infected ticks per infected host (range)
Mammals			
Blarina brevicauda	17/28 (61)	67 (13)	20 (4–56)
Didelphis virginiana	9/25 (36)	20 (4)	13 (4–50)
Glaucomys volans†	2/4 (50)	5 (8)	15 (14–16)
Mephitis mephitis†	1/1 (100)	2 (10)	10
Peromyscus leucopus	15/30 (50)	63 (11)	22 (4–50)
Procyon lotor	10/25 (40)	17 (4)	9 (4–20)
Sciurus carolinensis	14/20 (70)	19 (5)	8 (4–20)
Sorex cinereus†	2/6 (33)	4 (10)	23 (17–30)
Tamias striatus	10/19 (53)	40 (13)	24 (6–46)
Tamiasciurus hudsonicus	7/15 (47)	17 (6)	17 (4–73)
Birds			
Catharus fuscescens	9/21 (43)	19 (4)	10 (4–25)
Dumetella carolinensis	7/14 (50)	20 (9)	18 (4–33)
Hylocichla mustelina	14/28 (50)	27 (5)	10 (4–25)
Turdus migratorius	6/18 (33)	7 (2)	6 (4–11)

*Infected hosts are those that transmitted *A. phagocytophilum* to ≥1 *Ixodes scapularis* tick larva.
†Host species with <10 individual hosts sampled.

Dr Keesing is an educator and scientist in the Program in Biology at Bard College in Annandale, NY, and an adjunct scientist at the Cary Institute of Ecosystem Studies in Millbrook, NY. Her major research interest is the ecology of infectious diseases.

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Address for correspondence: Felicia Keesing, Bard College Program in Biology, Box 5000, Annandale-on-Hudson, NY, 12504, USA; email: keesing@bard.edu

MRSA Variant in Companion Animals

Birgit Walther, Lothar H. Wieler, Szilvia Vincze, Esther-Maria Antão, Anja Brandenburg, Ivonne Stamm, Peter A. Kopp, Barbara Kohn, Torsten Semmler, and Antina Lübke-Becker

Methicillin-resistant *Staphylocoocus aureus* (MRSA) harboring *mec*A_{LGA251} has been isolated from humans and ruminants. Database screening identified this MRSA variant in cats, dogs, and a guinea pig in Germany during 2008–2011. The novel MRSA variant is not restricted to ruminants or humans, and contact with companion animals might pose a zoonotic risk.

Worldwide, methicillin-resistant *Staphylococcus aureus* (MRSA) is a leading cause of infectious diseases in humans and animals (*1*). The staphylococcal cassette chromosome *mec* (SCC*mec*) harbors the *mec*A gene, which encodes an additional penicillin-binding protein 2a. In the presence of β-lactam antimicrobial drugs, this transpeptidase substitutes an essential cross-linking step in the process of cell-wall building (*2*). Eleven distinct SCC*mec* elements have been described (*3*). Recent reports of MRSA carrying a novel *mec*A homologue (*mec*A_{LGA251}) of a predicted amino acid identity of 62% with other *mec*A allotypes raised awareness about these pathogens, which possibly remain undetected by conventional PCR approaches (*3*–*5*). This lack of detection might have led to underestimation of the novel MRSA variant among clinical samples of human and animal origin.

High-level congruence between *S. aureus* of animal and human lineages has been demonstrated (*6*), and nearly every sequence type (ST) reported for MRSA associated with infections in companion animals was also commonly found in humans (*1*). Because previous reports indicated that MRSA harboring *mec*A_{LGA251} originated from either human or ruminant hosts (*3*–*5*), we searched our database for companion animal isolates that displayed a MRSA phenotype but had failed to give a positive PCR result for *mec*A.

Author affiliations: Freie Universität Berlin, Berlin, Germany (B. Walther, L. H. Wieler, S. Vincze, E.-M. Antão, B. Kohn, T. Semmler, A. Lübke-Becker); and Vet Med Labor GmbH, Ludwigsburg, Germany (A. Brandenburg, I. Stamm, P.A. Kopp)

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The Study

From November 2008 through December 2011, MRSA of companion animal origin was routinely isolated from specimens submitted for diagnostic purposes to Vet Med Labor GmbH in Ludwigsburg, Germany, or to the Institute of Microbiology and Epizootics, Freie Universität Berlin, in Berlin, Germany. *S. aureus* was confirmed as described (*7*) and stored in glycerol stocks at −80°C.

PCR routinely used to confirm methicillin resistance and species identity had failed to produce a positive signal for *mec*A in 10 MRSA isolates from companion animals (2 isolates from dogs, 7 from cats, and 1 from a guinea pig) (*8*). We screened these 10 isolates for the *mec*A homologue by using the PCR method published by Cuny et al. (*5*) and sent the amplicons obtained to LGC Genomics GmbH (Berlin, Germany) for sequencing. Automated antimicrobial drug susceptibility testing was performed by using the bioMérieux VITEK 2 system (Nürtingen, Germany) according to the manufacturer's instructions. The following drugs were tested according to Clinical and Laboratory Standards Institute guidelines M31–A3: penicillin, ampicillin–sulbactam, oxacillin, gentamicin, kanamycin, enrofloxacin, marbofloxacin, erythromycin, clindamycin, tetracycline, nitrofurantoin, chloramphenicol, and trimethoprim–sulfamethoxazole, (*9*). All isolates were further characterized by *spa* typing, multilocus sequence typing, and microarray hybridization by using the Alere Identibac *S. aureus* Genotyping chip (Alere Technologies GmbH, Jena, Germany) as described (*10*–*12*).

The presence of the *mec*A homologue was verified for all 10 isolates. All PCR amplicons demonstrated 100% identity with the DNA sequence of *mec*A_{LGA251} (National Center for Biotechnology Information no. FR821779.1). The strains originated from geographically diverse areas (5 federal states of Germany) and were isolated from different infection sites (Table). All strains were identified as MRSA by the VITEK 2 system (growth in the presence of 6 μg/mL cefoxitin according to the VITEK 2 Advanced Expert System), although oxacillin MICs were rather low (0.5 μg/mL) or moderately high (≈4 μg/mL) (Table). Phenotypic resistance toward non–β-lactams was not detected.

As has been described for atypical MRSA, 4 strains belonged to ST130 and 1 strain belonged to ST1945 (differs from ST130 by 1 allele) (*3*–*5*). The remaining 5 isolates were assigned to ST599 (differs from ST121 by 2 alleles) (Table). ST599 has been reported for methicillin-susceptible isolates from humans in Europe, Asia, and Africa (www.mlst.net). The Figure shows a minimum spanning tree based on 4,197 entries of the *S. aureus* multilocus sequence type database (http://saureus.mlst.net/) as of January 19, 2012 (Figure, panel A) and a detailed view of the branches and STs harboring strains with the novel *mec*A homologue published (Figure, panel B) (*3*–*5*).

Table. Characteristics of 10 methicillin-resistant *Staphylococcus aureus* isolates harboring *mecA*$_{LGA251}$ obtained from companion animals, Germany, 2008–2011*

IMT no.	Original no.	Year isolated	Host	Site	Clinical signs	Free-ranging animal	Federal state	OXA MIC†	*spa* type‡	ST§
17403	VB 999987	2008	Cat	Eye	Purulent infection	Yes	Rhineland-Palatinate	≥4	t10033	1945
21135	VB 964992	2010	Cat	Wound	Lymphadenitis	Yes	Bavaria	≥4	t843	130
21231	VB 971931	2010	Cat	Skin	Dermatitis	Unknown	Hessia	1	t1773	130
24068	VB 961584	2010	Cat	Tachea	Stridor	Yes	Hessia	0.5	t10006	599
25044	VB 969929	2010	Dog	Abscess	Tumor, dolor	No	Bavaria	2	t1694	599
25147	VB 969572–2	2010	Cat	Wound	Suture dehiscence	Yes	North Rhine-Westphalia	2	t278	599
25470	VB 972406	2010	Dog	Eye	Purulent infection	No	Bavaria	2	t1694	599
25715	VB 969935	2010	Guinea pig	Fistula	Purulent infection	No	Hessia	2	t843	130
28299	VB 952042	2011	Cat	Phlegmon	Dermatitis	Yes	Bavaria	1	t278	599
28429	IMT 2272/11	2011	Cat	Abscess	Fever	Yes	Berlin	≥4	t10009	130

*IMT, Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany; OXA, oxacillin; ST, sequence type; VB, Vet Med Labor GmbH, Ludwigsburg, Germany. All isolates were positive for *nuc* and negative for *mecA* according to PCR to detect MRSA (*8*), and all were positive for *mecA*$_{LGA251}$ according to PCR to detect the novel *mec*A homologue (*5*).
†Detected by the VITEK 2 system (bioMérieux, Nürtingen, Germany).
‡*Spa* repeats and *spa* type determined according to Harmsen et al. (*11*) and the Ridom SpaServer (http://spaserver.ridom.de).
§Determined according to Enright et al. (*10*).

Microarray hybridization data revealed that the *agr* type I and capsule type 5 seem to be associated with ST599 and *agr*III and that clonal complex (CC) 130 isolates harbor the capsule type 8 encoding gene. CC130 and ST599 isolates were positive for the surface-associated proteins *clf*A, *clf*B, *fnb*A, and *bbp*. All ST599 strains produced a positive hybridization result for 1 of the gene variants encoding the toxin responsible for toxic shock syndrome (*tst1* or *tst-bov*), and all but 1 of them harbored the enterotoxins C (*sec*) and L (*sel*), indicating the presence of an *S. aureus* pathogenicity island that encodes superantigens (*13*). Positive or ambiguous hybridization signals for *ccrB1*, *ccrA3*, and *ccrB3* were obtained for 5 isolates, suggesting the presence of the SCC*mec*XI in those strains, according to the results of Shore et al. (*4*).

Conclusions

Our findings of CC130 and ST599 MRSA harboring *mecA*$_{LGA251}$ in several companion animal species suggest that in Germany, the presence of the *mec*A homologue in MRSA is not exclusively associated with CC130. This finding supports the hypothesis that some, if not all, MRSA strains that harbor the novel *mec*A variant can cause infections among a broad variety of hosts, as has been shown for MRSA of human, equine, canine, and other companion animal origins (*1*,*7*). All currently known *mecA*$_{LGA251}$–

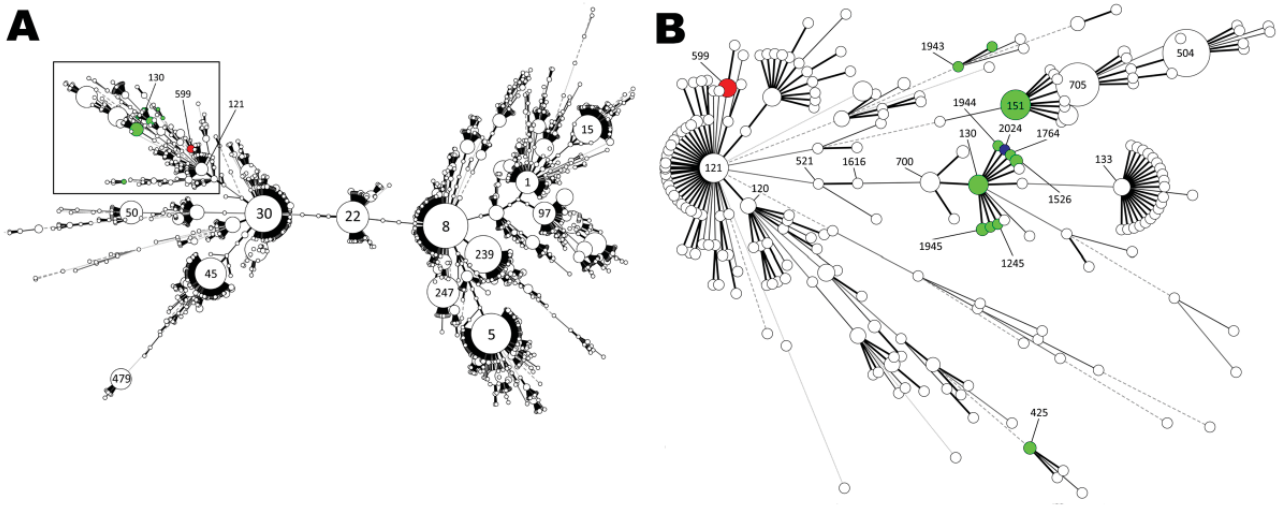

Figure. Minimum spanning tree based on multilocus sequence typing data from 4,197 *Staphylococcus aureus* strains (A) and an enlarged view of 1 phylogenetic group (B). Each circle represents a distinct sequence type (ST), and circle size is proportional to ST frequency. A color version of this figure, showing *mecA*$_{LGA251}$-positive *S. aureus* strains of companion animal origin reported in this study and sequence data from published multilocus sequence typing results (*3*–*5*); ST599 methicillin-resistant *S. aureus*; and ST2024 methicillin-sensitive *S. aureus* isolated from a wild rat, is available online (wwwnc.cdc.gov/EID/article/18/12/12-0238-F1.htm).

carrying MRSA were observed in a distinct section of the *S. aureus* population (Figure, panel B). Whether this phylogenetic group possesses the ability to integrate the novel *mec*A variant needs to be further investigated.

In the past, *mec*A_{LGA251}–carrying MRSA could have been misidentified as methicillin sensitive by routine PCR. However, all isolates were correctly identified as MRSA by the VITEK 2 system, as reported (*4*).

Of the 10 isolates, 7 were found in specimens from cats. A recent study identified cats as a potential natural reservoir for *S. aureus* of CC133, a genetic lineage that has also been reported for *S. aureus* of ruminant origin (*14*). Moreover, we have identified a CC130 strain (MSSA_ST2024, t8403) from a wild rat (IMT21250; ID4035) (www.mlst.net). In addition, CC130 MRSA containing the *mec*A homologue has only recently been reported for isolates from humans in Germany (*5*).

Although many investigators focus on livestock-associated MRSA, and because particular companion animal lineages of MRSA seem to be lacking, transmission of MRSA between companion animals and human family members in close proximity might be underestimated, especially in cases of recurrent infection (*15*). The emergence of MRSA harboring the novel *mec*A homologue has consequences for the verification methods for MRSA used in veterinary medicine; implementation of new methods will be inevitable. Their supposed restriction to only a few genetic lineages and the potential risk for interspecies transmission of atypical MRSA between companion animals and their owners in household environments needs further elucidation.

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Dr Walther is a veterinarian holding a postdoctoral position at the Institute of Microbiology and Epizootics in the Infectious Disease Diagnostics and Molecular Epidemiology Working Group. Her primary research interests focus on molecular epidemiology of MRSA and methicillin-resistant *S. pseudintermedius* in companion animals.

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Address for correspondence: Birgit Walther, Institute of Microbiology and Epizootics, Freie Universität Berlin, Philippstr. 13, 10115 Berlin, PO Box 040225, 10061 Berlin, Germany; email: walther.birgit@vetmed.fu-berlin.de

Arctic-like Rabies Virus, Bangladesh

Khondoker Mahbuba Jamil, Kamruddin Ahmed, Moazzem Hossain, Takashi Matsumoto, Mohammad Azmat Ali, Sohrab Hossain, Shakhawat Hossain, Aminul Islam, Mohammad Nasiruddin, and Akira Nishizono

Arctic/Arctic-like rabies virus group 2 spread into Bangladesh ≈32 years ago. Because rabies is endemic to and a major public health problem in this country, we characterized this virus group. Its glycoprotein has 3 potential *N*-glycosylation sites that affect viral pathogenesis. Diversity of rabies virus might have public health implications in Bangladesh.

Rabies virus causes severe encephalitis in a wide range of mammals, including humans. Conservative estimates suggest that 55,000 persons worldwide die of rabies each year (*1*). Although the case-fatality rate in humans is 100%, rabies is preventable by vaccination. Bangladesh has the world's third highest death rate for human rabies, an estimated 2,100 deaths per year (*2*). Dogs are the main reservoir of the virus and are responsible for spillover infections in humans (*2*). Therefore, dogs should be the principal target for successful rabies elimination.

With political will and solid global epidemiologic information, rabies elimination is possible. Molecular typing of circulating rabies viruses is necessary to identify and develop effective control measures, and to understand the spread of certain rabies virus variants and their incursion into new regions (*3*). For rabies elimination, this knowledge is needed for establishing cooperative approaches between neighboring countries to which the disease is endemic.

Bangladesh is one of several countries in which no molecular study has been conducted to identify types of rabies virus circulating within its boundaries. A lack of knowledge of phylogenetic relationships of Bangladesh rabies virus with viruses in other countries continues to hinder coordinated rabies control efforts in the region. This study was conducted to characterize rabies virus circulating in Bangladesh and to determine its relationship with viruses in neighboring countries to clarify its epidemiologic relationships, origin, and transmission dynamics.

Author affiliations: Institute of Epidemiology, Disease Control, and Research, Dhaka, Bangladesh (K.M. Jamil); Oita University, Oita, Japan (K. Ahmed, T. Matsumoto, A. Nishizono); Ministry of Health and Family Welfare, Dhaka (M. Hossain); Dhaka City Corporation, Dhaka (M.A. Ali, S. Hossain, A. Islam, M. Nasiruddin); and Tongi Municipality, Tongi, Bangladesh (S. Hossain)

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The Study

Seven brain samples were collected from animals with suspected rabies in 3 districts of Bangladesh (Dhaka, Narayanganj, and Narshingdi) in 2010 (Table 1). A portion of brainstem was removed from each sample and preserved in TRizol (Invitrogen, Carlsbad, CA, USA) at –20°C. Total RNA was extracted from brain homogenate, cDNA was synthesized by using random hexamer primers, reverse transcription PCR was conducted to amplify gene fragments, and nucleotide sequencing of genes was performed (*4*).

Full-length nucleoprotein (N) and glycoprotein (G) gene sequences from samples were determined. Nucleotide identities of N and G genes were 98%–100%. Amino acid identities of N and G genes were 100% and 98%–100%, respectively. Complete genomic sequencing (11,928 nt) of strain BDR5 was also conducted.

Evolutionary analysis was performed by using full-length N gene. We created a maximum clade credibility phylogenetic tree using the Bayesian Markov chain Monte Carlo method available in BEAST version 1.6.1 (*5*). Analysis was conducted by using a relaxed (uncorrelated lognormal) molecular clock and a generalized time reversible + Γ + proportion invariant model (*6*). All chains were run for 90 million generations and sampled every 3,000 steps and an effective sample size >1,383 was obtained for all estimated parameters. Posterior densities were calculated with 10% burn-in and checked for convergence by using Tracer version 1.5 in BEAST.

The mean rate of nucleotide substitution estimated for the N gene was 2.3×10^4 substitutions/site/year (95% highest posterior density [HPD] $1.4–3.1 \times 10^4$ substitutions/site/year). This rate is consistent with that of a previous study (*7*). The phylogenetic tree showed that rabies viruses in Bangladesh belong to Arctic/Arctic-like group 2 (AAL2) (*3*) also known as Arctic-like-1 (*8*), in close association with the strain from Bhutan.

Approximately 397.0 years ago (95% HPD 273.5–589.5 years), AAL and cosmopolitan rabies virus segregated from their most recent common ancestor (Figure 1). Approximately 225.6 years ago (95% HPD 157.4–324.2 years), AAL3 segregated. Approximately 187.4 years ago (95% HPD 129.0–271.9 years), AAL1 and AAL2 segregated. The AAL2 clade had a common progenitor that circulated ≈133.1 years ago (95% HPD 91.3–193.4 years), which has evolved into several different lineages. One lineage evolved 91.5 years ago (95% HPD 63.1–132.2 years) and currently circulates in Bangladesh, India, and Bhutan. Separate linages circulate in others

Table 1. Characteristics of 7 animal samples tested for rabies virus, Bangladesh

Sample no.	Animal	Age, y	District	History	Signs and symptoms	GenBank accession no.*
BDR1	Dog	Unknown	Dhaka	Unknown	Angry, biting tendency, excessive salivation, gradually became drowsy	Not determined
BDR2	Cow	8	Narsingdi	Calf died of suspected rabies 1 wk earlier	Angry, salivation, drooping of tongue, inability to drink or eat	AB699208
BDR3	Cow	10	Dhaka	Unknown	Angry, salivation, frequent micturition, inability to drink or eat	AB699209
BDR4	Goat	3	Narayanganj	Dog bite 2.5 mo earlier	Angry, inability to eat and drink, biting tendency	AB699210
BDR5	Goat	2	Narayanganj	Dog bite to head 2 mo earlier	Angry, salivation, inability to eat and drink	AB699220 (whole genome)
BDR6	Cow	6	Dhaka	Unknown	Angry, salivation, trying to attack	AB699212
BDR7	Cow	5	Narayanganj	Dog bite 2 mo earlier	Angry, salivation, trying to attack	AB699213

*For glycoprotein gene.

countries in this region, including Iran, Nepal, Pakistan, and Afghanistan. AAL2 spread into central Bangladesh 32.3 years ago (95% HPD 18.4–50.6 years) in ≈1978 (95% HPD range 1958–1991).

Compared with the AAL2 strain from India (AY956319), BDR5 had several amino acid substitutions (Table 2). Sizes of their 2 genomes, leader RNA, trailer RNA, and intergenic regions were similar. The *N*-glycosylation site was predicted by using the NetNGlyc 1.0 server (www.cbs.dtu.dk/server/netnglyc). With the exception of BDR6, the G gene of all strains had potential glycosylation sites at position 37, 146, and 319.

Conclusions

Genetic analysis and phylogenetic studies can contribute to understanding the epidemiology of rabies virus in disease-endemic countries. Molecular analysis of animal rabies viruses showed that AAL2 appeared in central Bangladesh only 32 years ago. A close association between N genes sequences from rabies viruses in Bangladesh and

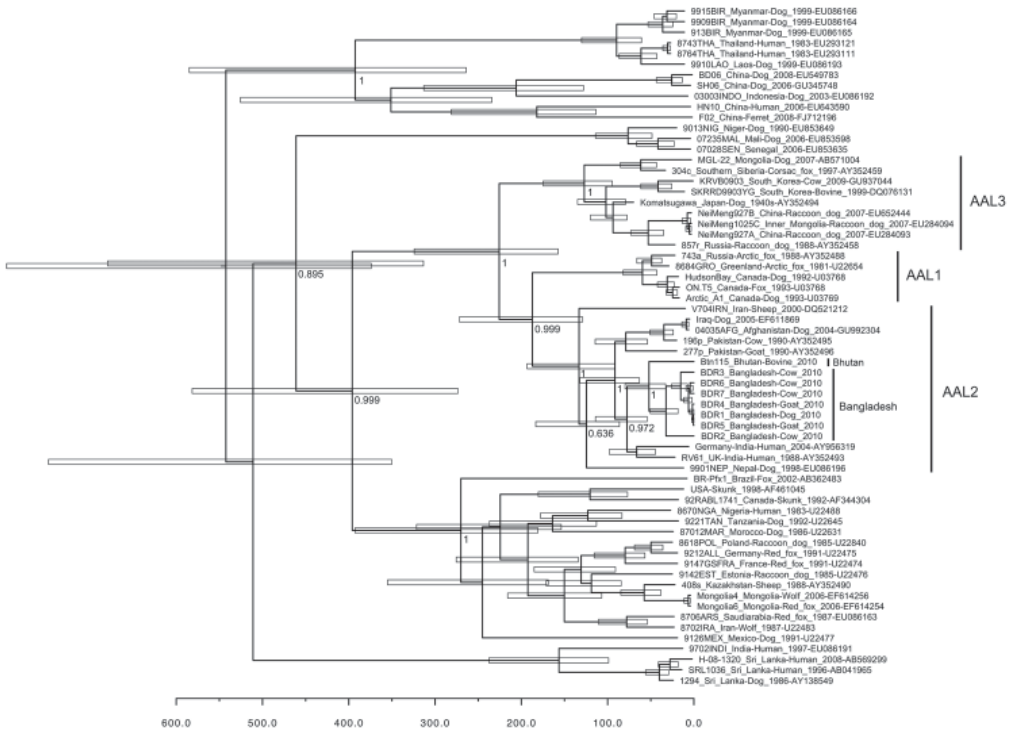

Figure 1. Bayesian maximum credibility tree showing genealogy of rabies virus obtained by analyzing nucleotide sequences of full nucleoprotein (N) gene sequences (1,350 nt), Bangladesh. Nodes indicate the mean age at which they are separated from the most recent common ancestor, and white horizontal bars at nodes indicate 95% highest posterior density values of the most recent common ancestor. Numbers at the main nodes indicate posterior values. Scale bar indicates time scale in years starting from 2010. Each strain name is followed by country of origin, host, year of detection, and GenBank accession number.

Nucleotide sequence data of the N gene of rabies viruses from Bangladesh appear in the DDBJ/EMBL/GenBank nucleotide sequence databases: accession nos.: AB699214 (rabies virus strain BDR1), AB699215 (strain BDR2), AB699216 (strain BDR3), AB699217 (strain BDR4), AB699218 (strain BDR6), AB699219 (strain BDR7), and AB699220 (whole genome of strain BDR5). AAL, Arctic/Arctic-like.

Table 2. Substitutions in genome sequence of rabies virus BDR5 from Bangladesh compared with genome sequence of strain from India (AY956319), 2010*

Protein, amino acid substitution	Site/domain/region of protein†
N	
$Asp_{378} \rightarrow Glu_{378}$	Antigenic site IV
$Gln_{422} \rightarrow Arg_{422}$	–
P	
$Ser_{64} \rightarrow Pro_{64}$	Variable domain I
$Gln_{71} \rightarrow Thr_{71}$	Variable domain I
$Asn_{90} \rightarrow Ser_{90}$	N protein binding site in variable domain II
$Pro_{159} \rightarrow Ser_{159}$	N protein binding site in variable domain II
$His_{162} \rightarrow Ser_{162}$	N protein binding site in variable domain II
$Asn_{166} \rightarrow Ser_{166}$	N protein binding site in variable domain II
$Ala_{174} \rightarrow Val_{174}$	N protein binding site in variable domain II
M	
$Leu_{21} \rightarrow Pro_{21}$	–
$Ser_{46} \rightarrow Gly_{46}$	–
$Ile_{168} \rightarrow Val_{168}$	–
G	
$Ala_{-(minus)15} \rightarrow Val_{-15}$	Signal peptide
$Val_{-(minus)6} \rightarrow Phe_{-6}$	Signal peptide
$Val_{7} \rightarrow Ile_{7}$	–
$Asp_{146} \rightarrow Asn_{146}$	Putative additional *N*-glycosylation: NKS
$Val_{427} \rightarrow Ile_{427}$	–
$Arg_{462} \rightarrow Gly_{462}$	Transmembrane domain
$His_{465} \rightarrow Arg_{465}$	Transmembrane domain
$Gly_{473} \rightarrow Ser_{473}$	Transmembrane domain
L	
$Asp_{18} \rightarrow Glu_{18}$	–
$Ala_{19} \rightarrow Thr_{19}$	–
$Arg_{315} \rightarrow Lys_{315}$	Conserved domain I
$Val_{361} \rightarrow Leu_{361}$	Conserved domain I
$His_{640} \rightarrow Gln_{640}$	Conserved domain III
$Lys_{657} \rightarrow Arg_{657}$	Conserved domain III
$Ala_{966} \rightarrow Thr_{966}$	Conserved domain IV
$Pro_{1133} \rightarrow Ser_{1133}$	Conserved domain V
Arg_{1307} Lys_{1307}	Conserved domain IV
$Asp_{1373} \rightarrow Gly_{1373}$	–
$Leu_{1626} \rightarrow Val_{1626}$	–
$Leu_{1654} \rightarrow Ser_{1654}$	–
$Val_{1755} \rightarrow Ile_{1755}$	–
$Cys_{1825} \rightarrow Tyr_{1825}$	–
$Asn_{1841} \rightarrow Lys_{1841}$	–
$Gln_{1845} \rightarrow His_{1845}$	–
$Cys_{1872} \rightarrow Phe_{1872}$	–
$Asn_{2091} \rightarrow Ser_{2091}$	–

*N, nucleoprotein; P, phosphoprotein; M, matrix protein; G, glycoprotein; L, polymerase.
†– indicates that the amino acid substitution was in a location that has no site/domain/region name. NKS, asparagine-lysine-serine.

Bhutan indicates that they originated from a common ancestor. If one considers the ease of human movement between countries, AAL2 most likely entered Bangladesh from India rather than from Bhutan.

Circumstantial evidence suggests that rabies virus spread from India to Bhutan (*9*). AAL2 circulates in many states of India. It has spread into southern India and has replaced older strains (*10*,*11*). It is likely that AAL2 is also circulating in states of India that are between Bhutan and Bangladesh. Estimated time of AAL2 spread is based on 7 samples that are representative of central Bangladesh (Figure 2). Therefore, further surveillance might identify the extent to which AAL2 has spread and the diversity of rabies viruses in other parts of Bangladesh that might alter the estimated date of spread. It has been reported that arctic rabies virus and other variants can co-circulate in the same region (*12*).

The G protein is the major factor responsible for the pathogenesis of rabies virus and contains 2 glycosylation sites (*13*). The G protein of strains from Bangladesh uniquely evolved to contain 3 potential glycosylation sites, which has been reported in only fixed (laboratory adapted) strains and proposed to be responsible for their reduced pathogenicity (*13*). However, the site for additional

Figure 2. Three districts of Bangladesh from which samples were tested for Arctic/Arctic-like rabies virus and strains were found. Black, Dhaka District, strains BDR1, BDR3, and BDR6; light gray, Narayanganj District, strains BDR4, BDR5, and BDR7; dark gray, Narshingdi District, strain BDR2.

glycosylation differs between Bangladeshi and fixed strains. Detection of an additional glycosylation site and amino acid substitutions deserve further investigations.

AAL viruses could have moved southward from Siberia or other northern regions of the former Soviet Union into Nepal, India, and other countries in Asia by a species jump from fox to dog at some point (*3*). Another possibility is that AAL viruses first emerged in dogs in southern Asia and subsequently spread to northern climes, where they are now maintained in fox populations (*3*,*8*). Extensive surveillance of viruses from Iran, Iraq, Afghanistan, and countries north of them is necessary to determine the origin and spread pattern of AAL rabies virus.

The timeline of divergence of different lineages determined in this study was similar to that previously reported (*8*). That study and our study used the full-length N gene to determine the time of divergence. Another study reported the timeline of divergence as a more recent event (*14*). This study used partial sequences of N genes, which might be responsible for different results. Rabies virus from Nepal also belongs to AAL2, and as reported in a previous study (*15*), seemed to be forming a different lineage. However, the speculation was not supported by a significant a posterior density value (0.6355). Thus, a network of countries is urgently needed to exchange information on molecular typing of circulating strains of rabies virus that might be useful in controlling rabies in this region.

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Dr Jamil is a physician and virologist in the Department of Virology, Institute of Epidemiology, Disease Control, and Research, Dhaka, Bangladesh. Her research interests are virology and molecular epidemiology of rabies virus.

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Address for correspondence: Kamruddin Ahmed, Research Promotion Institute, Oita University, Yufu 879-5593, Oita, Japan; email: ahmed@oita-u.ac.jp

No Evidence of Prolonged Hendra Virus Shedding by 2 Patients, Australia

Carmel Taylor, Elliott G. Playford, William J.H. McBride, Jamie McMahon, and David Warrilow

To better understand the natural history of Hendra virus infection and its tendency to relapse, 2 humans infected with this virus were monitored after acute infection. Virus was not detected in blood samples when patients were followed-up at 2 and 6 years. Thus, no evidence was found for prolonged virus shedding.

Most virus infections resolve after an acute phase. A small subset can cause persistent infection and result in continual shedding of virus, and others use a latency mechanism to evade the host immune response. Hendra and Nipah viruses (family *Paramyxoviridae*, genus *Henipavirus*) can cause respiratory disease and encephalitis in humans. Hendra virus infection is acquired by close contact with horses infected by spillover infection from fruit bats, which are the natural reservoir for these viruses (*1*).

In a small proportion of cases, relapse with encephalitis after a mild acute phase is a feature of henipavirus infection and is often fatal. For example, several cases of relapsing encephalitis caused by Nipah infection in humans have been reported (*2–4*), as well as 1 case caused by Hendra virus in a human (*5*,*6*). Whether virus shedding occurs after an acute infection of any severity is currently unclear. Because standard veterinary practice in Australia is to destroy animals that survive natural or experimental Hendra virus infection (*7*), long-term monitoring for virus shedding has not been possible.

To address the nature of viral persistence in cases of Hendra virus infection, virus shedding and serologic changes were monitored in 2 of 3 infected persons. In addition, multiple samples from 1 of these persons and from 2 persons who died were compared to determine the most appropriate specimen type for detection by quantitative reverse transcription PCR (qRT-PCR).

Author affiliations: Queensland Health Forensic and Scientific Services, Archerfield, Queensland, Australia (C. Taylor, J. McMahon, D. Warrilow); Princess Alexandra Hospital, Brisbane, Queensland, Australia (E.G. Playford); University of Queensland School of Medicine, Brisbane (E.G. Playford); and James Cook University School of Medicine and Dentistry, Cairns, Queensland, Australia (W.J.H. McBride)

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The Study

Of 7 recognized humans infected with Hendra virus, 3 survived. Of these 3 persons, clinical samples were available for 2 survivors. Patient 1 was a 25-year-old woman (veterinarian) in whom a self-limited influenza-like illness (ILI) developed in November 2004 (*8*). Patient 2 was a 21-year-old woman (veterinary nurse) who showed development of an ILI that progressed to acute encephalitis in July 2008 (*9*). Although she survived, patient 2 showed persistent, postencephalitic, high-level cognitive deficits. Currently, both patients do not show clinical evidence of relapse.

For this study, specimens were obtained from both patients at various times during acute-phase infection and during the convalescent phase. Blood was tested for IgG and IgM against Hendra virus. Nasopharyngeal aspirate (NPA), blood, urine, and cerebrospinal fluid were tested for viral RNA by using qRT-PCR.

Both patients showed IgM and IgG responses to Hendra virus. For patient 1, no specimens were collected in the months after the acute phase and by 12 months, no IgM could be detected. However, IgG reactivity has been maintained for 6 years (Figure). Patient 2 has maintained IgM and IgG reactivity for ≥1.5 years after initial examination. The prolonged IgM response in patient 2 might have been caused by persistent infection or associated with more severe acute encephalitic manifestations. A prolonged IgM response has been observed in persons with West Nile virus meningitis, and encephalitis and may be a general aspect of central nervous system involvement (*12,13*).

Viral RNA was not detected in any specimen from patient 1 during the acute phase or at the 6-year follow-up (Figure). Multiple samples from patient 2 were positive for virus during a 1-month period during the acute phase (Figure, Table). However, no samples showed positive results for Hendra virus RNA on subsequent follow-up. Thus, neither patient showed evidence of virus shedding after the acute phase (ILI or encephalitis).

To facilitate future PCR testing, we assessed various sample types for their suitability for detection of Hendra virus RNA during the acute phase of infection. In addition to patient 2, samples from 2 other case-patients with Hendra virus infection not described in this report (patients 3 and 4) were tested. Patient 1 was not included because virus shedding was not detected at any time during her infection. Times chosen were from the earliest to the latest time point at which a positive result was obtained by qRT-PCR (Table).

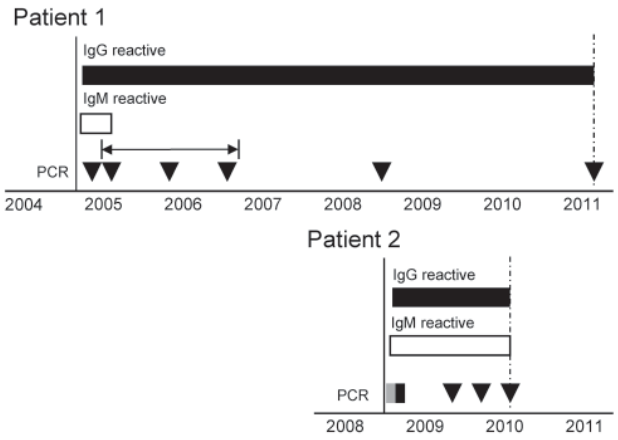

Figure. Serologic and quantitative reverse transcription PCR (qRT-PCR) results for samples from 2 patients infected with Hendra virus, Australia. Testing was performed from the time at which symptoms first developed (black vertical line) until the most recent sample indicated (dashed vertical arrows). IgG (black bars) and IgM (white bars) reactivity was determined by using a modified microsphere immunoassay (*10*), and a positive control serum sample to determine the cutoff value. Virus RNA was detected (gray box) or not detected (black box or triangles) by using a qRT-PCR (*11*). The horizontal arrow indicates 3 samples stored at −80°C and tested retrospectively for Hendra virus RNA. Positive and negative controls were included in all tests and showed expected results.

Hendra virus RNA was detected in blood, NPA (or swab specimen), and urine, confirming the suitability of these sample types. Frequency of detection varied for 3 patients. None of the 3 sample types was consistently better than another, and all were suitable choices for diagnostic purposes. Hendra virus RNA was also detected in cerebrospinal fluid, but there were too few samples to determine whether this material was a reliable sample type.

Conclusions

Serologic and PCR results were reported for the 2 patients during the early phase of infection (*8*,*9*). Our study followed-up survivors of Hendra virus infection, and found no evidence for prolonged shedding of virus after acute infection. Because of the small total number of human cases and the high case-fatality rate, there are only 3 known surviving patients with Hendra virus infection, 2 whom were included in this study. Therefore, our results provide useful information for clinicians and public health officials for treating individual patients and minimizing the risk for transmission.

However, because of the small number of samples tested in this study, prolonged shedding after acute Hendra virus infection cannot be ruled out. In 1995, a patient who had an episode of aseptic meningitis after caring for 2 Hendra virus–infected horses had a relapse 13 months after the initial episode (*5*). This patient also showed the potential for virus shedding during management of disease survivors. Thus, further monitoring of current and future survivors and experiments using a suitable animal model is required to answer this question.

The mechanism by which henipaviruses remain dormant is unknown. Virus has not been isolated from persons with relapse cases (*2*,*5*). This finding suggests that virus might persist in a noninfectious mutant form analogous to that which occurs with related measles virus during subacute sclerosing panencephalitis (*14*). In addition, the cellular reservoir for the virus and the mechanism by which it maintains latency are unknown.

It would be desirable to use PCR to test a variety of sample types for diagnosis of infection with Hendra virus. All sample types tested were suitable in terms of sensitivity. However, obtaining an NPA specimen during the acute phase of infection has a potential risk for aerosol contamination, and needle stick injury is a risk if a blood sample is obtained. Collection of a urine sample may be an acceptable risk without reducing the sensitivity of detection and risk to health care staff. This procedure should assist clinicians in management of henipavirus infections.

Table. Samples from patients infected with Hendra virus, Australia*

Sample type	No. positive/no. tested (%)		
	Patient 2†	Patient 3‡	Patient 4‡
Blood	7/18 (39)	6/11 (55)	22/22 (100)
Urine	9/17 (53)	7/7 (100)	19/22 (86)
NPA or swab specimen	10/13 (77)	3/11 (27)	19/ 20 (95)

*NPA, nasopharyngeal aspirate.
†Human infected with Hendra virus; virus shed 3–6 weeks postexposure.
‡Two other humans infected with Hendra virus; virus was shed 3–5 weeks postexposure.

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Ms Taylor is supervising scientist of the Public Health Virology Serology Laboratory of Queensland Health Forensic and Scientific Services. Her main area of expertise is serologic diagnosis of human mosquito-borne viral diseases, such as dengue, and other rare or exotic human viral infections, including Hendra virus.

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Address for correspondence: David Warrilow, Public Health Virology Laboratory, Queensland Health Forensic and Scientific Services, PO Box 594, Archerfield, Queensland 4108, Australia; email: david_warrilow@health.qld.gov.au

Differentiation of Prions from L-type BSE versus Sporadic Creutzfeldt-Jakob Disease

Simon Nicot, Anna Bencsik, Eric Morignat, Nadine Mestre-Francés, Armand Perret-Liaudet, and Thierry Baron

We compared transmission characteristics for prions from L-type bovine spongiform encephalopathy and MM2-cortical sporadic Creutzfeldt-Jakob disease in the Syrian golden hamster and an ovine prion protein–transgenic mouse line and isolated distinct prion strains. Our findings suggest the absence of a causal relationship between these diseases, but further investigation is warranted.

Among transmissible spongiform encephalopathies (TSEs), the L-type bovine spongiform encephalopathy (L-BSE) in cattle requires particular attention for public health. L-BSE is transmitted more efficiently than is classical BSE among primates (*1*–*3*) as well as among transgenic mice that express human prion protein (PrP) (*4*,*5*). We recently reported that L-BSE was readily transmissible by experimental oral inoculation in a nonhuman primate species, the grey mouse lemur (*Microcebus murinus*) (*3*). These findings raise the possibility that some human Creutzfeldt-Jakob disease (CJD) cases might result from exposure to the L-BSE agent; previous studies highlighted similarities between L-BSE and some human subtypes (type 2) of sporadic CJD (sCJD) (*1*,*6*).

To examine the possible relationship between L-BSE and sCJD, we evaluated a strain-typing strategy that relies on comparative transmission characteristics in the Syrian golden hamster and in a transgenic mouse line (TgOvPrP4) expressing ovine PrP (ARQ allele). Both of these species are susceptible to L-BSE prions from cattle (*7*,*8*). The transmission of L-BSE, including after a first passage in *Microcebus murinus* lemurs (*3*), was compared with that for the MM2-cortical subtype of sCJD (*9*); this subtype was chosen on the basis of a study that indicated higher levels of molecular similarities of L-BSE with this sCJD subtype than with the MV2 subtype (*1*).

Author affiliations: Agence Nationale de Sécurité Sanitaire (Anses), Lyon, France (S. Nicot, A. Bencsik, E. Morignat, T. Baron); INSERM U710, Montpellier, France; Université Montpellier 2, Montpellier; École Pratique des Hautes Études, Paris, France (N. Mestre-Francés); and Hôpitaux Civils de Lyon, Université Lyon 1, INSERM U1028, and Centre National de la Recherche Scientifique, Lyon (A. Perret-Liaudet)

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The Study

The TSE brain inocula used in this study, conducted during November 2010–December 2011, were derived from 2 natural L-BSE isolates from France (02-2528 and 08-0074); a lemur injected intracerebrally (i.c.) with the 02-2528 L-BSE cattle isolate (*3*); and a human patient with MM2-cortical sCJD. Consent was obtained for using tissues from the human patient in research, including genetic analyses. Animal experiments were performed in the biohazard prevention area (A3) of the Anses-Lyon animal facilities, in accordance with the guidelines of the French Ethical Committee (decree 87-848) and European Community Directive 86/609/EEC.

Six-week-old TgOvPrP4 mice and 4-week-old Syrian golden hamsters were injected i.c. with 20 and 30 μL, respectively, of 10% (wt/vol) brain homogenates in 5% sterile glucose. Serial passages were performed in TgOvPrP4 mice by i.c. inoculation of 1% (wt/vol) homogenates from mice positive for protease-resistant PrP (PrP^{res}). At the terminal stage of the disease, animals were euthanized, and their brains and spleens were collected for PrP^{res} analyses by Western blot and for histopathologic studies (*8*).

In hamsters, transmission of the MM2-cortical sCJD agent was inefficient. Clinical signs were absent up to 876 days postinoculation (dpi) (Table), and disease-associated PrP (PrP^{d}) in brain samples was not detected by paraffin-embedded tissue blot (PET-blot) (Figure 1, panel A), immunohistochemical (Figure 1, panel C), or Western blot (Figure 1, panels E, F) analyses. PrP^{res} was also undetectable in spleen tissues by Western blot (Table).

In contrast, the L-BSE agent passaged in a lemur was efficiently transmitted to hamsters, with a mean survival period of 529 ± 117 dpi, similar to that for L-BSE from cattle (622 ± 64 dpi) (Table). PET-blot analysis (Figure 1, panel B) showed widespread PrP^{res} distribution in the brain; immunohistochemical analysis (Figure 1, panel D) showed a granular type of PrP^{d} deposition that redefined the periphery of most of the blood vessels. Western blot analysis (Figure 1, panels E, F) showed PrP^{res} in the brains of hamsters inoculated with L-BSE from cattle and lemur and in 1/4 spleens of hamsters injected with L-BSE passaged in lemur (Table). Brain PrP^{res} was characterized by low apparent molecular mass (≈19 kDa for the unglycosylated band) associated with a lack of reactivity toward the N terminal 12B2 antibody, in contrast to that for the control animal with scrapie (Figure 1, panels E, F).

Table. Comparison of transmission of sCJD and L-BSE in hamsters and mice

Hosts and inoculum	Passage	Mean survival time, dpi ± SD	No. brain PrP^d positive/no. tested	No. spleen PrP^{res} positive/no. tested
Syrian golden hamsters				
sCJD MM2-cortical	1	833 ± 33	0/4	0/4
L-BSE lemur	1	529 ± 177	5/5	1/4
L-BSE cattle (02-2528)	1	622 ± 64†	4/5†	0/5
TgOvPrP4 mice				
sCJD MM2-cortical	1	639 ± 49	3/4	0/4
L-BSE lemur	1	509 ± 97	7/7	7/7
L-BSE cattle (02-2528)	1	627 ± 74‡	9/10‡	0/5§
L-BSE cattle (08-0074)	1	497 ± 49	6/8	0/9
sCJD MM2-cortical	2	111 ± 25	12/12	12/12
L-BSE lemur	2	194 ± 7	12/12	12/12
L-BSE cattle (02-2528)	2	202 ± 26‡	9/9‡	3/5
L-BSE cattle (08-0074)	2	186 ± 37	12/12	9/11

*Isolate identification numbers are shown in parentheses. sCJD, sporadic Creutzfeldt-Jakob disease; L-BSE, L-type bovine spongiform encephalopathy; TgOvPrP4, ovine prion protein–transgenic; dpi, days postinoculation; PrP^d, disease-associated prion protein; PrP^{res}, protease-resistant prion protein.
†Data from (*7*).
‡Data from (*8*).
§Data from (*10*).

In TgOvPrP4 mice, all TSEs were efficiently transmitted, as confirmed by PrP^d accumulation in the mouse brains (Table). After serial passages in additional TgOvPrP4 mice, the survival periods in each experiment became considerably shorter (Table; online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/pdfs/12-0342-Techapp.pdf). No statistically significant differences in results were identified between the L-BSE sources ($p>0.6$). Mean survival period decreased to 111 ± 25 dpi at second passage in mice inoculated with the agent of MM2-cortical subtype sCJD, which differed significantly from that of mice inoculated with L-BSE ($p<0.0001$). A third passage of both cattle L-BSE and human sCJD did not reduce the survival periods in TgOvPrP4 mice (data not shown).

Western blot analyses of PrP^{res} from mouse brains showed partially similar features for MM2-cortical sCJD and L-BSE, including low molecular mass (≈19 kDa for the unglycosylated band) (Figure 2, panel A) and similar conformational stability of PrP^d after treatment with guanidinium hydrochloride (online Technical Appendix Figure 2). However, the proportions of diglycosylated, monoglycosylated, and unglycosylated bands of brain PrP^{res} differed between sCJD and L-BSE (Figure 2, panel C); higher proportions of diglycosylated PrP^{res} were found in sCJD-infected mice (mean 67% of the total signal) compared with L-BSE–infected mice (≈18% lower; $p<0.0001$). PrP^{res} was readily identified in the spleens of TgOvPrP4 mice at the second passage for sCJD and L-BSE

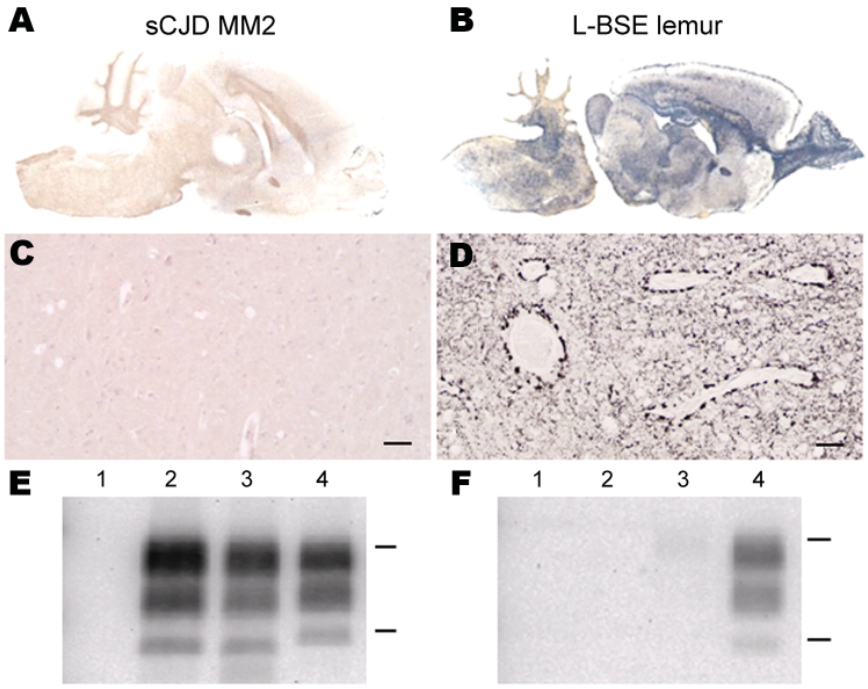

Figure 1. Susceptibility of Syrian golden hamsters to MM2-cortical subtype sporadic Creutzfeldt-Jakob disease (sCJD) and L-type bovine spongiform encephalopathy (L-BSE) prions. Disease-associated prion protein (PrP^d) was analyzed in brains of hamsters injected with human MM2-cortical sCJD and L-BSE from a mouse lemur by paraffin-embedded tissue blot (A, B), immunohistochemistry (C, D), or Western blot (E, F). Monoclonal antibodies against prion protein were SAF84 (A–D), SHa31 (E), and 12B2 (F). C, D) Scale bars = 200 μm. E, F) Controls were hamsters infected with L-BSE from cattle (isolate 02-2528) and with scrapie (experimental isolate SSBP/1 after a first passage in ovine prion protein–transgenic mice). Lane 1, sCJD MM2; lane 2, L-BSE from lemur; lane 3, L-BSE from cattle control; lane 4, scrapie control. Bars to the right indicate the 29.0- and 20.1-kDa marker positions.

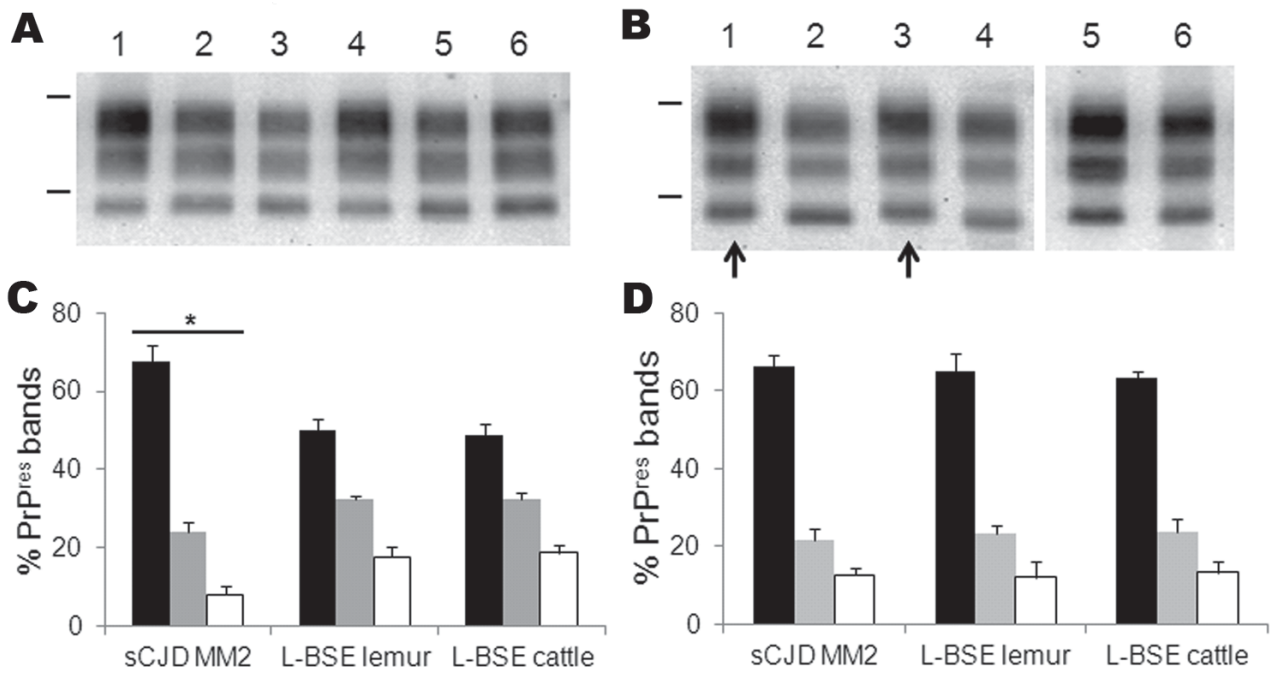

Figure 2. Western blot molecular typing of protease-resistant prion protein (PrP^{res}) in brain and spleen tissues of ovine prion protein–transgenic (TgOvPrP4) mice at second passage. PrP^{res} from mice infected with MM2-cortical subtype sporadic Creutzfeldt-Jakob disease (sCJD), L-type bovine spongiform encephalopathy (L-BSE) from lemur, and L-BSE from cattle (02-2528) were compared in brain (A) and spleen (B) tissues (monoclonal antibody SHa31). Bars to the left of Western blots indicate the 29.0- and 20.1-kDa marker positions. A) Lanes 1, 4, sCJD MM2; lanes 2, 5, L-BSE from lemur; lanes 3, 6, L-BSE from cattle control; B) lanes 1, 3, sCJD MM2; lanes 2, 4, 6, L-BSE from lemur; lane 5, L-BSE from cattle control. C, D) Proportions of PrP^{res} glycoforms in brain (C) and spleen (D) tissues. Error bars indicate SD. *Indicates $p<0.0001$ when comparing PrP^{res} proportions from mice infected with MM2-cortical sCJD with those infected with L-BSE.

from cattle and at the first passage for L-BSE from lemur (Table). No significant differences in the proportions of PrP^{res} glycoforms for sCJD-infected versus L-BSE–infected mice were observed in the spleens (Figure 2, panel D), but PrP^{res} was ≈0.5 kD higher in mice injected with sCJD (Figure 2, panel B, arrows).

Histopathologic analysis showed severe vacuolar lesions in TgOvPrP4 mice infected at second passage with sCJD and lemur-passaged L-BSE (online Technical Appendix Figure 3). However, in sCJD-infected mice, vacuolar lesions were mostly observed in the anterior parts of the brain (except the parietal cortex), whereas in mice infected with lemur-passaged L-BSE, the lesions were more widely distributed, involving the colliculi and the hypothalamus. In mice infected with sCJD and lemur-passaged L-BSE, PET-blot analyses showed that most of the PrP^{res} occurred in the frontal parts of the brain, but the intensity and appearance of PrP^{res} in the cortex, thalamus, and hippocampus were distinctly different. Immunohistochemical analyses of the hippocampus showed PrP^{d} deposition in the dentate gyrus in sCJD-infected mice, in contrast to a lack of deposition in lemur-passaged L-BSE–infected mice.

Conclusions

We report the isolation of 2 prion strains derived from L-BSE and MM2-cortical sCJD after transmission in Syrian hamsters and ovine PrP–transgenic mice. In hamsters, we did not transmit any disease with sCJD, but the transmission of L-BSE from lemur was efficient, as previously reported for L-BSE from cattle (*7*,*11*). This result suggests that L-BSE did not undergo major modifications after this cross-species transmission and could indicate a clear biologic difference between MM2-cortical sCJD and L-BSE. We also demonstrated the efficient transmission of both L-BSE and MM2-cortical sCJD in TgOvPrP4 mice, which enabled us to compare these diseases in a single model. Unexpectedly, during serial passages, we observed that the agent of MM2-cortical sCJD causes a much more rapidly fatal disease. Despite similar molecular features in sCJD and L-BSE, including the PrP^{res} electrophoretic mobility and the conformational stability of PrP^{d}, sCJD and L-BSE differed in PrP^{res} glycosylation for the mouse brains and gel migrations for the mouse spleens. Mice infected with MM2-cortical sCJD versus those infected with L-BSE also showed distinct lesion profiles and PrP^{d} distribution,

which confirms clear biologic differences between these diseases.

Although only 1 case of sCJD of a unique molecular subtype was examined in our study, our observations do not support the hypothesis of a causal relationship between L-BSE and this human sCJD subtype. Our study thus encourages further investigations using the proposed bioassay approach for a more complete evaluation of possible relationships between L-BSE and human prion diseases.

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Mr Nicot is a PhD student at the Agence Nationale de Sécurité Sanitaire in Lyon. His primary research interests include characterization of the infectious agents and prion protein during intra- and interspecies transmission of animal and human prion diseases.

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Address for correspondence: Thierry Baron, Anses-Lyon, 31 Avenue Tony Garnier, 69364 Lyon Cedex 07, France; email: thierry.baron@anses.fr

Hepatitis E Virus Outbreak in Monkey Facility, Japan

Hiroshi Yamamoto, Juri Suzuki, Atsushi Matsuda, Takafumi Ishida, Yasushi Ami, Yuriko Suzaki, Isao Adachi, Takaji Wakita, Naokazu Takeda, and Tian-Cheng Li

An outbreak of hepatitis E virus occurred in an outdoor monkey breeding facility in Japan during 2004–2006. Phylogenetic analysis indicated that this virus was genotype 3. This virus was experimentally transmitted to a cynomolgus monkey. Precautions should be taken by facility personnel who work with monkeys to prevent infection.

Wild or reared monkeys have been used as disease models in animal facilities worldwide. Because disease caused by hepatitis E virus (HEV) is a zoonosis (*1*–*4*), monkeys might be infected. We examined the prevalence of antibodies against HEV in serum and fecal samples collected from monkeys in animal facilities at the Primate Research Institute of Kyoto University in Japan for 6 years (2004–2009). We found that spontaneous infection and transmission of HEV occurred in a monkey facility.

The Study

There are 9 monkey colonies (A–I) at the Primate Research Institute of Kyoto University. Colonies A–G contained Japanese monkeys (*Macaca fuscata*), and colonies H and I contained rhesus monkeys (*Macaca mulatta*). Each colony was bred in a separate outdoor breeding facility. A total of 588 monkey serum samples were collected during September–November 2004–2009 and tested for IgG and IgM against HEV and for HEV RNA by ELISA or reverse transcription PCR (RT-PCR) as described (*5*–*7*). Samples from colonies G and F were collected during 2004–2006, whereas in 2009 samples were collected from colonies A, C, D, and I.

The prevalence of IgG against HEV was 0% in 2004, 20.0% in 2005, and 78.5% in 2006, followed by a gradual decrease to 35.9% in 2009 (Table 1). The prevalence of IgM against HEV increased from 0% in 2004 to 2.5% in 2005 and to 6.6% in 2006, and then decreased to 1.1% in 2007 and 0% in 2008 and 2009.

Table 1. Prevalence of IgG and IgM against hepatitis E virus in monkeys at monkey facility, Japan, 2004–2009

Year	No. positive/no. tested (%)	
	IgG	IgM
2004	0/110	0/110
2005	24/120 (20.0)	3/120 (2.5)
2006	96/121 (78.5)	8/121 (6.6)
2007	73/96 (76.0)	1/96 (1.1)
2008	47/90 (52.2)	0/90
2009	18/51 (35.3)	0/51

IgG against HEV was not detected in any of the 9 colonies in 2004, indicating that HEV infection did not occur before October 2004. However, in 2005, the prevalence of IgG reached 100% in colony D and 20% in colony G (Figure 1). ELISA titers were high, ranging from 0.293 to 1.641 in colony D and from 0.230 to 0.845 in colony G. These results suggested that HEV infection occurred after October 2004 in the monkey facility. The prevalence of IgG was higher in colony D than in colony G, and IgM was not detected in colony D, suggesting that HEV infection occurred earlier in colony D than in colony G. These colonies adjoined each other, indicating that the first HEV infection occurred in colony D and was then transmitted to colony G. Colonies A, C, D, E, and H each had an IgG prevalence of 90%–100%, and colonies B and G had an IgG prevalence >80% in 2006 (Figure 1). These results demonstrated that infection spread over a large area, except for colony F, during 2005 and 2006.

To compare the kinetics of IgG formation during 2004–2009, serum samples from 25 monkeys awhose peak ELISA optical density (OD) values for IgG against HEV were each higher than 1.0 were selected. In most monkeys, OD values for IgG increased rapidly and then decreased gradually year by year. The kinetic pattern of monkey M1543 was different from those of other monkeys that had high OD values (2.568–2.738). IgM was detected exclusively in this monkey in 2006 (OD value 0.620).

Serum samples from the 25 monkeys were used to detect HEV RNA by RT-PCR. Four serum samples were positive for HEV RNA; all were from the same monkey (M1543) from which samples were collected in 2006, 2007, 2008, and 2009. Nucleotide sequences of 348 bp coding the partial open reading frame 2 showed 100% identity. This result indicated that monkey M1543 was infected persistently with HEV and produced virus continuously.

To examine whether HEV was present in feces, 2 fecal samples were collected from monkey M1543 in September and November 2009 for detection of HEV RNA. Both samples were positive for HEV RNA. Nucleotide sequences of these samples were identical to those detected from serum samples.

Author affiliations: University of Toyama, Toyama, Japan (H. Yamamoto, I. Adachi); Kyoto University, Aichi, Japan (J. Suzuki); University of Tokyo, Tokyo, Japan (A. Matsuda, T. Ishida); National Institute of Infectious Diseases, Tokyo (Y. Ami, Y. Suzaki, T. Wakita, T.-C. Li); and Osaka University, Osaka, Japan (N. Takeda)

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Primers were designed on the basis of sequences of swine HEV (GenBank accession no. AB248522), and RT-PCR was performed to amplify the viral genome except for the N terminus noncoding region. This strain was designated the monkey HEV Inuyama strain (JQ026407). Phylogenetic analysis of its genome indicated that this strain belongs to HEV genotype 3 (Figure 2). Infectivity of the monkey HEV strain was examined ex vivo with a human hepatocarcinoma cell line (PLC/PRF/5), and in vivo with 2 HEV-negative cynomolgus monkeys. Both experiments showed that the virus was infectious (online Technical Appendix Figures 1 and 2, wwwnc.cdc.gov/EID/pdfs/12-0884-Techapp.pdf).

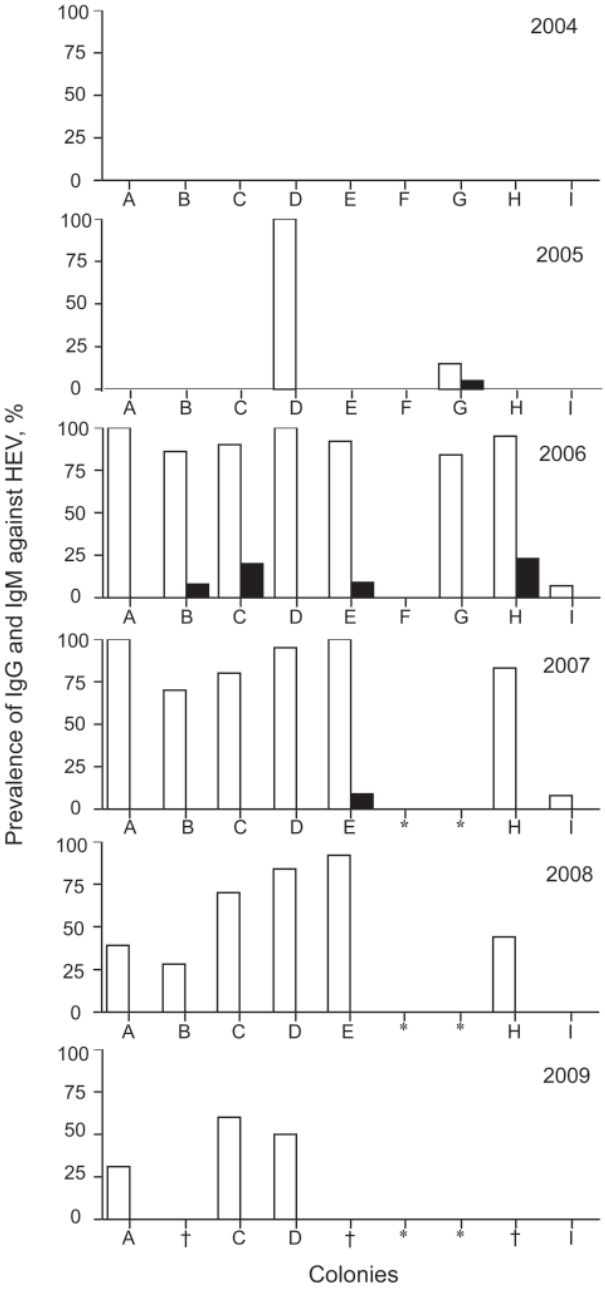

Figure 1. Prevalence of IgG (white bars) and IgM (black bars) against hepatitis E virus (HEV) in monkey facility, Japan, 2004–2009. *Monkeys were moved to another animal facility; †specimen not available.

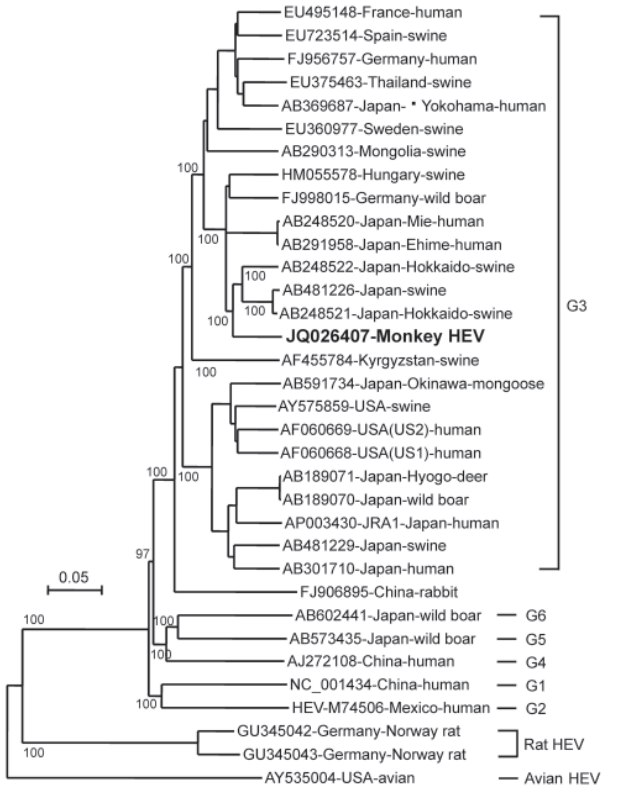

Figure 2. Phylogenetic analysis of monkey hepatitis E virus (HEV) Inuyama strain on the basis of nucleotide sequence of the HEV genome except for a 5′ noncoding region (7,206 nt) by using avian HEV as an outgroup. Values along the branches are bootstrap values determined on the basis of 1,000 resamplings of datasets. **Boldface** indicates strain isolated in this study. Genotypes are indicated on the right. Scale bar indicates nucleotide substitutions per site.

A total of 94 human serum samples were collected from staff of the Primate Research Institute and subjected to ELISA for detection of IgG and IgM against HEV. All serum samples were negative for IgM against HEV, but the prevalence of IgG was 6.9% in 2007, 9.7% in 2008, and 11.8% in 2009, although differences among these years were not significant ($p>0.05$) (Table 2). No HEV RNA was detected in serum samples, and none of the staff had symptomatic hepatitis E during the 6-year study.

Conclusions

We conducted long-term monitoring of HEV infection in monkeys and report natural infection and transmission of HEV in a monkey facility. We sought to determine the source of the HEV outbreak and where HEV was intro-

Table 2. Prevalence of IgM against hepatitis E virus in serum samples from animal handlers at monkey facility, Japan, 2007–2009*

Year	No. positive/no. tested (%)
2007	2/29 (6.9)
2008	3/31 (9.7)
2009	4/34 (11.8)

*All samples were negative for IgG against hepatitis E virus and for virus RNA.

duced to colony D. At our research institute, each monkey colony is bred in a separate outdoor breeding facility built on a mountain, and the monkeys live in an environment similar to their natural habitat. Because each outdoor feeding facility is isolated by a double fence, natural reservoirs of HEV (wild boars and deer) cannot enter it. Phylogenetic analysis of monkey HEV strains indicated that this virus was genotype 3, and BLAST analysis showed that the monkey isolate is closest to HEV strains isolated from pigs in Japan. Nucleotide identities were 92%–93% (AB248521, AB248522, and AB481226). However, no evidence indicates that HEV is transmitted from pigs or wild boars to monkeys.

A notable finding in this study was the persistence of HEV infection. Generally, HEV infection is self-limiting and symptoms are transient. Persistent HEV infection occurs in solid-organ transplant recipients who have received immunosuppressive drugs (*8*) or in patients with other conditions associated with immunosuppression, such as HIV infection (*9*) and hematologic malignancies (*10*,*11*). However, there is no evidence of immunosuppression in monkey M1543, and the cause of the persistent HEV infection in this monkey is unknown.

The fact that the infectious HEV strain was detected in a monkey facility and caused an HEV outbreak cast doubt and apprehension on the safety of handling monkeys. Although no staff member showed development of symptomatic hepatitis E, precautions should be taken by facility workers who work with monkeys to prevent infection with HEV.

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Dr Yamamoto is an associate professor at the University of Toyama, Toyama, Japan. His research interests are zoonoses, especially surveys of simian herpes B virus and hepatitis E virus infections of laboratory animals; and mouse and monkey Mx genes.

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Address for correspondence: Tian-Cheng Li, Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan; email: litc@nih.go.jp

Group 2 Vaccinia Virus, Brazil

Felipe Lopes Assis, Iara Apolinario Borges, Paulo César Peregrino Ferreira, Cláudio Antônio Bonjardim, Giliane de Souza Trindade, Zélia Inês Portela Lobato, Maria Isabel Maldonado Guedes, Vaz Mesquita, Erna Geessien Kroon, and Jônatas Santos Abrahão

In 2011, vaccinia virus caused an outbreak of bovine vaccinia, affecting dairy cattle and dairy workers in Brazil. Genetic and phenotypic analyses identified this isolate as distinct from others recently identified, thereby reinforcing the hypothesis that different vaccinia virus strains co-circulate in Brazil.

Throughout most of Brazil, vaccinia virus (VACV), family *Poxviridae*, is the etiologic agent of bovine vaccinia (*1*). Outbreaks often occur on unhygienic rural properties and cause mild to severe rashes on teats and udders of dairy cows and various locations on humans (*1*,*2*). Dairy workers usually seek medical care for the painful lesions, but rarely are they hospitalized. Some studies suggest an association between these outbreaks of bovine vaccinia and the VACV strains used during the World Health Organization smallpox eradication campaign (*3*). Since 1999, VACV strains in Brazil have been investigated (*2*–*8*); biological and molecular approaches indicated 2 distinct groups of these viruses (*9*,*10*). In 2011, a bovine vaccinia outbreak occurred in Serro County, Minas Gerais state, in southeastern Brazil, one of the largest milk-producing regions in Brazil. The outbreak affected 91 dairy cows and 3 dairy workers, 1 of whom was hospitalized (online Technical Appendix Figure 1, panel A, wwwnc.cdc.gov/EID/pdfs/12-0145.pdf). Our aim was to elucidate the genetic and phenotypic characteristics of this VACV isolate.

The Study

The outbreak affected 2 farms, 91 cows, and 3 humans (Figure 1). On day 1, on farm 1, the index case was a sick cow with ulcerative lesions on the teats and udder. On day 3, the owner of farm 1 (patient A), who had had direct contact with the sick cow, noticed lesions on his hand. On day 5, another 6 cows on farm 1 became sick, and patient A went to farm 2 and handled healthy cattle. On day 7, all 14 cows on farm 1 were sick. On day 9, some cows on farm 2 became sick. On day 12, the owner of farm 2 (patient B) and his employee (patient C) became sick. On day 15, patient C was hospitalized with high fever, lymphadenopathy, prostration, and painful vesicular–pustular lesions on his hands and arms. He received clinical support and remained hospitalized for 10 days. He had no immunologic disorders and took no medications that could be associated with his severe clinical condition. According our investigation, only those 3 patients had direct contact with the infected cattle during the outbreak.

By day 18, all 77 cows on farm 2 were sick. On day 24, veterinary surveillance teams isolated these farms for 8 days. On day 26, patient C returned to work although lesions remained on his hands and arms (online Technical Appendix Figure 1, panel B). By day 28, all cattle were recovering.

To identify the etiologic agent responsible for the outbreak, on day 27 we collected swab samples from lesions of patients B and C (not from patient A, whose lesions were healing) and from 1 infected cow (from farm 2). Samples were placed in Vero cells for virus isolation as described (*2*) and then purified in a sucrose gradient (*11*). The isolates from the patients B and C and the cow were named VACV Serro human 1/2011 (SH1V/2011), VACV Serro human 2/2011 (SH2V/2011), and VACV Serro bovine 1/2011 (SB1V/2011), respectively.

The isolates were examined by PCR for the *A56R* gene (hemagglutinin [HA]), and the fragments obtained (950 bp) were sequenced and analyzed as described (*2,12,13*). The nucleotide sequences showed 100% identity among

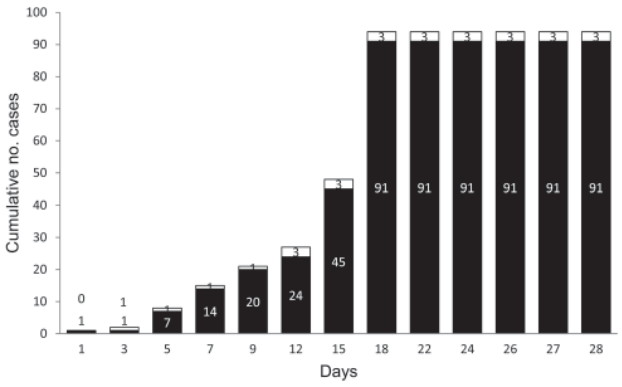

Figure 1. Timeline of 2011 vaccinia virus (VACV) outbreak in Serro County, Minas Gerais state, southeastern Brazil, involving 2 neighboring farms, 91 dairy cattle, and 3 dairy farm workers. Day 1, first case of bovine vaccinia in cow, farm 1; day 3, first case of human infection, patient 1, farm 1; day 5, more cases in cattle, farm 1; day 7, entire herd of cattle sick, farm 1; day 9, first 6 cows sick, farm 2; day 12, second and third human cases (patients B and C); day 15, patient C hospitalized; day 18, entire herd of cattle sick, farm 2; day 22, cumulative (both farms) mild production decrease of 70%; day 24, both farms quarantined; day 26, patient C returns to work, with lesions; day 27, lesion samples collected from patients B and C and 1 cow, farm 2; day 28, all cattle recovering.

Author affiliation: Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

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all VACV Serro 2011 isolates and exhibited high identity with VACV strains from Brazil, particularly SPAn232 (99.8% identity) and GuaraniP1 (99.5% identity) viruses. The HA phylogenetic tree clustered most VACV isolates from Brazil together, mainly because of the presence of the deletion signature (group 1). However, VACV Serro 2011 isolates did not exhibit this signature and instead clustered with VACV strains that are less frequently isolated during outbreaks in Brazil (group 2) (Figure 2, panel A).

To further characterize the virus, we also sequenced the *A26L* gene (*ati*) (*14*). Because HA sequences were identical for SH1V/2011, SH2V/2011, and SB1V/2011 (and hypothetically represent the same isolate), we selected SH2V/2011 to analyze for *ati* and virulence in BALB/c mice. The *ati* gene is highly polymorphic in VACV strains from Brazil, and some strains exhibit a large deletion in the *ati* gene (*15*). Therefore, we used *ati* to characterize the VACV strains from Brazil. A 1,600-bp fragment that also did not contain the deletion was amplified from SH2V/2011. This virus exhibits no deletion in either the HA or *ati* genes in the analyzed regions of these genes. The *ati* phylogenetic tree (Figure 2, panel B) showed that SH2V/2011 also clustered with SPAn232 virus (group 2) and was segregated from the other group 1 VACV strains.

Given the atypical genetic profile of SH2V/2011 and the long-term hospitalization of patient C, we investigated the virulence of this isolate in mice (following the rules of Committee of Ethics for Animal Experimentation, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil). A total of 16 BALB/c mice were divided into 4 groups of 4 mice each. We intranasally inoculated 4 mice with 10-mL doses of viral suspensions containing 10^6 PFUs, as described (*10*). Two groups were inoculated with VAVC-GuaraniP1 and VACV-GuaraniP2 as virulent and

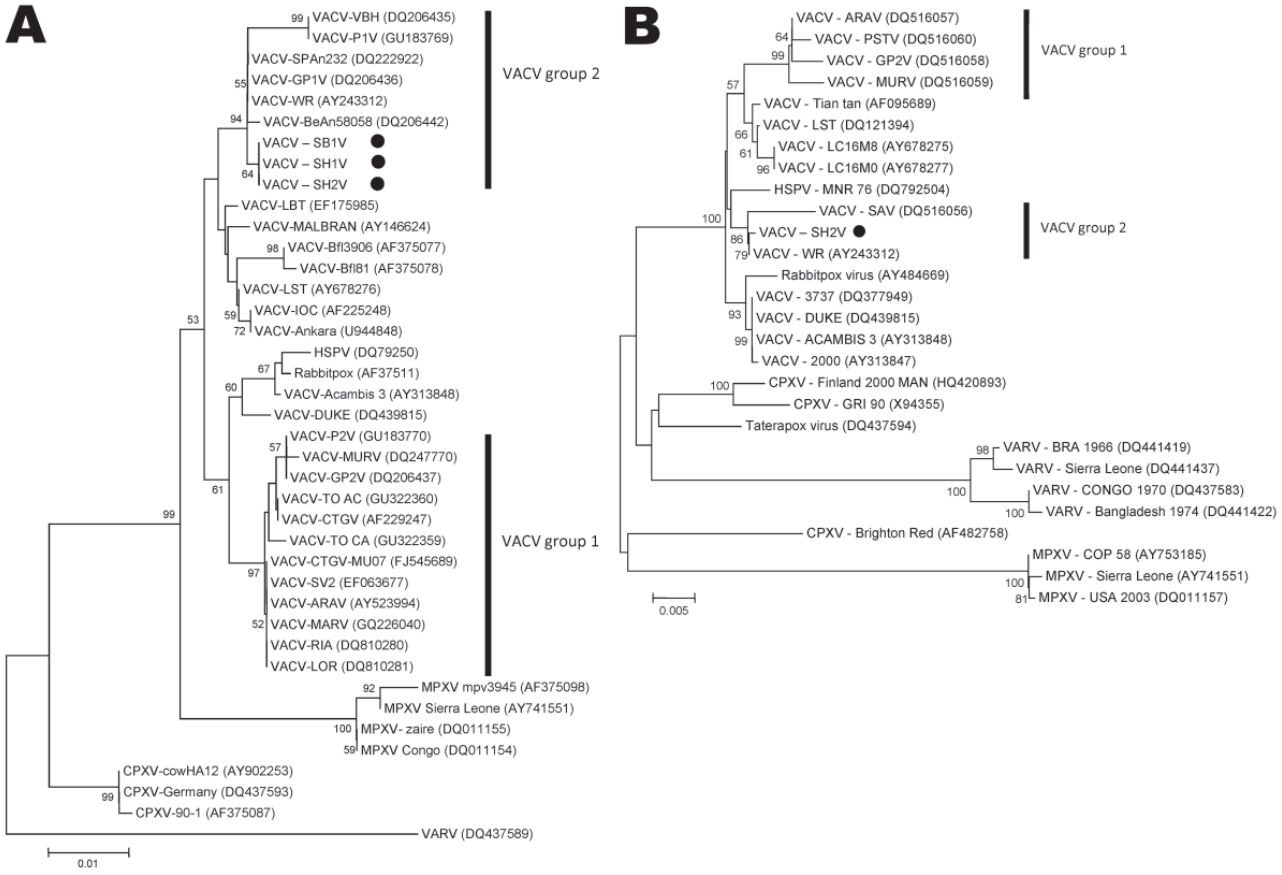

Figure 2. Phylogenetic analysis of vaccinia virus (VACV) isolates. A) Phylogenetic tree based on the nucleotide sequence of the orthopoxvirus hemagglutinin gene. VACV Serro bovine (SB1V), VACV Serro human 1 and 2 (SH1V and SH2V) grouped with VACV group 2 isolates, far from other VACV group 1 members. These isolates grouped far from (outliers) Serro-2 virus, a VACV isolated in the same geographic region. B) Phylogenetic tree based on the nucleotide sequence of the orthopoxvirus *ati* gene. The hemagglutinin tree shows SH2V grouping with VACV Western Reserve (WR) and SPAn232 virus, members of VACV group 2, not close to other isolates from Brazil. The neighbor-joining method with the Tamura-Nei model of nucleotide substitutions in the MEGA4 software program (www.megasoftware.net/) was used. Bootstrap confidence intervals are shown on branches (1,000 replicates) with GenBank accession numbers. MPXV, monkeypox virus; CPXV, cowpox virus; HSPV, horsepox virus; VARV, variola virus. Black dots indicate samples isolated during the 2011 outbreak of bovine vaccinia in Brazil. Scale bars indicate nucleotide substitutions per site.

nonvirulent controls, respectively. Another group was inoculated with phosphate-buffered saline. The SH2V/2011 sample was highly virulent in BALB/c mice; morbidity rate was high (online Technical Appendix Figure 2), thereby supporting the grouping of this sample in the virulent cluster. The animals that were infected with SH2V/2011 exhibited ruffled fur, arched backs, and weight loss, much like those infected with VACV-GuaraniP1. No clinical signs were observed in mice that had been inoculated with either VACV-GuaraniP2 or phosphate-buffered saline.

To further characterize the virus, we also performed a plaque phenotype assay in BSC-40 cell cultures. The VACV strains from Brazil that exhibited virulence in a BALB/c model usually formed large plaques in BSC-40 cell cultures. This assay showed that, in contrast to VACV-GuaraniP2, in BSC-40 cell cultures, SH2V/2011 induces the formation of large plaques that are similar to those induced by VACV-Western Reserve and VACV-GuaraniP1 (online Technical Appendix Figure 3).

Conclusions

Our results indicate that VACV Serro-2011 is a new mouse-virulent VACV strain associated with an outbreak that affected cows and humans. Recently, several VACV strains have been isolated in Brazil, most exhibiting a signature deletion in *A56R*, few or no deletions in the *ati* gene, and low virulence in mouse models. In contrast, the VACV isolated from the outbreak reported here, affecting cattle and humans, exhibited virulence in mice but no deletions in either the *A56R* or *ati* genes. This strain is genetically and phenotypically distinct from the Serro-2 strain that was isolated from the same region in 2005 (*7*); thus, >1 VACV might be circulating in Serro and possibly other regions of Brazil (*7,8*).

During outbreaks of bovine vaccinia, hospitalization of humans, especially for several days, is unusual. Unfortunately, despite the development of in vivo models for study and differentiation of VACV from Brazil, no clear association between viral genetics and disease severity in humans and cattle has been shown. Although preliminary, the data presented here indicate a possible association between these 2 factors, considering the hospitalization of patient C to be long term. Although the surveillance and characterization of VACV have advanced in recent years, observation and description of any clinical characteristics of infected humans and cattle are still helpful. Such observations might be associated with genotypic and phenotypic features of VACV, which could influence surveillance and control strategies for the management of VACV outbreaks.

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Mr Assis is a biologist and doctoral candidate at the Laboratório de Vírus, Microbiology Department, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais. His research interests focus on monitoring, phylogeny, and preventing emerging infectious diseases.

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Address for correspondence: Jônatas Santos Abrahão, Universidade Federal de Minas Gerais–Microbiology, Av. Antônio Carlos, 6627 Belo Horizonte, Minas Gerais 31270-901, Brazil; email: jonatas.abrahao@gmail.com

Porcine Reproductive and Respiratory Syndrome Virus, Thailand, 2010–2011

Dachrit Nilubol, Thitima Tripipat, Tawatchai Hoonsuwan, and Khampee Kortheerakul

Characterization of porcine reproductive and respiratory syndrome virus (PRRSV) isolates from pigs in Thailand showed 30-aa discontinuous deletions in nonstructural protein 2, identical to sequences for highly pathogenic PRRSV. The novel virus is genetically related to PRRSV from China and may have spread to Thailand through illegal transport of infectious animals from bordering countries.

Porcine reproductive and respiratory syndrome (PRRS) has a substantial economic effect on the swine industry worldwide. PRRS virus (PRRSV), a member of the family *Arteriviridae*, is the etiologic agent of the syndrome. PRRSVs are divided into 2 distinct genotypes: type 1 and type 2. The genotypes have a similar genomic organization, and 10 open reading frames (ORFs) have been identified (*1*–*3*). Nonstructural protein 2 (Nsp2) and ORF5 are the most variable regions (*4*,*5*), coding for replicase protein and neutralizing epitope, respectively.

In general, PRRSV causes a disease characterized by reproductive failure in sows and respiratory infection in growing pigs. However, in June 2006, a disease characterized by high fever and associated with a high mortality rate emerged in the People's Republic of China (PRC), resulting in the death of >20 million pigs (*6*). The disease, referred to as porcine high fever disease (PHFD), was caused by a new PRRSV variant with a unique hallmark: 2 discontinuous 30-aa deletions in Nsp2. The variant, identified as a highly pathogenic (HP) PRRSV, has subsequently become endemic in PRC (*7*), and it has spread to other countries, including Vietnam (*8*) and Lao People's Democratic Republic (Lao PDR) (*9*).

It is thought that HP-PRRSV spread to Thailand early in 2010. Pigs on a small farm in Nong Khai, a border province in northeastern Thailand located near Lao PDR, showed signs of illness identical to those for PHFD. Within 2 weeks of the initial outbreak, similar clinical features were observed in pigs on 19 small farms in a nearby village. Since then, pigs exhibiting similar clinical signs have been observed in >100 herds in >20 provinces throughout Thailand. The causative agent was isolated from sick pigs and determined to be PRRSV.

To further our knowledge about PRRSV in Thailand, we genetically characterized partial Nsp2 and complete ORF5 genes of PRRSV isolates. In addition, we determined sickness and mortality rates on affected farms.

The Study

During August 2010–June 2011, outbreaks of disease consistent with PHFD were investigated on 4 pig farms located in geographically separate regions of Thailand (Table 1). Herds were selected for study if farm owners agreed to participate. Pigs in all 4 herds had similar clinical signs. In 3 herds, the outbreak was initially observed in the breeding herd and lasted for ≈1 month; most deaths occurred in the third week. In those 3 herds, the initial signs of illness in sows were inappetence and high fever (40°C–42°C), followed by reddened skin and abortion. Illness rates among sows were 100%, 50%, and 60%, respectively for the 3 herds. The highest number of deaths among the sows occurred within 1 week of onset of the first symptoms. The percentage of culled sows on the 3 farms was 20.4%, 13.6%, and 6.7%, respectively; abortion rates were 52.8%, 8.4%, and 8.7%, respectively (Table 1). The outbreak in the fourth herd was confined to nursery facilities housing ≈4,000 pigs; nearly all pigs were sick within 1 week, and the mortality rate approached 60% within 2 weeks.

We performed PCR on serum samples from sick pigs to determine the presence of PRRSV; previously reported primers (*7*,*10*) were used to amplify partial Nsp2 and complete ORF5 genes. Products were cloned and sequenced at Bio Basic Inc. (Markham, Ontario, Canada). ClustalW (*11*) was used to align nucleotide and deduced amino acid sequences; 18 partial Nsp2 and 58 complete ORF5 genes were analyzed (Table 2).

To determine the relationship of PRRSV from herds in Thailand to HP-PRRSV, we compared the partial Nsp2 amino acid sequences corresponding to aa 404–640 of ORF1a from the isolates from Thailand with sequences for HP-PRRSV from PRC and Vietnam and for strain VR2332. PRRSV isolates from Thailand possess 2 discontinuous 30-aa deletions (aa 482 and 534–562) that are identical to those in HP-PRRSV (Figure 1).

To analyze the ORF5 genes of isolates from Thailand, PRC, and Vietnam, we constructed a phylogenetic tree by using the distance-based neighbor-joining method as implemented in MEGA4 (*12*). Bootstrap analysis was performed with 1,000 replicates. The tree showed the co-existence of HP-PRRSV types 1 and 2 in pigs in Thailand (Figure 2). Type 1 isolates from all 4 examined

Author affiliation: Chulalongkorn University, Bangkok, Thailand

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Table 1. Characteristics of pig farms with herds infected by PRRSV, Thailand, 2010–2011*

Herd ID, geographic location in country	Production system	Herd size, no. sows	Used attenuated North American PRRSV vaccine	No. (%) sow losses†		No. (%) sows that aborted
				Died	Culled	
UDT, northeast	Farrow-to-wean	500	No	48 (9.6)	102 (20.4)	264 (52.8)
UD, north	Farrow-to-wean	1,500	NK	ND	ND	ND
SCP, west	Farrow-to-finish	500	Yes	153 (30.6)	68 (13.6)	42 (8.4)
FDT, central	Farrow-to-finish	1,200	Yes	29 (2.4)	80 (6.7)	104 (8.7)

*PRRSV, porcine reproductive and respiratory syndrome virus; ID, identification; NK, not known; ND, no data available.
†Data are for the 4 weeks following the start of the outbreak on each farm.

herds clustered with previously reported clusters (*13*,*14*) distinct from type 1 modified live vaccine viruses (Porcillis PRRSV and Amervac PRRS). In contrast, some of the type 2 isolates from affected herds in Thailand had formed a novel cluster distinct from previously reported clusters (*13*,*14*). The novel type 2 isolates from Thailand clustered with isolates from PRC and Vietnam that were associated with PHFD. Genetic similarities between the novel type 2 isolates and HP-PRRSV were 97.8%–98.5% and 96.5%–99.0% homologous at the nucleotide and amino acid levels, respectively. However, the novel type 2 isolates from Thailand were more closely related to the 07QN isolate from Vietnam (98.5% nt and 99.0% aa similarities) than to the isolates from PRC.

We further investigated routes by which the virus spread. Before the outbreaks in Thailand, dead pigs were illegally transported from Lao PDR to an illegal slaughterhouse located not far from the farm where the first outbreak occurred, and the owner of the farm often visited the slaughterhouse. These findings suggest the movement of infected pigs in neighboring countries might play a role in introducing HP-PRRSV to new regions.

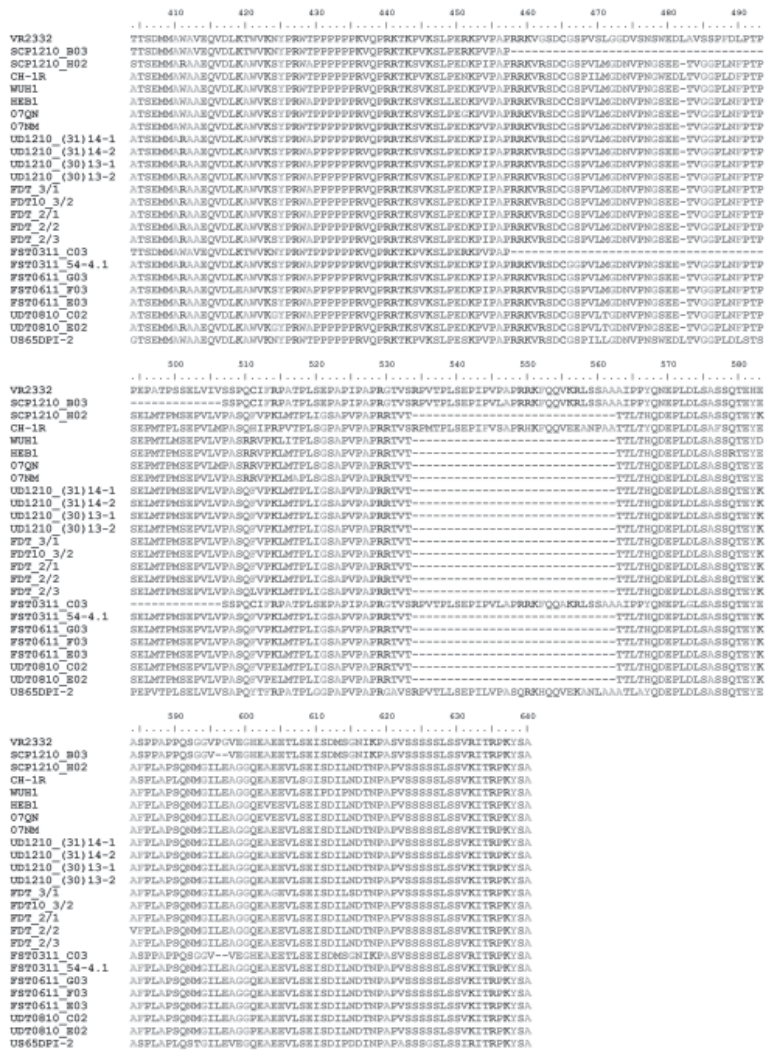

Figure 2. Phylogenetic analysis of types 1 and 2 porcine reproductive and respiratory syndrome virus (PRRSV) isolates constructed by the neighbor-joining method and based on the nucleotide sequences of complete ORF5 genes. The analysis included the following: previous and recent isolates (solid circles) from herds in Thailand that had an outbreak of HP-PRRSV; European reference isolates, including Lelystad virus (solid triangle) and 2 type 1 modified live vaccines (Porcilis PRRS, MSD Animal Health, Boxmeer, the Netherlands; and AMERVAC PRRS, Hipra, Girona, Spain) from Europe (open triangles); North American reference isolates, including VR2332 (solid diamond) and North American modified live vaccines (Ingelvac PRRS MLV, Boehringer Ingelheim, St Joseph, MO, USA) (open diamonds); modified live vaccines from PDR (CH1R) (open square); isolates from the People's Republic of China (**boldface**); and isolate from Vietnam (light gray font). Scale bar indicate nucleotide substitutions per site; numbers at nodes represent the percentage of 1,000 bootstrap replicates. A color version of this figure is available online (wwwnc.cdc.gov/eid/article/18/12/11-1105-F2.htm).

Infected pigs that were transported across the country and illegal slaughterhouses were the most likely routes of the spread of PRRSV within Thailand. The owners of several of the herds we investigated reported that pigs showed clinical signs within 1–2 days after trucks hauling dead pigs arrived at their farms. It was reported that dead pigs from herds in outbreak areas had been loaded on the trucks the day before they arrived at these farms. In Thailand, unlike in the United States, dead pigs are not composted, buried, or incinerated; instead, they are sold to

Table 2. PRRSV isolates obtained for sequence analysis from infected pig herds, Thailand, 2010–2011*

Isolate no.	Isolate name	Year and month collected	Genotype	Genes analyzed	GenBank accession no.
1	UD1210EU24/3	2010 Dec	I	ORF5	JX183110
2	UD1210EU23/2	2010 Dec	I	ORF5	JX183111
3	UD1210EU24/1	2010 Dec	I	ORF5	JX183112
4	SCP1210EU7/79-A07	2010 Dec	I	ORF5	JX183113
5	UD1210EU24/2	2010 Dec	I	ORF5	JX183114
6	UD1210EU24/1	2010 Dec	I	ORF5	JX183115
7	UD1210EU25/2	2010 Dec	I	ORF5	JX183116
8	UD1210EU25/1	2010 Dec	I	ORF5	JX183117
9	SCP0311EU1/3	2011 Mar	I	ORF5	JX183118
10	SCP0311EU1/2	2011 Mar	I	ORF5	JX183119
11	FDT0111EU2/3	2011 Mar	I	ORF5	JX183120
12	FDT0111EU2/2	2011 Mar	I	ORF5	JX183121
13	UD1210EU23/3	2010 Dec	I	ORF5	JX183122
14	SCP0311EU1/1	2011 Mar	I	ORF5	JX183123
15	FDT0111EU1/2	2011 Mar	I	ORF5	JX183124
16	SCP0311EU3/1	2011 Mar	I	ORF5	JX183125
17	FDT0111EU1/1	2011 Mar	I	ORF5	JX183126
18	FDT0111EU2/1	2011 Mar	I	ORF5	JX183127
19	SCP0311EU3/2	2011 Mar	I	ORF5	JX183128
20	UDT0810US_5/28–160	2010 Dec	II	ORF5	JN255819
21	UDT0810US_5/28–161	2010 Dec	II	ORF5	JN255820
22	UDT0810US_5/28–162	2010 Dec	II	ORF5	JN255821
23	UDT0810US_5/28–163	2010 Dec	II	ORF5	JN255822
24	UDT0810US_5/28–164	2010 Dec	II	ORF5	JN255823
25	UDT0810US_5/28–165	2010 Dec	II	ORF5	JN255824
26	UDT0810US_5/28–166	2010 Dec	II	ORF5	JN255825
27	UDT0810US_5/28–167	2010 Dec	II	ORF5	JN255826
28	UD1210US/61-E03	2010 Dec	II	ORF5	JN255827
29	UD1210US/61-F03	2010 Dec	II	ORF5	JN255828
30	UD1210US/61-G03	2010 Dec	II	ORF5	JN255829
31	UD1210US/62-H03	2010 Dec	II	ORF5	JN255830
32	UD1210US/62-A04	2010 Dec	II	ORF5	JN255831
33	UD1210US/62-B04	2010 Dec	II	ORF5	JN255832
34	UD1210US-25–1	2010 Dec	II	ORF5	JN255833
35	FDT10US-2–1	2010 Dec	II	ORF5	JN255834
36	FDT10US-2–2	2010 Dec	II	ORF5	JN255835
37	FDT10US-2–3	2010 Dec	II	ORF5	JN255836
38	SCP1210-U.S.-7–79–1	2010 Dec	II	ORF5	JN255837
39	SCP1210-U.S.-7–79–2	2010 Dec	II	ORF5	JN255838
40	UDT0810_E02	2010 Dec	II	Partial Nsp2	JN255839
41	UDT0810_C02	2010 Dec	II	Partial Nsp2	JN255840
42	SCP1210_H02	2010 Dec	II	Partial Nsp2	JN255842
43	SCP1210_B03	2010 Dec	II	Partial Nsp2	JN255841
44	FST0311_C03	2010 Dec	II	Partial Nsp2	JN255843
45	UD1210 (31)14–1	2010 Dec	II	Partial Nsp2	JN255844
46	UD1210 (31)14–2	2010 Dec	II	Partial Nsp2	JN255845
47	1–13(30)UD-1	2010 Dec	II	Partial Nsp2	JN255846
48	UD1210 (31)13–2	2010 Dec	II	Partial Nsp2	JN255847
49	FDT10_3/2	2010 Dec	II	Partial Nsp2	JN255848
50	FDT_3/1	2010 Dec	II	Partial Nsp2	JN255849
51	FDT_2/1	2010 Dec	II	Partial Nsp2	JN255852
52	FDT_2/2	2010 Dec	II	Partial Nsp2	JN255851
53	FDT_2/3	2010 Dec	II	Partial Nsp2	JN255850
54	FST0311_54–4.1	2010 Dec	II	Partial Nsp2	JN255853
55	FST0611_G03	2010 Dec	II	Partial Nsp2	JN255854
56	FST0611_F03	2010 Dec	II	Partial Nsp2	JN255855
57	FST0611_E03	2010 Dec	II	Partial Nsp2	JN255856
58	US65DPI-2	2010 Dec	II	Partial Nsp2	JN255857

*PRRSV, porcine reproductive and respiratory syndrome virus; ORF, open reading frame; Nsp2, nonstructural protein 2.

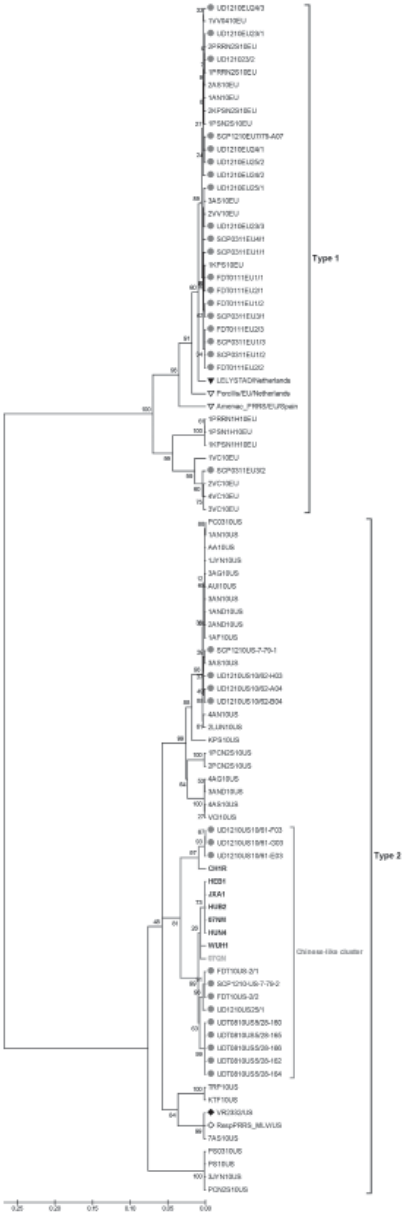

Figure 2. Phylogenetic analysis of types 1 and 2 porcine reproductive and respiratory syndrome virus (PRRSV) isolates constructed by the neighbor-joining method and based on the nucleotide sequences of complete ORF5 genes. The analysis included the following: previous and recent isolates (solid red circles) from herds in Thailand that had an outbreak of HP-PRRSV; European references, including Lelystad virus (solid triangle) and 2 type 1 modified live vaccines (Porcilis PRRS, MSD Animal Health, Boxmeer, the Netherlands; and AMERVAC PRRS, Hipra, Spain) from Europe (open triangles); North American references, including VR2332 (solid diamond) and North American modified live vaccines (Ingelvac PRRS MLV, Boehringer Ingelheim, USA) (open diamonds); modified live vaccines from the People's Republic of China (CH1R) (open square); isolates from the People's Republic of China (**boldface**); and isolate from Vietnam (purple font). Scale bar indicate nucleotide substitutions per site; numbers at nodes represent the percentage of 1,000 bootstrap replicates.

feed catfish. Truckers associated with this trade visit pig farms to buy and transport dead pigs. These trucks are not washed, so they are a potential source of contamination on farms.

Another source for the introduction of the novel PRRSV into Thailand could be an unapproved vaccine from PRC. The phylogenetic tree demonstrated that 3 recent isolates from Thailand (UD1210US/61-F03, UD1210US/61-G03, and UD1210US/61-E03) were more genetically related to CH-1R (an attenuated vaccine strain used in PRC) than HP-PRRSV (Figure 2). CH-1R is a classical PRRSV from PRC that does not possess the 2 discontinuous 30-aa deletions in Nsp2 (*15*). Furthermore, CH-1R is an attenuated PRRSV vaccine strain in PRC, and there is evidence that it has been illegally smuggled into Thailand. Thus, it is possible that this modified live virus from PRC may have been administered to the herd involved in the initial outbreak in Thailand and may have been the source of the novel PRRSV strain that caused the outbreak.

Conclusions

A novel PRRSV, which is genetically related to PRRSV isolates from PRC, has been introduced into Thailand. Sequences of Nsp2 revealed a unique 30-aa discontinuous deletion in the novel virus, a hallmark of HP-PRRSV. The virus may have been introduced into Thailand through the illegal transport of infected materials from bordering countries, more specifically, from Vietnam to Thailand through Lao PDR. This scenario is supported by our finding that PRRSV isolates from Thailand are more homologous with an isolate from Vietnam than with isolates from PRC. The cause of viral spread within Thailand may have been the movement of infected live and dead pigs across the country.

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Dr Nilubol is an assistant professor in the Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University. His research interests are PRRSV and porcine epidemic diarrhea.

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Address for correspondence: Dachrit Nilubol, Chulalongkorn University, Veterinary Microbiology, Faculty of Veterinary Science, Henry Dunant Rd, Pathumwan, Bangkok 10230 Thailand; email: dachrit@gmail.com

Cygnet River Virus, a Novel Orthomyxovirus from Ducks, Australia

Allan Kessell,[1] Alex Hyatt, Debra Lehmann, Songhua Shan, Sandra Crameri, Clare Holmes, Glenn Marsh, Catherine Williams, Mary Tachedjian, Meng Yu, John Bingham, Jean Payne, Sue Lowther, Jianning Wang, Lin-Fa Wang, and Ina Smith

A novel virus, designated Cygnet River virus (CyRV), was isolated in embryonated eggs from Muscovy ducks in South Australia. CyRV morphologically resembles arenaviruses; however, sequencing identified CyRV as an orthomyxovirus. The high mortality rate among ducks co-infected with salmonellae suggests that CyRV may be pathogenic, either alone or in concert with other infections.

In May 2010, an outbreak of disease at a duck farm at Cygnet River on Kangaroo Island, South Australia, Australia, occurred in 4-month-old Muscovy ducks (*Cairina moschate*). The ducks had been incorrectly fed during a week-long absence of the owner from the farm. The ducks were lethargic and had diarrhea, and the mortality rate among infected ducks was high. Of 150 ducks, 128 died in a 3-day period. Despite treatment with tetracycline, only 5 of the remaining 22 ducks survived.

The Study

After disease was discovered among Muscovy ducks on a farm in South Australia, 5 ducks with signs of infection were submitted for investigation to Gribbles Veterinary Pathology (Glenside, SA, Australia); 2 ducks died during transit. The 3 remaining ducks were euthanized, and postmortem examination showed severe necrotising fibrinous enteritis, multifocal piecemeal hepatitis, and severe fibrinous multifocal splenitis. *Salmonella enterica* serovar Typhimurium (phage type 9) was isolated from the ducks and was detected in at least 2 of the following from each animal: feces, spleen, bone marrow, and liver. Histologic lesions were consistent with a disseminated *Salmonella* infection; thus, a diagnosis of septicemic salmonellosis was made.

Pooled samples (liver, brain, lung, spleen, and gastrointestinal tissues) from 2 ducks were submitted for disease exclusion at the Australian Animal Health Laboratory, Commonwealth Scientific and Industrial Research Organization (Geelong, VIC, Australia). Results of nucleic detection assays excluded Newcastle disease virus and influenza virus as causative agents.

Isolation in 9- to 11-day-old embryonated eggs was performed on liver, brain, lung, spleen, and gastrointestinal tissues from 2 ducks after the samples were treated with antimicrobial drugs. In each of 3 passages in the embryonated eggs, embryo death occurred on passage days 4–5 for all tissues except the gastrointestinal tissues. However, when red blood cells from chicks and guinea pigs were used, hemagglutination that would indicate the presence of paramyxoviruses or influenza viruses was not observed in the allantoic fluid. In addition, the agent was filterable, suggesting the presence of a virus.

We observed hemolysis on the heads of the embryos and processed the embryos and chorioallantoic membranes for histopathologic studies. Multifocal necrosis was observed in the liver and lung of the embryos and in the chorioallantoic membranes. These necrotic lesions were consistent with an infectious agent. No influenza or Newcastle disease virus antigens were detected in the embryo and chorioallantoic membrane by immunohistochemical testing.

We observed cytopathic effects in tissues during additional passages in chicken embryo fibroblasts, Muscovy duck embryo fibroblasts, and Vero and Vero E6 cells. The cytopathic effect observed in Vero cells was minimal in comparison with that observed in other cell lines. We analyzed cultured samples from the allantoic fluid, chicken embryo fibroblasts, and Vero cells by using negative-contrast electron microscopy with nano-W stain (Nanoprobes, Yaphank, NY, USA). Vero cell monolayers were grown on sapphire disks, lightly fixed, and frozen under high pressure (Leica EM HPM100 high-pressure freezer; Leica Microsystems, North Ryde, NSW, Australia) before being freeze-substituted (*1*), infiltrated, and embedded in HM20 resin. After polymerization, the blocks were sectioned to 90 nm and counterstained with uranyl acetate and lead citrate. PCR for the causative agents of duck virus hepatitis and duck virus enteritis and for paramyxoviruses and mycoplasmae did not amplify any products.

Author affiliations: Gribbles Pathology, Glenside, South Australia, Australia (A. Kessell); Commonwealth Scientific and Industrial Research Organization, Geelong, Victoria, Australia (A. Hyatt, S. Shan, S. Crameri, C. Holmes, G. Marsh, C. Williams, M. Tachedjian, M. Yu, J. Bingham, J. Payne, S. Lowther, J. Wang, L.-F. Wang, I. Smith); and Kangaroo Island Veterinary Clinic, Kangaroo Island, South Australia, Australia (D. Lehmann)

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[1]Current affiliation: Charles Sturt University, Wagga Wagga, New South Wales, Australia.

The virus grew to low titer in the allantoic fluid of eggs and in culture (in chicken embryo fibroblasts; 50% tissue culture infectious dose, $10^{4.8}$/mL), which made initial identification by electron microscopy difficult. Despite minimal cytopathic effect, virus propagation in cell lines was highest in Vero cells (50% tissue culture infectious dose, $10^{7.9}$/mL), most likely because this cell line lacks interferon. The virus particles had ultrastructural characteristics of arenaviruses and orthomyxoviruses (Figure 1).

PCR amplification using panarenavirus PCR primers (*2*) did not identify arenavirus sequences. Therefore, we performed high-throughput sequencing by using the 454 Genome Sequencer FLX system (Roche Diagnostic Australia Pty Ltd, Castle Hill, NSW, Australia) to identify the virus. We pooled the allantoic fluid and tissue culture supernatant from chicken embryo fibroblasts for clarification at 10,000 rpm (JA 25.50 rotor; Beckman Coutler, Gladesville NSW, Australia) for 20 min and then centrifugation at 36,000 rpm (SW41 rotor; Beckman Coulter) for 90 min on a 20% sucrose cushion in TNE buffer (10 mM Tris, 0.1 M NaCl, 1 mM EDTA) buffer. The virus was resuspended in 350 mL of Buffer RLT (RNeasy lysis buffer) and extracted by using the RNeasy Mini Kit (QIAGEN, Doncaster, VIC, Australia) according to manufacturer's instructions. We performed additional processing of the sample as described (*3*).

Sequencing resulted in 15.5 Mb of sequence, which was assembled into 1,796 contigs. We used the 30 longest contigs in a BLAST search to match sequences available in the GenBank database (*4*). No similarity was observed at the nucleotide level, so we performed a blastx (www.ncbi.nlm.nih.gov/BLAST) search. Of the 30 sequences, 3 shared sequence identity with Quaranfil virus, Johnston Atoll virus, and Lake Chad virus, respectively; these viruses are members of the proposed genus *Quarjavirus*, family *Orthomyxoviridae* (*5*). All matches were within the polymerase genes of orthomyxoviruses; Quaranfil virus was the closest match (≈60% sequence identity).

The complete gene encoding the matrix gene was amplified by using PCR with published primers for Quaranfil virus (*5*) and then cloned and sequenced (GenBank accession no. JQ693418). The deduced amino acid sequence for the matrix protein was compared with sequences of other orthomyxoviruses and showed 31% identity with Quaranfil virus, 19% with influenza B(B/Yamagata), 18% with influenza A(A/PR/8/34), and 15% with infectious salmon anemia virus. We conducted phylogenetic analysis of the matrix protein of amino acid sequences from representative orthomyxoviruses by using the maximum likelihood method with MEGA5 (*6*) (Figure 2). Bootstrapping at 1,000 replicates was conducted. Analysis showed that this virus was novel; thus it was designated Cygnet River virus (CyRV). Further analysis is ongoing to determine the full genome sequence.

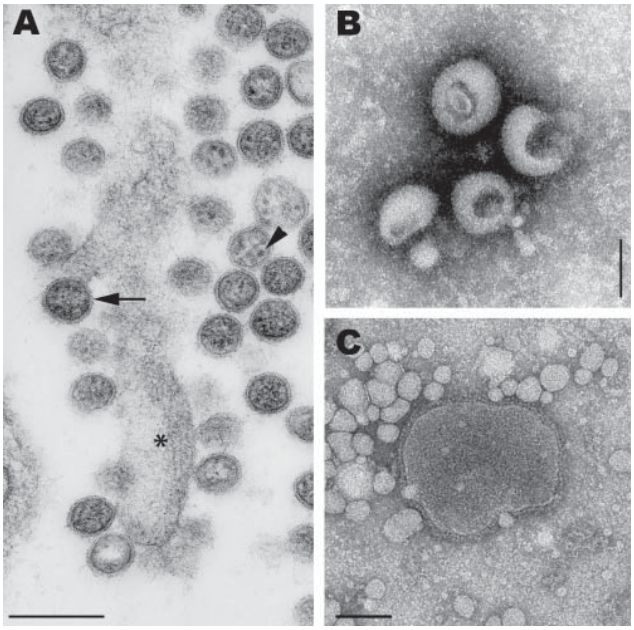

Figure 1. A) Transmission electron micrograph of an ultrathin section of Vero cells infected with Cygnet River virus (CyRV) from a Muscovy duck, Australia. Arrow, virus budding from the plasma membrane; arrowhead, sand-like structures. *Host cell projection. Scale bar = 200 nm. B, C) Transmission electron micrographs of CyRV prepared by negative-contrast electron microscopy. Scale bars = 100 nm. Preparations were derived from supernatant of CyRV-infected Vero cells (B) and from allantoic fluid of CyRV-infected eggs (C).

Conclusions

We identified a novel orthomyxovirus virus isolated from Muscovy ducks in South Australia. Examination by electron microscopy showed that the virus has a strong morphologic resemblance to arenaviruses and orthomyxoviruses. Next-generation sequencing enabled identification of the virus as an orthomyxovirus (member of the family *Orthomyxoviridae*). The implications of this discovery extend to 3 areas.

First, the discovery of this novel virus will enable the development of diagnostic reagents for the future detection of the virus. The isolation of the virus also enables us to conduct in-depth pathogenesis studies (ongoing) and to assess the potential role of this virus in disease outbreaks among ducks.

Second, the discovery of this virus provided supportive evidence for the creation of a new genus within the family *Orthomyxoviridae*. The family comprises 5 known genera (*Influenzavirus A*, *B*, and *C*, *Isavirus,* and *Thogotovirus*) and 1 tentative genus (*Quarjavirus*). The proposed genus *Quarjavirus* contains the 3 virus species: Quaranfil, Johnston Atoll, and Lake Chad viruses (*5*), and, now, a

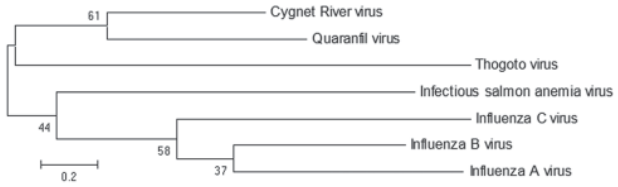

Figure 2. Maximum-likelihood tree showing phylogenetic relationships between Cygnet River virus isolate 10–01646 (GenBank accession no. JQ693418) and other orthomyxoviruses: Quaranfil virus isolate EG T 377 (accession no. GQ499304), Thogoto virus strain PoTi503 (accession no. AF527530), infectious salmon anemia virus isolate RPC/NB (accession no. AF435424), influenza C virus C/Yamagata/8/96 (accession no. AB064433), influenza B virus B/Wisconsin/01/2010 (accession no. CY115184), and influenza A virus A/California/07/2009(H1N1) (accession no. CY121681). Tree was based on deduced amino acid sequences of the complete matrix protein of orthomyxoviruses, applying 1,000 bootstrap replicates (*6*). Numbers at nodes indicate percentage of 1,000 bootstrap replicates. Scale bar indicates nucleotide substitutions per site.

fourth member—CyRV. Quaranfil virus, a human pathogen that caused a mild febrile illness in children in Egypt (*7*), and Johnston Atoll virus, a tick-borne virus of birds, were previously identified as arenaviruses on the basis of morphologic characteristics (*8*); however, on the basis of sequence identity, they were subsequently determined to be orthomyxoviruses (*5*). Quarjaviruses are tick-borne, so ticks may play a role in the transmission of CyRV.

Third, our findings highlight the value of undertaking a thorough disease investigation. To ensure that all potential causative agents are identified during an investigation, the presence of >1 agent should not be discounted. This notion was elegantly demonstrated a few years ago when Reston Ebola virus was discovered in pigs in the Philippines after an initial diagnosis of porcine reproductive and respiratory syndrome (*9*). Although the pathogenicity of CyRV infection alone in ducks remains to be investigated, the high mortality rate (97%) among ducks co-infected with salmonellae and CyRV raises the possibility that CyRV may be pathogenic, either alone or in concert with other infections. In the ducks we investigated, insufficient feeding may also have played a role in the infection dynamics and the clinical outcome.

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Dr Kessell works as a pathologist at Charles Sturt University. His areas of interest are diagnostic pathology and practical veterinary pathology.

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Address for correspondence: Ina Smith, CSIRO Australian Animal Health Laboratory, PO Bag 24, Geelong, Victoria 3220, Australia; email: ina.smith@csiro.au

High Diversity of RNA Viruses in Rodents, Ethiopia

Yonas Meheretu,[1] Dagmar Čížková,[1] Jana Těšíková, Kiros Welegerima, Zewdneh Tomas, Dawit Kidane, Kokob Girmay, Jonas Schmidt-Chanasit, Josef Bryja, Stephan Günther, Anna Bryjová, Herwig Leirs, and Joëlle Goüy de Bellocq

We investigated synanthropic small mammals in the Ethiopian Highlands as potential reservoirs for human pathogens and found that 2 rodent species, the Ethiopian white-footed mouse and Awash multimammate mouse, are carriers of novel Mobala virus strains. The white-footed mouse also carries a novel hantavirus, the second Murinae-associated hantavirus found in Africa.

Most emerging infectious diseases of humans or domestic animals are zoonoses, and among emerging pathogens, RNA viruses are highly represented (*1*). The synanthropic nature of some rodent species makes them important reservoirs of RNA viruses pathogenic to humans, such as hantaviruses (e.g., Seoul virus in black and Norway rats worldwide) and arenaviruses (e.g., Lassa virus in the multimammate mouse in western Africa or lymphocytic choriomeningitis virus in the house mouse worldwide). In Africa, members of the rodent genera *Mastomys* and *Arvicanthis* are linked to human activity; these rodents are widespread throughout sub-Saharan Africa and are crop pests and zoonotic reservoirs for human pathogens. Histories of synanthropy are likely longest for rodents in areas of early human sedentism, making RNA virus richness in early centers of domestication such as the Ethiopian Highlands of particular interest.

Hantaviruses (family *Bunyaviridae*) are RNA viruses primarily carried by rodents and soricomorphs (shrews and moles), although 2 new species have recently been described in bats (*2*,*3*). Arenaviruses (family *Arenaviridae*) are primarily rodent-borne RNA viruses. Members of both genera can cause life-threatening diseases in humans: arenaviruses cause hemorrhagic fevers in the Americas and Africa, and hantaviruses cause hemorrhagic fever with renal syndrome in Asia and Europe and hantavirus cardiopulmonary syndrome in the Americas. In Africa, only Lassa and Lujo arenaviruses are known to be highly pathogenic to humans. In contrast, hantaviruses have not yet been found to cause life-threatening human diseases in Africa, but hantavirus-specific antibodies have been found in human serum samples from several countries in Africa (*4*,*5*). To investigate the role of synanthropic small mammals as potential reservoirs of emerging pathogens in Ethiopia, we sampled rodent and shrew species in areas near human habitations and screened them for hantavirus and arenavirus RNA.

The Study

Small mammals from domestic and peridomestic areas were trapped during August–December 2010 in 2 high-altitude localities, Golgolnaele (13°52′N, 39°43′E, elevation 2,700 m) and Mahbere Silassie (13°39′N, 39°08′E, elevation 2,600 m), and in 1 lower-altitude locality, Aroresha (12°25′N, 39°33′E, elevation 1,600 m), in the Tigray region of the Ethiopian Highlands. Kidney samples preserved in RNAlater reagent (QIAGEN, Hilden, Germany) and stored at –80°C were used for total RNA extraction by using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany). Samples were pooled in pairs by locality and host species. RNA was reverse transcribed by using random hexamers as primers. Screening for arenaviruses was performed by using a pan–Old World arenavirus PCR targeting the large (L) gene (*6*). Screening for hantaviruses was performed by using a nested PCR assay targeting the hantavirus L gene (*7*).

A total of 201 small mammals from 6 species were screened for arenaviruses and hantaviruses (Table). Among them, 1 Ethiopian white-footed mouse (*Stenocephalemys albipes*) from Golgolnaele and 2 Awash multimammate mice (*Mastomys awashensis*) from Aroresha were positive for arenavirus RNA; 10 white-footed mice from the 2 highland localities (6 from Golgolnaele, 4 from Mahbere Silassie) were positive for hantavirus RNA. Amplicons were purified and sequenced, and nucleotide sequences were aligned on the basis of the amino acid alignment. Phylogenetic analyses were performed on the nucleotide sequences by using a maximum-likelihood (ML) approach (*8*).

After sequencing of the 3 arenavirus-positive samples, 3 distinct arenavirus sequences were obtained, and an ML tree was constructed for these 3 arenavirus sequences and the partial L gene (340 bp) of representatives of Old World arenaviruses (Figure 1). The 3 sequences cluster with Mo-

Author affiliations: Academy of Sciences of the Czech Republic Institute of Vertebrate Biology, Brno, Czech Republic (Y. Meheretu, D. Čížková, J. Těšíková, J. Bryja, A. Bryjová, J. Goüy de Bellocq); Mekelle University, Mekelle, Ethiopia (Y. Meheretu, K. Welegerima, Z. Tomas, D. Kidane, K. Girmay); University of Antwerp, Antwerp, Belgium (Y. Meheretu, H. Leirs, J. Goüy de Bellocq); and Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany (J. Schmidt-Chanasit, S. Günther)

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[1]These authors contributed equally to this article.

Table. Small mammal species screened for arenaviruses and hantaviruses, Ethiopian Highlands, August–December 2010*

Species	No. animals by locality and elevation			Total no. animals
	Aroresha, 1,600 m	Golgolnaele, 2,700 m	Mahbere Silassie, 2,600 m	
Ethiopian white-footed mouse (*Stenocephalemys albipes*)	0	33	23	56
Awash multimammate mouse (*Mastomys awashensis*)	16	1	1	18
Mus (*Nannomys*) sp. mice	11	20	2	33
Black rat (*Rattus rattus*)	37	2	5	44
African giant shrew (*Crocidura olivieri*)	6	2	17	25
Dembea grass rat (*Arvicanthis dembeensis*)	8	13	4	25
Total	78	71	52	201

*Species identification was confirmed by sequencing the partial mitochondrial cytochrome b gene (*11*). Representative sequences are available in GenBank (accession nos. JQ956464-JQ956479). Voucher specimens from representative rodents have been deposited at the Evolutionary Ecology group, University of Antwerp, and are available from the authors on request.

bala virus (80% bootstrap support), an arenavirus discovered in *Praomys* sp. in the Central African Republic in 1983 (*10*). However, the 3 sequences from Ethiopia are not monophyletic; the 2 sequences from multimammate mice cluster together (94% bootstrap support), but the sequence from the white-footed mouse from Golgolnaele is basal to the clade (Mobala + *M. awashensis* virus sequences), with the Menekre virus, found in *Hylomyscus* sp. in Guinea (*11*), used as outgroup. The sequences from multimammate mice on average differ from those of Mobala virus and the sequence from the white-footed mouse by the same order of magnitude in terms of amino acids: 5.0 ± 2.1% and 5.9 ± 2.2%, respectively. The average amino acid difference between the sequence from the white-footed mouse and that from Mobala virus was 8.1 ± 2.6%. Therefore, these arenaviruses seem to be 2 strains of Mobala virus carried by 2 rodent species and found in 2 localities ≈250 km apart from each other and with an altitude difference of 1,100 m.

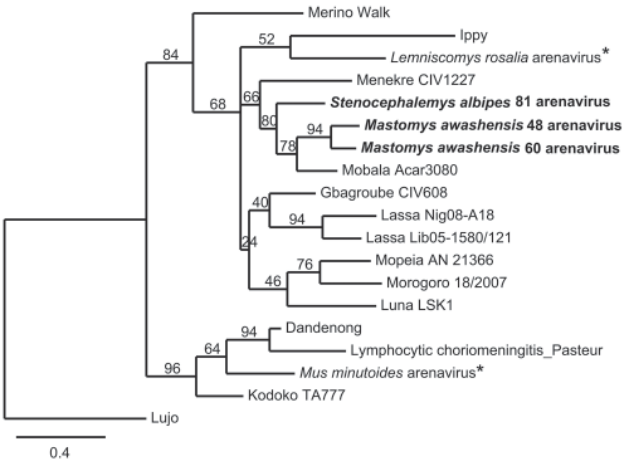

Figure 1. Maximum-likelihood tree of Old World arenaviruses showing the position of 3 arenaviruses (**boldface**; GenBank accession nos. JQ956481–JQ956483) found in kidney samples of Awash multimammate mice (*Mastomys awashensis*) and Ethiopian white-footed mice (*Stenocephalemys albipes*). The tree was constructed on the basis of analysis of partial sequences of the RNA polymerase gene; phylogeny was estimated by using the maximum-likelihood method with the GTR + I + Γ (4 rate categories) substitution model to account for rate heterogeneity across sites as implemented in the PhyML program (*8*). Lujo arenavirus was used as an outgroup. Numbers represent percentage bootstrap support (1,000 replicates). *Arenaviruses from Tanzania that have not yet been named (*9*). Scale bar indicates nucleotide substitutions per site. GenBank accession numbers of the virus strains: EU136039, GU830849, AY363902, EF179864, GU979511, GU481071, DQ868486, GU182412, FJ952385, AB586645, GU830863, GU078661, DQ328876, AY363904, EU914110, GU182413.

After sequencing of the 10 hantavirus-positive samples, 4 distinct hantavirus sequences were obtained, 2 from Golgonaele and 2 from Mahbere Silassie. Figure 2 shows the ML tree for these 4 sequences and the partial L gene (347 bp) of representatives of hantaviruses. The tree is not well resolved, and shrew- and mole-associated hantaviruses do not cluster. Two rodent-associated clades are supported: the previously known Murinae-associated hantaviruses (69% bootstrap support) and the Cricetidae-associated hantaviruses (92% bootstrap support, with 1 exception, Rockport, Soricomorpha-associated hantavirus). Although all 4 sequences were found in *S. albipes* mice, a Murinae species endemic to Ethiopia, they do not group with the Murinae-associated hantaviruses or with hantaviruses found in other African small mammals, such as bats (*2*,*3*) or shrews (*12*,*13*). The 4 sequences form a unique, divergent clade with the 2 sequences from Mahbere Silassie basal to the sequences from Golgonaele, which cluster together. The average amino acid difference between the sequences from Ethiopia and those from Murinae-associated hantaviruses was 27.0 ± 4.0%. Because the new amino acid sequences are at least 21.0 ± 4.0% divergent from those of other hantaviruses, we conclude that *S. albipes* mice are carrying a novel hantavirus. We propose the name Tigray virus for this virus because it was found in the Tigray region of Ethiopia. Additional genetic characterization, in particular of the small and medium segments, will be conducted to further clarify the evolutionary relationship of this virus within the hantavirus genus.

Conclusions

Two rodent species living in close proximity to human settlements in Ethiopia are carriers of arenaviruses and hantaviruses. Recently, several new arenaviruses and hantaviruses have been described in small mammals in Af-

rica, but no clear association with human diseases has been found (*2*,*3*,*9*,*11*–*13*). However, arenavirus and hantavirus infections are likely severely underreported because symptoms may resemble those of many other febrile infections (*2*). Investigating the presence of antibodies for Mobala virus and the proposed Tigray virus in humans in the Ethiopian Highlands is the next step in evaluating their pathogenicity. A recent study in Guinea showed that 2/68 patients with fever of unknown origin had antibodies for Sangassou hantavirus (*5*); a case of putative hantavirus disease (hemorrhagic fever with renal syndrome) was also reported in the Central African Republic (*14*). Hantavirus infections may thus be an unrecognized medical problem in Africa and deserve more attention.

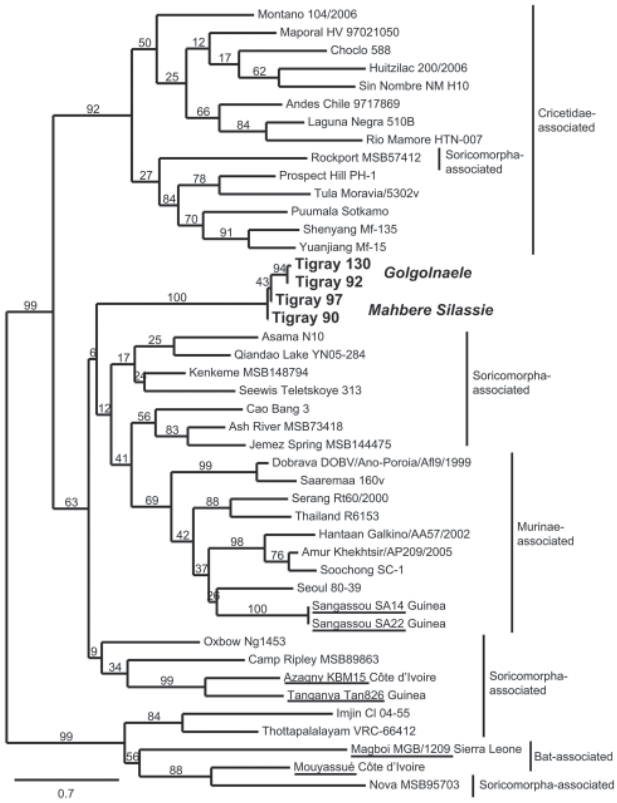

Figure 2. Maximum-likelihood tree of hantaviruses showing the position of the 4 sequences of Tigray hantavirus (**boldface**; GenBank accession nos. JQ956484–JQ956487) found in kidney samples of Ethiopian white-footed mice (*Stenocephalemys albipes*). The tree was constructed on the basis of analysis of partial sequences of the RNA polymerase gene; phylogeny was estimated by using the maximum-likelihood method with the general time reversible + I + Γ (4 rate categories) substitution model to account for rate heterogeneity across sites as implemented in the PhyML program (*8*). Numbers represent percentage bootstrap support (1,000 replicates). Underlining indicates hantaviruses found in Africa. Scale bar indicates nucleotide substitutions per site. GenBank accession numbers of the virus strains: AB620030, NC_003468, EU929078, EF619961, JF276228, EF540771, EF543525, EF397003, NC_005235, AB620033, JN037851, FJ170809, FJ170812, AB620108, EF641807, FJ593501, GQ306150, AF005729, EU788002, AB620102, FJ593498, FJ593497, EF646763, NC_005225, GU566021, FJ809772, HM015221, AJ410618, DQ268652, JQ082305, EU424336, NC_005238, AM998806, NC_005217, DQ056292, EF050454, JN116261, EU001330, AJ005637, JQ287716.

In conclusion, our screening of 201 small mammals led to the identification of 2 novel strains of Mobala arenavirus and a novel hantavirus in 2 rodent species found in Ethiopia, *M. awashensis* and *S. albipes*. These rodents belong to the exclusively African Praomyini tribe (*15*), which hosts 5/11 arenaviruses (Lassa, Mopeia, Luna, Mobala, and Menekre viruses) and the only Murinae-associated hantavirus (Sangassou virus) described in Africa. Our results support a major role for Praomyini as hosts in the evolutionary history of arenaviruses and hantaviruses in Africa.

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Dr Meheretu is a lecturer at Mekelle University and a postdoctorate researcher at the Academy of Sciences of the Czech Republic Institute of Vertebrate Biology. His research interests are rodent pests that cause agricultural damage and act as natural reservoirs of human pathogens in Ethiopia.

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Address for correspondence: Joëlle Goüy de Bellocq, Evolutionary Ecology Group, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belgium; email: joelle.gouydebellocq@ua.ac.be

West Nile Virus Neurologic Disease in Humans, South Africa, September 2008–May 2009

Dewald Zaayman and Marietjie Venter

We investigated West Nile virus (WNV) as a possible disease etiology in persons hospitalized in South Africa. Of 206 specimens tested, 36 had WNV neutralizing antibodies, significantly more than in similar earlier serosurveys. Seven probable acute WNV cases were identified, suggesting WNV may be overlooked as an etiology of severe disease in South Africa.

West Nile virus (WNV), a mosquito-borne flavivirus (*1*), is a reemerging pathogen of global concern (*2*). Febrile illness occurs in ≈20% of WNV-infected persons; neurologic complications (e.g., meningitis, encephalitis, flaccid paralysis) occur in <1% (*3*).

Detection of IgM in serum or cerebrospinal fluid (CSF) is the preferred method for diagnosing WNV infection; however, because of cross-reactivity between flaviviruses, positive results should be confirmed by virus neutralization assay. Early WNV infection can be diagnosed by PCR and virus isolation (*4*), but success has been limited in diagnosing more advanced disease with these techniques.

WNV is endemic to southern Africa. In 1947, one of the largest WNV epidemics recorded occurred in the Karoo region of South Africa (*5*), and another occurred in combination with a Sindbis virus epidemic in 1983–84 in the Witwatersrand–Pretoria region of South Africa. The most recent seroprevalence data for WNV in the Pretoria region of South Africa is from the 1970s (reviewed in [*6*]). To update that information, we determined whether WNV is being overlooked as a possible cause of disease in persons hospitalized in South Africa. The University of Pretoria Research Ethics Committee approved this study.

The Study

Serum and CSF samples were obtained from the National Health Laboratory Service, Thswane Academic Division, Thswane, South Africa, which serves public sector hospitals in northern South Africa. To select samples for testing, we reviewed laboratory submission requests for patients with clinical conditions consistent with WNV infection: fever, headache, rash, or neurologic signs (*7*,*8*). A total of 206 patient samples (15 CSF and 191 serum) were selected. During September 2008–May 2009, we screened samples for the presence of WNV by using real-time reverse transcription PCR (rRT-PCR) (*9*), virus neutralization assay, and IgM ELISA.

We detected WNV neutralizing antibodies in serum and CSF samples by using a modified method (*10*). In brief, we mixed 50% tissue culture infective doses of Kunjin virus strain MRM61C (100 U/mL) in 2% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) with 2-fold dilutions of heat-inactivated patient serum (1:10–1:640) in equal volumes and incubated the mixture for 1 h at 37°C in 5% CO_2. We then added 1 volume of Vero cells (1×10^5 cells/mL) in 2% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Lonza, Basel, Switzerland) and incubated the mixture for 72 h at 37°C in 5% CO_2. Samples were considered positive for WNV neutralizing antibodies if <25% of the cells/well displayed cytopathic effect. Comparative testing with Wesselsbron virus, a closely related flavivirus, did not show cross-reactivity within the parameters of what we considered positive.

Of the 206 specimens, 40 (19.42%, 95% CI 14.02%–24.82%) were positive for neutralizing antibodies. Of these, 36/191 serum samples had antibody titers of <160. The positive CSF samples (4/15) had antibody titers of 4 (Table 1). Positive serum samples were also tested by WNV IgM capture ELISA (Panbio; Alere, Sinnamon Park, QLD, Australia); 2 had positive results (Table 1).

Of the 206 specimens, 190 were of sufficient quantity to be subjected to RNA extraction (QIAamp Viral RNA Mini Kit; QIAGEN, Valencia, CA, USA) and subsequent WNV nested rRT-PCR (*9*). The presence of lineage 2 WNV RNA was identified in 1 CSF specimen and confirmed by sequencing (GenBank accession no. JX974605) (Figure).

To evaluate the sensitivity of these molecular and serologic tests for diagnosing WNV in humans, we used the same methods to test 9 archived sequential serum samples in parallel (Table 2). The samples were from a patient with WNV encephalitis who became infected with neuroinvasive lineage 2 WNV strain in 2003 after a needlestick injury (*11*). Samples were collected 0–30 days after exposure. Initial symptoms developed on postexposure day 7 and persisted for 19 days; the patient completely recovered by day 26 (*11*).

Conclusions

We conducted a retrospective investigation of patients hospitalized with febrile illness or neurologic disease of unknown etiology in the Pretoria region of South Africa

Author affiliations: University of Pretoria, Pretoria, South Africa (D. Zaayman, M. Venter); and National Institute for Communicable Diseases, Johannesburg, South Africa (M. Venter)

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Table 1. Characteristics and clinical information for 7 acutely ill patients with WNV infection, South Africa*

Sample no.	Date sample collected	Patient age, y/sex	Sample type	WNV antigen/antibody test			Clinical information
				AN (titer)	IgM	PCR	
4562	2008 Nov	45/M	CSF	Pos (4)	–	–	Suspected HIV encephalopathy or PML, TPHA neg, HIV neg, paraparesis
6208	2008 Nov	35/F	CSF	Pos (4)	–	–	Hepatomegaly, lymphadenopathy, fever, vomiting, epigastric pain, EBV IgM neg, EBV IgG pos, malaria neg, hepatitis neg
8785	2009 Jan	36/M	CSF	Pos (4)	–	–	Acute paresis of lower limbs, delirium, HSV-1 and HSV-2 neg (PCR), HTLV-1 neg, TPHA neg
3111	2009 Feb	5/M	CSF	Pos (4)	–	–	Meningitis, enterovirus pos (PCR)
0269	2009 Apr	11/M	Serum	Pos (40)	Pos	Neg	Rash, fever, *Brucella* neg (PCR), coxsackieviruses B1–B6 neg, CMV IgM neg, CMV IgG pos
0312	2009 Apr	26/M	Serum	Pos (80)	Pos	Neg	Severe headache, fever, suspected enterovirus, coxsackieviruses B1–B5 neg, *Rickettsia conorii* neg, EBV IgM neg
SAH 5238	2008 Oct	2/M	CSF	Neg	Neg	Pos	Decreased level of consciousness, rash, fever, meningitis, measles neg, mumps neg

*WNV, West Nile virus; AN, antibody neutralization; CSF, cerebrospinal fluid; pos, positive; –, insufficient sample for testing; PML, progressive multifocal leukoencephalopathy; TPHA, *Treponema pallidum* hemagglutination assay; neg, negative; EBV, Epstein-Barr virus; HSV-1 and -2, herpes simplex virus types 1 and 2; HTLV, human T-lymphotropic virus; CMV, cytomegalovirus.

to determine whether some of the cases could be ascribed to WNV infection. Evidence of acute WNV infection was identified in samples for 7 patients (Table 1). For 2 of the patients, WNV infection was identified by the presence of IgM and neutralizing antibodies in serum samples; these patients had been hospitalized for febrile illness. For the other 5 patients, infection was identified by a WNV–positive (by PCR) CSF sample (1 patient) and by the presence of neutralizing antibodies in CSF samples (4 patients); these patients had been hospitalized for neurologic signs and symptoms.

The 4 patients with neutralizing antibodies in CSF all had severe neurologic complications (Table 1). Samples from these patients were insufficient for performing IgM testing; thus, WNV infection cannot be definitively determined. However, the presence of WNV neutralizing antibody in CSF samples plus acute clinical signs and symptoms of WNV infection provide a high index of suspicion for WNV infection in these patients. Factors such as increased blood–brain barrier permeability and the persistence of WNV antibodies long after infection may also serve as explanations for the presence of neutralizing antibodies in their CSF.

Nested rRT-PCR results and phylogenetic analysis confirmed the presence of lineage 2 WNV in the CSF sample from 1 patient (Figure); sequencing showed that the

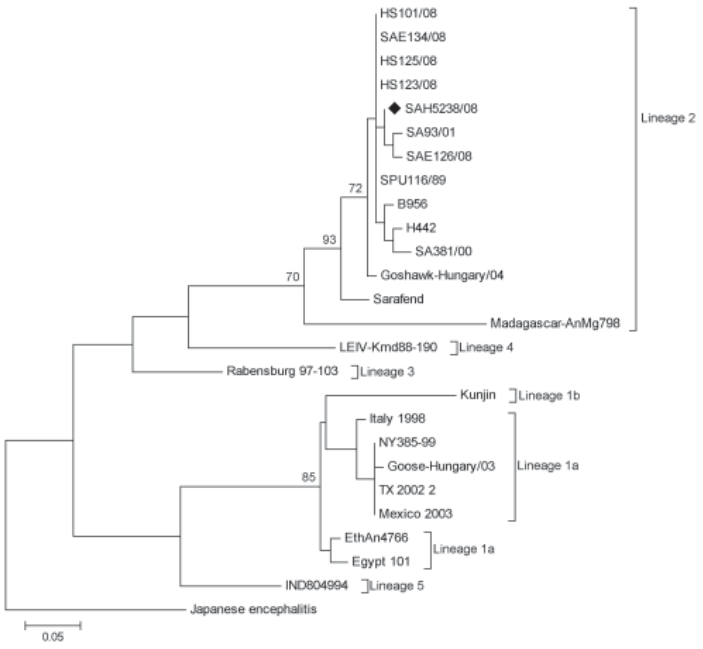

Figure. Maximum-likelihood tree of an ≈200-bp fragment of the nonstructural 5 gene of a reverse transcription PCR–positive West Nile virus (WNV) specimen SAH5238/08 (GenBank accession no. JX974605; black diamond) isolated from a human in South Africa in 2008. The tree shows the relationship of the strain to representative sequences of 5 WNV lineages, including 5 WNV lineage 2 strains isolated from horses in South Africa in 2008 (*15*). The scale bar indicates nucleotide substitutions per site. Bootstrap statistics of >70% are indicated on the tree branches. WNV strains (accession numbers): B956 (AY532665), SA381/00 (EF429199), SA93/01 (EF429198), SPU116/89 (EF429197), Goshawk-Hungary/04 (DQ116961), H442 (EF429200), Sarafend (AY688948), Madagascar AnMg798 (DQ176636), HS123/08 (FJ464376), HS101/08 (FJ464378), SAE126/08 (FJ464379), SAE134/08 (FJ464380), HS125/08 (FJ464377), Rabensburg97103 (AY765264), LEIV-Krnd88-190 (AY277251), Kunjin (D00246), Egypt101 (AF260968), EthAn4766 (AY603654), Italy1998 (AF404757), Goose-Hungary/03 (DQ118127), NY385-99 (EF571854), TX2002 (DQ164205), Mexico2003 (AY660002), IND804994 (DQ256376), Japanese encephalitis (HM228921).

Table 2. Results of a time-trial experiment with serum samples from a WNV-infected person, South Africa*

Days after exposure to WNV	IgM ELISA†	Neutralization assay (titer)	Nested PCR
0	Neg	Neg	Neg
8	Neg	Neg	Pos
9	Neg	Neg	Pos
10	Neg	Neg	Neg
11	Neg	Neg	Neg
13	Neg	Pos (20)	Neg
16	Pos	Pos (40)	Neg
26	Pos	Pos (40)	Neg
30	Pos	Pos (80)	Neg

virus is closely related to 2 neuroinvasive WNV lineage 2 strains identified in South Africa (*11*,*12*) (Table 1). The low rate of PCR-positive cases was not entirely unexpected and may be explained by 2 factors: 1) PCR has limited success for detecting arboviruses because CSF contains low levels of virus and arbovirus-associated viremia is brief (*13*), and 2) false-negative test results may occur if samples are not properly stored to protect the integrity of potential viral RNA.

To evaluate the sensitivity of the 3 diagnostic methods used in our study, we conducted a time-trial experiment by using retrospective serum samples from a patient in whom WNV meningoencephalitis developed after a needlestick injury (*11*). Early samples (postexposure days 8 and 9) were positive for WNV by rRT-PCR only. Results for samples obtained ≥13 and ≥16 days after exposure were positive by neutralization assays and IgM ELISA, respectively (Table 2). Experiments with horses have indicated that WNV neutralizing antibody assays show a positive result earlier than IgM ELISAs; the reasons for this are undetermined (*14*). Although our time-trial experiment reflects findings from only 1 patient and should ideally be performed on a cohort, the results, considered with those from the studies in horses (*14*), may imply that some cases of WNV infection in humans and animals may be missed if IgM ELISA is the only serologic test used.

Using virus neutralization assays, we identified the presence of WNV antibodies in 36/204 serum samples from patients with febrile and neurologic illness in South Africa. This finding indicates that the patients were exposed to WNV. In addition, results were negative for the patients in our study who were tested for herpes simplex virus types 1 and 2, measles, mumps, and enteroviruses, and no other etiologic agent was found. Thus, infection with WNV should be included in the differential diagnosis of patients in this region with neurologic disease, especially considering the frequent detection of severe neurologic disease in horses in the region (*15*).

Our findings confirm that WNV is being overlooked as a cause of severe neurologic disease in South Africa, and they suggest a need for increased clinical awareness, enhanced prospective surveillance, and a more current serosurvey of WNV infection in humans. PCR may be a useful diagnostic method during early infection, but after seroconversion has taken place, serologic tests (e.g., IgM ELISA in conjunction with virus neutralization) are more likely to yield accurate results.

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Mr Zaayman is pursuing a PhD degree in the Zoonosis Unit, Department of Medical Virology, University of Pretoria. He is conducting research on the development and application of molecular and immunological tools for the differential diagnosis of West Nile virus in South Africa.

Prof Venter is head of the Zoonoses Research Unit in the Department of Medical Virology, University of Pretoria, Pretoria, South Africa, and head of the Center of Respiratory Diseases and Meningitis, National Institute for Communicable Diseases, Sandringham, South Africa. Her primary research interests are arboviruses and their association with neurological diseases in humans and animals in Africa and viral causes of severe acute respiratory infections.

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Address for correspondence: Marietjie Venter, Zoonoses Research Unit, Department Medical Virology, Pathology Bldg, Room 2.72, Faculty of Health Science, 5 Bophela Rd, University of Pretoria, South Africa, 2000; email: marietjiev@nicd.ac.za

etymologia

Ehrlichia

[ār-lik′e-ə]

Named in honor of German scientist Paul Ehrlich, *Ehrlichia* is a genus of gram-negative bacteria of the family *Anaplasmataceae*. Although Ehrlich was not a bacteriologist and was primarily known for his work in hematology, immunology, and chemotherapy, the species *Ehrlichia kurlovi* was proposed in 1937 by Russian rickettsiologist Sh. D. Moshkovsky.

In 1889, Mikhail Georgiyevich Kurloff, perhaps working with Ehrlich, published a description of atypical granules in guinea pig leukocytes. These granules were assumed to be normal; rickettsiae would not be discovered for several decades. In his 1937 paper, Moshkovsky recognized the guinea pig granules as rickettsial inclusion bodies and named the genus "in honor of Paul Ehrlich, since it was in his laboratory that the first representatives of this group were discovered, and because he has contributed so much to the study of the morphology of the blood and of the agents of infectious diseases."

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Address for correspondence: Ronnie Henry, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E03, Atlanta, GA 30333, USA; email: boq3@cdc.gov

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Antimicrobial Drug–Resistant *Escherichia coli* in Wild Birds and Free-range Poultry, Bangladesh

Badrul Hasan, Linus Sandegren, Åsa Melhus, Mirva Drobni, Jorge Hernandez, Jonas Waldenström, Munirul Alam, and Björn Olsen

Multidrug resistance was found in 22.7% of *Escherichia coli* isolates from bird samples in Bangladesh; 30% produced extended-spectrum β-lactamases, including clones of CTX-M genes among wild and domestic birds. Unrestricted use of antimicrobial drugs in feed for domestic birds and the spread of resistance genes to the large bird reservoir in Bangladesh are growing problems.

Dissemination of *Enterobacteriaceae* that produce extended-spectrum β-lactamases (ESBLs) is increasing in humans and animals globally (*1*,*2*). Clinically relevant sequence and ESBL types have been reported among wild birds (*3*). *Escherichia coli* strains from domestic animals and poultry tend to carry the same CTX-M enzyme variants that are locally dominant in human isolates (*4*). Using birds as sentinels of the spread of antimicrobial drug resistance in the environment could indicate a wider prevalence of drug-resistant disease in humans (*3*,*5*).

In Bangladesh, the problem of antimicrobial drug resistance in humans and poultry is augmented by the uncontrolled use of unprescribed antimicrobial drugs (*6*). A high prevalence of resistant phenotypes has recently been reported in poultry and human *E. coli* isolates from Bangladesh (*6*,*7*). ESBL-producing *E. coli* and *Klebsiella pneumoniae* are common in clinical settings (*8*), but data quantifying the prevalence of different ESBL genotypes are limited. We screened fecal samples from wild birds and from poultry in the Rajshahi district of Bangladesh for antimicrobial-resistant and ESBL-producing *E. coli*.

Author affiliations: Uppsala University Department of Medical Sciences, Uppsala, Sweden (B. Hasan, Å. Melhus, M. Drobni, B. Olsen); Linnaeus University Centre for Ecology and Epidemiology in Microbial Model Systems, Kalmar, Sweden (B. Hasan, J. Hernandez, J. Waldenström, B. Olsen); International Center for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh (M. Alam); and Uppsala University Department of Medical Biochemistry and Microbiology, Uppsala (L. Sandegren).

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The Study

Samples from 96 birds (41 wild ducks, 29 chickens, 23 ducks, and 3 geese) were collected from the Padmachar area of Rajshahi District during January 2009. In this area, a lake hosts several thousand wintering wild birds; that lake also is frequented by poultry from surrounding households. Each fecal sample, collected by swirling a cotton swab in a bird's cloaca or droppings, was submerged in a bacterial freeze medium and handled as described (*5*). Each sample was placed on an Uriselect 4 agar plate (Bio-Rad Laboratories, Marnes-La-Coquette, France), and assessed for *E. coli* by biochemical testing and API 20E biochemical strips (bioMérieux SA, Marcy-l'Etoile, France). One *E. coli* isolate per positive bird sample was tested by disk diffusion against 15 antimicrobial drugs (Table 1) according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org). Multidrug resistance was defined as resistance to at least 3 classes of antimicrobial drugs.

Each sample was also enriched in brain-heart infusion broth (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with vancomycin 16 μg/mL (ICN Biomedicals Inc. Aurora, OH, USA) for 18 h at 37°C. For detection of ESBL-producing bacteria and genes ($bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM}) in putative ESBL isolates, described methods were used (*5*,*9*). Carbapenem-resistant isolates were screened on Mueller-Hinton agar plates supplemented with 2 μg/mL or 8 μg/mL meropenem and incubated overnight at 37°C.

The genetic profiles of the ESBL-producing *E. coli* isolates were determined by using repetitive element PCR. The reaction mixture contained 1× Taq PCR buffer, 0.625 μmol/L primer ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′), 1.9 mmol/L $MgCl_2$, 50 μmol/L dNTPs, 0.6 U Taq polymerase, and template in a total volume of 20 μL. Cycling parameters were 1 min at 94°C; 1 min at 36°C and 2 min at 72°C for 45 cycles, and a final extension for 5 min at at 72°C. Isolates that had identical strong band patterns but an addition or a loss of a weak band were assigned subtype numbers.

One representative for each repetitive element PCR genotype and subtype (n = 18) was characterized by multilocus sequence typing (MLST) (*10*). After sequencing, allele profiles and sequence types were determined by using the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli/#). One representative sample for each genotype was tested for transferability of the ESBL plasmid by conjugation to recipient *E. coli* DA11782 (*mcrA, Δmrr-hsdRMS-mcrBC*, *ΔlacX74, deoR, recA1, araD139Δ* [*ara-leu*] *7697, galK, rpsL, endA1, nupG*, *rif*^{R}). Equal amounts

Table 1. Antimicrobial drug resistance phenotypes in randomly isolated *Escherichia coli* from wild and domestic birds, Bangladesh

Drug	Domestic birds, n = 52	Wild birds, n = 14
Tetracycline	24	1
Trimethoprim/sulfamethoxazole	17	2
Ampicillin	15	4
Cephradine	0	1
Cefuroxime	2	1
Cefadroxil	0	1
Nalidixic acid	11	1
Ciprofloxacin	3	1
Streptomycin	3	1
Gentamicin	0	1
Fosfomycin	0	1
Chloramphenicol	4	0
Nitrofurantoin	1	0
Tigecycline	0	0
Mecillinam	0	0

of donor and recipient overnight cultures in Luria-Bertani broth were mixed and incubated, without shaking, overnight at 37°C. Approximately 10^9 CFU of conjugation mixture was placed on selective plates containing 10 μg/mL cefotaxime, 100 μg/mL rifampin, and 50 μg/mL nalidixic acid and incubated overnight at 37°C.

E. coli was isolated from 66 samples, yielding an isolation rate of 73.3% regardless of bird species. Thirty-five (53%) of the 66 isolates were resistant to ≈1 antimicrobial compounds. The most common resistance was to tetracycline. The 3 next most common resistances were to ampicillin, trimethoprim/sulfamethoxazole, and nalidixic acid (Table 1). Multidrug resistance was found in 22.7% (15/66) of the isolates, and 13.6% (9/66) of the isolates were resistant to 4 or 5 classes of antimicrobial drugs. Screening for carbapenamase producers yielded no isolates.

The overall prevalence of ESBL carriage among birds was 30% (27/90); 36 *E. coli* isolates produced ESBL. Thirty-four of them belonged to the CTX-M-1 group (2 $bla_{\text{CTX-M-1}}$ and 32 $bla_{\text{CTX-M-15}}$) and 2 to the CTX-M-9 group, the latter of which were CTX-M-14–like. Combinations of $bla_{\text{CTX-M-15}}$ or $bla_{\text{CTX-M-1}}$ and $bla_{\text{TEM-1}}$ were detected in 50% of the isolates, whereas none harbored SHV-genes.

The genetic fingerprints of the ESBL-producing *E. coli* isolates identified 15 genotypes, of which 19 (53%) of 36 were type A (Table 2).This genotype was found in wild and domestic birds. MLST analysis revealed 15 different sequence types (STs) and 1 nontypeable isolate (Table 2). Four isolates had new allele types or a new combination of allele types and were given novel STs (ST2690–ST2693). STs found in wild birds differed from those in poultry. One CTX-M-14–producing isolate from chicken belonged to the internationally recognized ST131 clone. Conjugation was successful for 9/18 isolates, indicating the transferability of plasmids carrying ESBL genes.

Conclusions

The carriage rate of ESBLs was high and the predominating antimicrobial-resistant phenotypes of wild birds and poultry appeared to correlate with antimicrobial prescription patterns in Bangladesh (*6*). Most ESBL-positive samples originated from poultry, and household poultry was the predominant carrier of the $bla_{\text{CTX-M-15}}$ genotype and the CTX-M-14–like enzymes. However, the

Table 2. Antimicrobial drug resistance genotype classification of ESBL-producing *Escherichia coli* isolates from wild birds and free-range poultry, Bangladesh*

Host	Isolate	ESB type	Repetitive element PCR genotype	Sequence type
Common teal (*Anas crecca)*	B51	CTX-M-15	A4	ST1408
Common teal	B53	CTX-M-15	A	ST1408
Tufted duck (*Aythya fuligula)*	B66	CTX-M-15	A3	ST1312
Domestic duck *(Anas platyrhynchos)*	B93	CTX-M-15	E	ST2141
Domestic duck	B97	CTX-M-15	G	ST2690†
Domestic duck	B98	CTX-M-15	F	ST448‡
Domestic duck	B100	CTX-M-15	D	ST405§
Domestic duck	B102	CTX-M-15	H	ST2691†
Domestic duck	B106	CTX-M-15	I	ST648
Domestic chicken *(Gallus domesticus)*	B125	CTX-M-15	A2	ST206¶
Domestic chicken	B127	CTX-M-1	J	ST744
Domestic chicken	B129	CTX-M-15	K	ST648
Domestic chicken	B130w	CTX-M-14 like	L	ST131
Domestic chicken	B133	CTX-M-15	M	ST2450
Domestic chicken	B136P	CTX-M-15	N	ST2692†
Domestic chicken	B137P	CTX-M-1	O	ST744
Domestic chicken	B140	CTX-M-15	A	ST2693†
Domestic chicken	B143	CTX-M-15	B	ST224

*ESBL, extended-spectrum β-lactamase.
†New sequence type.
‡Clonal complex ST448.
§Clonal complex ST405.
¶Clonal complex ST206.

$bla_{\text{CTX-M-15}}$ genotype was retrieved from wild birds. The CTX-M-15 gene shows a global distribution in clinical settings but has been reported from poultry in the United Kingdom (*11*) and from wild birds in Sweden (*5*), which indicates that this ESBL type also is widely disseminated in the environment.

The PCR-based genotyping showed the diversity of the ESBL-producing *E. coli* isolates. Wild birds and domestic poultry harbored the same strains, and some of the ducks had the same strains as chickens. This commonality of strains might be caused by a common use of natural water resources, and shows with what ease *E. coli* can travel between species.

MLST analysis identified several human-associated genotypes, including ST448, ST405, ST744, ST648, and ST131. The epidemic *E. coli* strain O25bST131 did not carry the more common CTX-M-15 gene but a CTX-M-14–like gene, a frequent finding in hospitals in Taiwan (*12*). Metallo-β-lactamases of the New Delhi metallo-β-lactamase type have not been found in the environment of Bangladesh (*13*), but ST405 and ST648 are associated with New Delhi metallo-β-lactamase-1–producing organisms on the subcontinent of India (*14*). Finally, *E. coli* ST744 carried in this study CTX-M-1. ESBL-producing *E. coli* ST744 has been reported previously in humans in Laos (*15*).

We showed that *E. coli* that produces CTX-M-15 is endemic to birds in Bangladesh. Our findings suggest that wild birds and free-range poultry might be crucial environmental indicators of antimicrobial drug resistance. They also might take a more active part than previously thought as spreaders and as long-term reservoirs of medically threatening pathogens and resistance genes. Several factors are likely to contribute to the widespread dispersal of ESBLs in Bangladesh, including dense population, poor sanitation, and close contact with livestock combined with a high selective pressure created by unrestricted use of antimicrobial drugs in human medicine, veterinary medicine, and aquaculture. Development of a countrywide antimicrobial resistance surveillance system in livestock, wildlife species, and humans in Bangladesh, as well as other measures, are needed immediately to control the situation.

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Dr Hasan is a veterinarian and doctoral student at the Department of Medical Sciences, Sections of Clinical Microbiology and Infectious Diseases at the Faculty of Medicine at Uppsala University, Sweden. His main research interests are molecular epidemiology with focus on antimicrobial drug resistance (ESBL/MBL-producing enterobacteria, vancomycin-resistant enterococcus, methicillin-resistant *Staphylococcus aureus*), wildlife diseases, and bacterial zoonotic diseases in developing countries.

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Address for correspondence: Badrul Hasan, Department of Medical Sciences/Section of Infectious Diseases, Uppsala University, SE-751 85 Uppsala, Sweden; email: badrul.hasan@medsci.uu.se

Westward Spread of *Echinococcus multilocularis* in Foxes, France, 2005–2010

Benoît Combes,[1] Sébastien Comte, Vincent Raton, Francis Raoul, Franck Boué, Gérald Umhang, Stéphanie Favier, Charlotte Dunoyer, Natacha Woronoff, and Patrick.Giraudoux[1]

During 2005–2010, we investigated *Echinococcus multilocularis* infection within fox populations in a large area in France. The parasite is much more widely distributed than hitherto thought, spreading west, with a much higher prevalence than previously reported. The parasite also is present in the large conurbation of Paris.

Echinococcus multilocularis is the causative agent of the parasitic zoonosis alveolar echinococcosis. The adult stage of this cestode is found mostly in the digestive tract of the red fox (*Vulpes vulpes*) (*1*). Parasite eggs, expelled in feces, are the only external living stage of the parasite life cycle. Once ingested by small mammals, they migrate to the liver and proliferate, forming protoscolices in multivesicular cysts. The life cycle is completed when a definitive host (usually canid) preys on an infected intermediate host (mostly rodent). Epidemiologic studies indicate that humans can be infected by eating raw vegetables contaminated by infected fox or dog feces or by direct contact with an infected fox or dog (*2*). Despite the low incidence of human alveolar echinococcosis in Europe (0.02–0.18 cases/100,000 inhabitants [*3*]) the zoonotic potential of the fox tapeworm, in terms of persistence and pathogenicity, poses a major parasitic threat to human health in nontropical regions (*4*).

Author affiliations: Entente for the Control of Zoonoses, Nancy, France (B. Combes, S. Comte, V. Raton, S. Favier); University of Franche-Comté/National Center for Scientific Research, Besançon, France (F. Raoul, P. Giraudoux); Institut Universitaire de France, Paris, France (P. Giraudoux); French Agency for Food, Environmental and Occupational Health and Safety, Nancy (F. Boué, G. Umhang); National Game Federation, Issy-les-Moulineaux, France (C. Dunoyer); and Departmental Veterinary Laboratories Manager Association, Besançon (N. Woronoff)

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Three main trends have been reported in the past decade in Europe. First, *E. multilocularis* prevalence has increased in foxes within areas to which it is known to be endemic (*5*), seemingly linked with the increase of fox population densities in Germany and Switzerland (*6*). Second, the geographic distribution of *E. multilocularis* in foxes has extended toward southern, northern, and eastern countries where it had not previously been detected; the most recent are northern Italy (*7*); Svalbard, Norway (*8*); and Sweden in 2011 (*9*). Third, the geographic distribution of echinococcosis has extended toward Russia and neighboring countries (*10*), including the Baltic states.

Until now, the eastern part of the French territory was considered the western limit of the European echinococcosis-endemic area. At the end of the 1990s, *E. multilocularis* in foxes was reported in only 10 of the 95 French departments (Figure 1). Studies conducted in the neighboring departments (departments 08, 21, 38, 52, 69, and 74) by sedimentation and counting technique (*11*) did not detect infection in foxes. However, since 1997, new cases of human echinococcosis have been recorded in areas without known infection of local fox populations (departments 01, 03, 07, 08, 12, 21, 23, 31, 35, 61, 44, 59, 61, 76, and 95) (*2*).

We present the results of a large-scale survey of *E. multilocularis* infection in foxes in France. Our study was conducted in 42 departments covering an area of 239,178 km^2 representing almost all of northeastern France.

The Study

During 2005–2010 (time span needed to cover the study area) and during the months more favorable for infection (October–April [*3*]), foxes were either shot at night or trapped. The sampling size was chosen to collect ≈100 foxes from each department. Therefore, a grid of 5 km × 5 km to 10 km × 10 km, depending on the department size, was superimposed over the sampling area, and no more than 1 fox was collected in each square. The geographic district where the sample was taken was then noted, and each fox was randomly allocated geographic coordinates within the commune (a French administrative division of 10–100 km^2).

Adult *E. multilocularis* worms were identified in departmental veterinary laboratories. Staff were trained by the Anses-Nancy laboratory (National Reference Laboratory for echinococcoses); that laboratory also confirmed any unrecognized specimens. For time- and cost-effectiveness during the analysis, we used the segmental and sedimentation counting technique (*12*).

We used the χ^2 test to compare *E. multilocularis* prevalence between departments. The distribution of *E.*

[1]These authors contributed equally to this article.

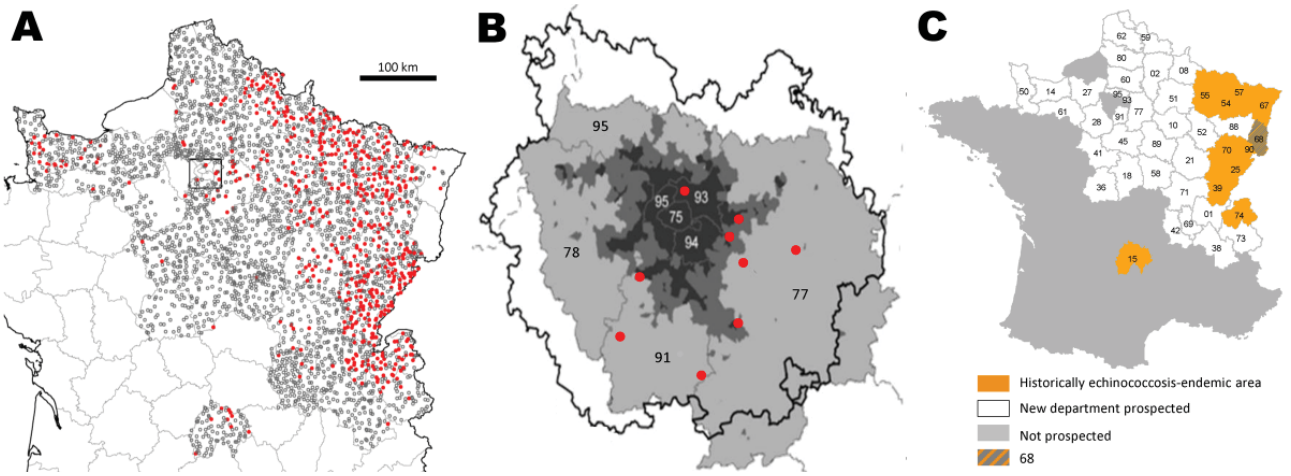

Figure 1. Fox locations (A, B) and department map (C), France, 2005–2010. Numbers in B and C are department national identification numbers. Panel B shows a close-up view of the departments of the Paris conurbation. Solid circle, *Echinococcosus multilocularis*–positive fox; open circle, *E. multilocularis*–negative fox; dark gray, area totally urbanized (75 is Paris intra muros); medium gray, area intensively urbanized; light gray, periurban landscapes. C) Department 68 belongs to the historically echinococcosis-endemic area but could not be explored for the current study. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/12/12-0219-F1.htm).

multilocularis prevalence in foxes was modeled against geographic coordinates by using a generalized additive model with a logistic link function and a thin plate regression spline on 300 knots (*13*). Analyses and graphic displays were conducted by using ArcGIS 9.3, R 2.14.0 and the R packages maptools 0.8–10, mgcv 1.7–12, sp. 0.9–91, and splancs 2.01–29.

A total of 3,307 foxes were collected (Table 1). Eighty-five could not be assigned a commune code and were not kept for further analysis, except to compute *E. multilocularis* prevalence in departments. The mean number of foxes collected by department was 84.95 (± SD 25.76), which represents a mean of 1.56 foxes per 100 km^2 (± SD 0.57). For 4 departments, (36, 61, 67, and 69), full sampling could not be completed because of technical and/or administrative reasons. Urban areas, such as departments 93, 95, and 91, also were undersampled because of human population density and high urbanization, all factors preventing easy fox sampling.

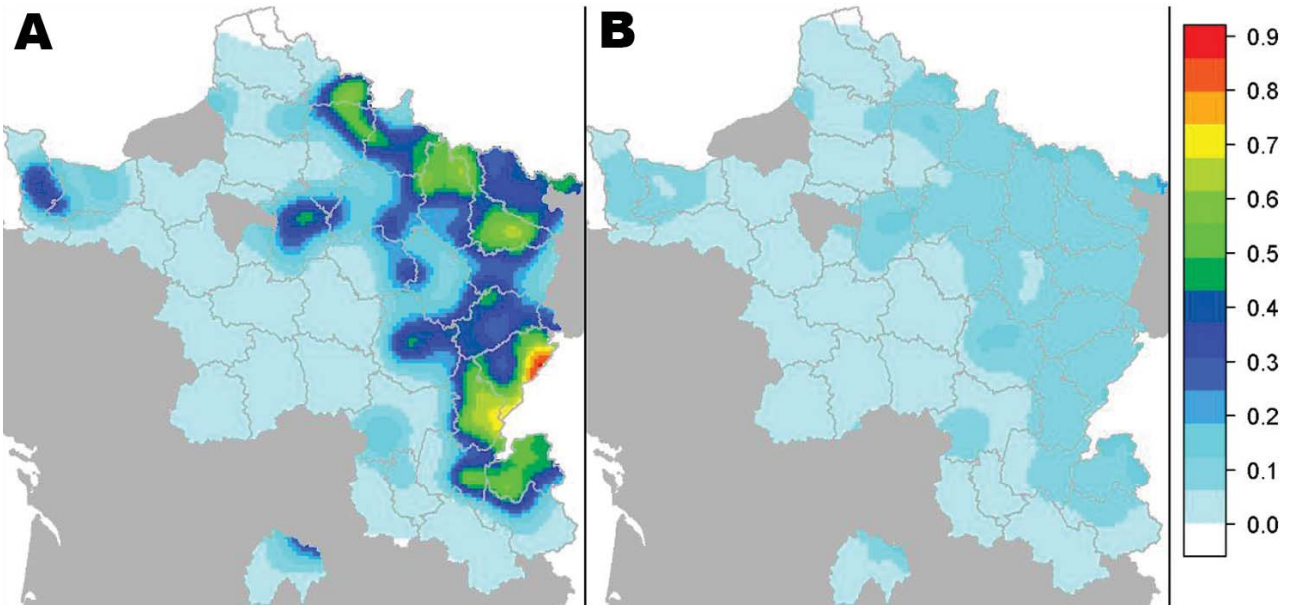

Figure 2. Model-predicted prevalence (A) and standard error (B) of *Echinococcus multilocularis* in foxes, France, 2005–2010. 1 = 100%. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/12/12-0219-F2.htm).

We confirmed *E. multilocularis* in foxes in 35 departments (Figure 2). The prevalence varied widely among departments, from 0 (95% CI 0–5%) to 54% (95% CI 42%–64%) (Table 1) but was locally higher in some areas (Figure 2). The mean prevalence in the entire studied area was 17% (n = 3,307; 95% CI 16%–19%). The prevalence in the historically echinococcosis-endemic area was 41% (n = 789; 95% CI 37%–44%) and represented >55% of all infected foxes and <21% of the total area studied. Furthermore, in comparing our results with those of earlier similar studies during the same season with the same technique, we detected a significant increase of *E. multilocularis* prevalence in foxes over time in most of these departments (Table 2).

Conclusions

Our study confirms the presence of *E. multilocularis* in areas where it is known to be endemic and indicates its presence in 25 additional departments. However, we cannot discard the possibility that *E. multilocularis* was present but remained undetected during the 1980s–1990s. That *E. multilocularis* could have remained undetected if it were not already at a very low prevalence in general is doubtful. Isolated human cases recorded in the early 2000s outside areas to which it is known to be endemic corroborate this possibility (*3*). The same uncertainty applies in other parts of Europe (*14*). Taken as a whole, these findings indicate that the transmission intensity of *E. multilocularis* through fox populations in the occidental part of the European focus area is likely to have increased

Table 1. Fox prevalence by department, France, 2005–2010

Department no., name	Total no. foxes	Prevalence, % (95% CI)	Density of collected foxes, no./100 km^2
01-Ain	98	20 (13–30)	1.7
02-Aisne	89	20 (13–30)	1.22
08-Ardennes	91	36 (27–47)	1.85
10-Aube	99	12 (7–21)	1.68
14-Calvados	96	14 (8–22)	1.73
15-Cantal*	97	9 (5–17)	1.68
18-Cher	74	1 (0–8)	1.55
21-Cote d'Or	72	21 (12–32)	0.85
25-Doubs*	113	53 (44–62)	2.21
27-Eure	93	0 (0–5)	1.66
28-Eure et Loire	42	0 (0–10)	0.97
36-Indre	52	0 (0–9)	1.03
38-Isere	89	1 (0–7)	1.2
39-Jura*	102	52 (42–62)	2.02
41-Loire et Cher	86	2 (0–9)	1.47
42-Loire	97	1 (0–6)	2.06
45-Loiret	100	0 (0–5)	1.53
50-Manche	81	15 (8–25)	1.35
51-Marne	103	19 (13–29)	1.26
52-Haute Marne	94	14 (8–23)	1.51
54-Meurthe et Moselle*	84	54 (42–64)	1.8
55-Meuse*	104	41 (32–51)	1.67
57-Moselle*	103	34 (25–44)	1.65
58-Nievre	110	1 (0–6)	1.74
59-Nord	96	20 (13–29)	1.74
60-Oise	87	7 (3–15)	1.53
61-Orne	55	4 (1–14)	0.93
62-Pas de Calais	90	0 (0–5)	1.34
67-Bas Rhin*	7	29 (5–70)	0.44
69-Rhone	48	8 (3–21)	1.69
70-Haute Saone*	81	36 (26–47)	1.54
71-Saone et Loire	79	9 (4–18)	1.13
73-Savoie	75	11 (5–20)	1.26
74-Haute Savoie*	73	49 (38–61)	1.76
77-Seine et Marne	55	29 (18–43)	1
80-Somme	89	8 (3–16)	1.68
88-Vosges	90	24 (16–35)	1.7
89-Yonne	97	0 (0–5)	1.75
90-Territoire de Belfort*	25	32 (16–54)	4.09
91-Essonne†	41	7 (2–21)	2.37
93-Seine Saint Denis†	6	17 (1–64)	2.53
95-Val d'Oise†	44	0 (0–10)	3.59
Historical endemic area	789	41 (35–41)	1.56
Total	3307	17 (16–19)	1.38

*Department belonging to the historically echinococcosis-endemic area.
†Department of the Paris capital conurbation (Figure 1).

Table 2. Changes in fox prevalence over time, France

Department	Total no. foxes collected		Prevalence, %		p value*
	1984–1987	2006–2010	1984–1987	2006–2010	
54/57	153	187	28	43	0.05
39	146	102	18	52	0.0002
25	37	116	46	52	0.85
67	192	21	4	19	0.04

*p value of the χ^2 comparing the 2 periods.

during the late 1990s and led to a much higher average prevalence than previously reported. Furthermore, infected foxes close to large-scale conurbations, such as Paris and its large suburban surrounding departments (93, 91, and 77) (Figure 1) amounting to 11,728,240 inhabitants, may create new conditions for human exposure similar to those already described in other highly urbanized cities, such as in Switzerland, Germany, and eastern France (Nancy), but on a much larger scale.

We believe that the public needs to be proactively informed and protected, including through awareness initiatives among urban residents and, in specific areas (*15*), more direct action toward the parasite may be considered. Monitoring the possible further extension of the parasite westward and southward and the evolution of prevalence in foxes in the historically and the newly echinococcosis-endemic areas also are essential.

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Dr Combes is head of the Entente for the Control of Zoonoses, Nancy, France. His research interests include epidemiologic surveillance and control of zoonoses.

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Address for correspondence: Patrick Giraudoux, Chrono-environment, University of Franche-Comte, place Leclerc, 25030 Besançon Cedex, France; email: patrick.giraudoux@univ-fcomte.fr

Candidatus Neoehrlichia mikurensis in Bank Voles, France

Muriel Vayssier-Taussat, Danielle Le Rhun, Jean-Philippe Buffet, Narimane Maaoui, Maxime Galan, Emmanuel Guivier, Nathalie Charbonnel, and Jean-François Cosson

To further assess the geographic occurrence, possible vectors, and prevalence of *Candidatus* Neoehrlichia mikurensis, we analyzed spleen tissues from 276 voles trapped close to human settlements in France; 5 were infected with the organism. Sequencing showed the isolates carried the same genotype as the bacteria that caused disease in humans and animals elsewhere in Europe.

Emerging infectious diseases substantially affect public health. Analysis of a database of 335 emerging infectious disease events indicated that 60.0% were zoonotic diseases, most (71.8%) of which originated in wildlife, and that zoonoses are increasing over time (*1*). Half of these emerging infection events involved bacteria belonging, for the most part, to the proteobacterial order *Rickettsiales* (*1*).

In Western Europe, the widespread and abundant *Ixodes ricinus* tick is the most common vector for human and animal pathogens and is also a major vector of pathogens responsible for rodent-borne diseases (*2*). Lyme borreliosis (Lyme disease), which is caused by infection with the bacteria *Borrelia burgdorferi* sensu lato, is the most prevalent tick-borne and rodent-borne illness. However, persons bitten by ticks can also be infected by other bacteria belonging to 3 main genera: *Anaplasma* spp., *Bartonella* spp. and *Rickettsiae* spp. Over the past 10 years, these bacterial species have been associated with tick-borne infections in humans (*2*).

During the last decade, DNA of a new species of intracellular bacteria belonging to the family *Anaplasmataceae* was sequenced from isolates from ticks and rodents originating in Europe and Asia (*3*–*5*). The first isolate of this organism was obtained in 2004 from wild *Rattus norvegicus* rats and *I. ovatus* ticks from Japan (*6*). A comparison of the morphologic and molecular characterization of that isolate with earlier, closely related sequences in the GenBank database supported classification of the isolate in a novel genetic cluster within the family *Anaplasmataceae*; thus, the nomenclature "*Candidatus* Neoehrlichia mikurensis" was proposed for all current organisms in this group (*6*).

Since the 2004 discovery of *Candidatus* N. mikurensis, the bacterium has been identified in different tick species, including *I. ricinus* ticks in Europe, and in small rodents (other than *R. norvegicus* rats) suspected of being the main reservoir for the bacterium (*7*–*10*). In 2010, human infection with C*andidatus* N. mikurensis was reported in a Swedish patient (*11*). In that same year, infections were described in 5 persons in Germany, Switzerland, and the Czech Republic (*12*). More recently, *Candidatus* N. mikurensis infection was reported in a dog in Germany (*13*). Signs and symptoms described in all cases were general and nonspecific (e.g., fever, cough, anemia, headache, pulmonary infiltrate, malaise, myalgia, joint pain, extreme fatigue, erythema), making diagnosis difficult, particularly in the absence of serologic tests. Thus, it is likely that the actual incidence of human *Candidatus* N. mikurensis infection in Europe is much higher than currently reported.

Previous studies of *Candidatus* N. mikurensis in Europe have advocated for an assessment of the geographic occurrence, possible vectors, and prevalence of this microorganism. In this study we demonstrate the presence of *Candidatus* N. mikurensis in France, specifically in the bank vole (*Myodes glareolus*), a suspected reservoir for the microorganism.

The Study

During the course of a 2008 study of the epidemiology of Puumala hantavirus, we trapped voles in the French Ardennes, a forested region on the border with Belgium, along a transect line of ≈80 km (*14*). Along this transect, we sampled 6 sites in forested areas and 4 sites in fragmented habitats (i.e., hedge networks). We euthanized the voles by cervical dislocation and determined their weight and sex. The animals were then dissected. Spleens were placed in RNA*later* Storage Solution (Sigma-Aldrich, St Louis, MO, USA) and stored at –20°C for further analyses. We used the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions to extract genomic DNA from a portion of the spleen into a final elution volume of 100 μL.

To test the spleen DNA for the presence of *Candidatus* N. mikurensis, we used PCR with specific primers targeting the *Candidatus* N. mikurensis *groEL* gene, as described (*13*): results were positive for 5 (1.8%) of 276 vole spleens tested. This prevalence lies within the range (0%–11.5%) found among small rodents trapped in other European countries (*7*,*9*). Rodents positive for *Candida-*

Author affiliations: Institut National de la Recherche Agronomique, Maisons-Alfort, France (M. Vayssier-Taussat, D. Le Rhun, J.-P. Buffet, N. Maaoui); and Institut National de la Recherche Agronomique, Montferrier-sur-Lez, France (M. Galan, E. Guivier, N. Charbonnel, J.-F. Cosson)

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tus N. mikurensis were trapped in forested patches in Woiries (49.903°N, 4.763°E), Elan (49.655°N, 4.770°E), and Croix-aux-Bois (49.419°N, 4.824°E), France, and also in a hedge network in Cliron (49.802°N; 4.619°E), France, where the *I. ricinus* tick is also abundantly present (E. Ferquel, pers. comm.). All *Candidatus* N. mikurensis–positive bank voles were trapped in close proximity (300 m–1 km) to human settlements and villages.

All PCR products (a 1,024-bp fragment of the *groEL* gene) were sequenced in forward and reverse directions by Eurofins MWG Operon (Ebersberg, Germany). Sequences were aligned and analyzed for phylogenetic purposes by using SeaView version 4 (*15*). All sequences were identical and matched those of the *Candidatus* N. mikurensis *groEL* gene. Sequences showed the highest identity with gene sequences of *Candidatus* N. mikurensis isolates in Europe. In particular, the sequences showed 99% nt identity with sequences obtained for isolates from 2 humans (GenBank accession nos. EU810407.1 and EU810406.1) and a dog (GenBank accession no. EU432375) residing in Germany. The lowest sequence identity was shared with isolates obtained outside of Europe: 95% nt identity with isolates from wild rodents in the People's Republic of China (GenBank accession nos. JQ359066, JQ359067, and JQ359068); 94% identity with isolates from wild rodents and *I. ovatus* ticks in Japan (GenBank accession nos. AB074461 and AB084583, respectively); and 91% identity with isolates from raccoons in the United States (GenBank accession no. EF633745).

We constructed the phylogenetic tree by using the neighbor-joining method and a Kimura 2-parameter distance. Bootstrap analysis was performed on 1,000 replicates.

Phylogenetic analysis of the comparatively long *groEL* gene sequence showed highly significant clustering of the samples into 3 groups supported by high bootstrap values (Figure), suggesting the existence of at least 3 types of *Candidatus* N. mikurensis sequence variants. Clustering was not related to the host or to the geographic origin of the sample. All sequences obtained from *Candidatus* N. mikurensis isolates from bank voles in France clustered together with sequences that had been amplified from samples from sick humans or animals in Europe.

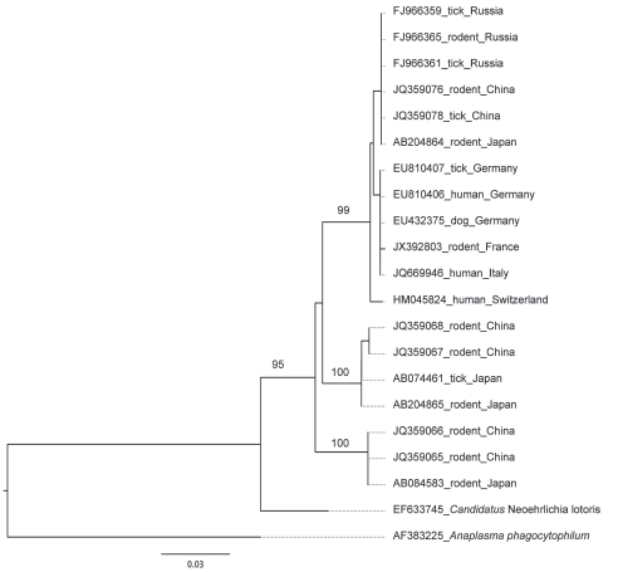

Figure. Phylogenetic relationships, as determined on the basis of the sequence of the *groEL* gene, between the unique *Candidatus* Neoehrlichia mikurensis genotype detected in a population of bank voles from the French Ardennes and other *Candidatus* N. mikurensis genotypes from other geographic regions. The phylogenetic tree was constructed by using the neighbor-joining method with the Kimura 2-parameter distance model. Bootstrap analysis was performed on 1,000 replicates; values are indicated at the nodes. GenBank accession numbers are indicated for each sequence used. The *groEL* sequence of *Anaplasma phagocytophilum* (accession no. AF383225) was chosen as an outgroup in the phylogenetic tree. Scale bar indicates estimated evolutionary distance.

Conclusions

Our results show that *Candidatus* N. mikurensis is present in bank voles in France; furthermore, the bacteria carried by the rodents in this study were of the same genotype as the *Candidatus* N. mikurensis that caused disease in humans and animals elsewhere in Europe. These findings have implications for public health because small rodents are the main source of blood meals for the larvae and nymphs of *I. ricinus* ticks and because the *Candidatus* N. mikurensis–positive rodents in our study were collected in close proximity to human dwellings. Thus, we are currently collecting *I. ricinus* ticks in the same region of France where we trapped the bank voles to determine whether ticks are a possible vector and a source of transmission of *Candidatus* N. mikurensis to humans.

In addition, nephropatia epidemica, a hemorrhagic fever caused by Puumala hantavirus, is endemic in the geographic region from which the bank voles in our study were collected. The symptoms for nephropatia epidemica (body aches, chills, sweats, fatigue, and high fever) are mild and could be confused with those caused by infection with *Candidatus* N. mikurensis. Diagnosis of *Candidatus* N. mikurensis infection relies only on PCR amplification of the bacterial DNA. The absence of serologic tests for *Candidatus* N. mikurensis, combined with the lack of knowledge relating to these bacteria among medical practitioners, makes diagnosis of *Candidatus* N. mikurensis infection particularly difficult. Thorough surveillance and improved diagnostic tools are required to gain more insight into the relevance of *Candidatus* N. mikurensis to public health.

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Dr Vayssier-Taussat is the head of a research team at Institut National de la Recherche Agronomique. Her area of interest is tick-borne bacteria, especially the development of molecular tools to identify tick and animal pathogens and to elucidate their mechanisms of infection.

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Address for correspondence: Muriel Vayssier-Taussat, INRA, USC Bipar, Anses, 23 Avenue du Général de Gaulle, Maisons-Alfort, France; email: mvayssier@vet-alfort.fr

Reemergence of Chikungunya Virus in Cambodia

Veasna Duong, Anne-Claire Andries, Chantha Ngan, Touch Sok, Beat Richner, Nima Asgari-Jirhandeh, Steve Bjorge, Rekol Huy, Sovann Ly, Denis Laurent, Bunheng Hok, Maria Concepcion Roces, Sivuth Ong, Meng Chuor Char, Vincent Deubel, Arnaud Tarantola, and Philippe Buchy

Chikungunya virus (CHIKV), probably Asian genotype, was first detected in Cambodia in 1961. Despite no evidence of acute or recent CHIKV infections since 2000, real-time reverse transcription PCR of serum collected in 2011 detected CHIKV, East Central South African genotype. Spatiotemporal patterns and phylogenetic clustering indicate that the virus probably originated in Thailand.

Chikungunya virus (CHIKV; family *Togaviridae*; genus *Alphavirus*) is an arthropod-borne virus transmitted to humans by *Aedes* spp. mosquitoes (*1*). It is an enveloped, positive-sense, single-stranded RNA virus with a genome of ≈11.8 kb (*2*). Three genotypes have been identified: Western African, East Central South African (ECSA), and Asian (*3*).

First identified in Tanzania in the mid-1950s, CHIKV circulated in the 1960s in sub-Saharan Africa and several Asian countries (*4*,*5*). Reemergence of CHIKV (ECSA genotype) was reported in Democratic Republic of Congo in 1999–2000 (*6*,*7*) and in Kenya in 2004 (*4*). This genotype emerged in Comoros followed by Réunion Island, the Seychelles, Mauritius, Mayotte, and India in 2005 (the Indian Ocean outbreak); in Sri Lanka and Malaysia in 2006; in Singapore and Thailand in 2008; and in China in 2010 (*8*).

In Cambodia, CHIKV was first detected in 1961, probably the Asian genotype that was circulating in the region at that time (*9*). Since 2000, all blood specimens collected by the National Dengue Control Program, Ministry of Health Cambodia, during hospital-based surveillance of dengue and investigation of suspected dengue cases have been screened for IgM against CHIKV, dengue virus (DENV), Japanese encephalitis virus (JEV), and other arboviruses. Despite this testing, no evidence of acute or recent CHIKV infections has been found. To confirm CHIKV infection in samples positive by serologic testing, we conducted real-time reverse transcription PCR (RT-PCR) and complete genome sequencing of the samples. In 2011, we detected CHIKV ECSA genotype in patients in Cambodia and analyzed the phylogenetic origin of the strains.

The Study

We obtained samples from 3 sources: national dengue-like surveillance, an encephalitis study, and the outbreak investigation. From surveillance, during 2000–2011, an average of >700 paired serum samples were collected annually from patients admitted to sentinel hospitals for dengue-like syndrome (Battambang, Siem Reap, Kampong Cham, Takeo, Phnom Penh; Figure 1) in the National Dengue Control Program (*10*). From the encephalitis study, conducted July 2010 through July 2011, samples from 196 patients were collected as part of a surveillance study of central nervous system infections in Jayavarman VII hospital in Siem Reap (with written consent from patients or legal guardians and study approval by the National Ethics Committee in Cambodia). During the outbreak investigation, serum was collected during investigations by National Health authorities in Preah Vihear Province on August 16 (n = 9) and December 9–10, 2011 (n = 8), of outbreaks of suspected measles-like or dengue-like illnesses (Table).

All serum was tested at the Institut Pasteur in Cambodia. Acute-phase and/or convalescent-phase specimens

Figure 1. Spatiotemporal pattern of chikungunya cases showing the 6 provinces in which cases occurred and the most likely route of virus introduction into Cambodia, 2011. Adapted, with permission, from www.nationsonline.org/oneworld/map/cambodia-administrative-map.htm.

Author affiliations: Institut Pasteur in Cambodia, Phnom Penh, Cambodia (V. Duong, A.-C. Andries, S. Ong, V. Deubel, A. Tarantola, P. Buchy); Ministry of Health, Phnom Penh (C. Ngan, T. Sok, R. Huy, S. Ly, M.C. Char,); Jayavarman VII Hospital, Siem Reap, Cambodia (B. Richner, D. Laurent); World Health Organization, Phnom Penh (N. Asgari-Jirhandeh, S. Bjorge, M.C. Roces); and Preah Vihear Provincial Health Department, Preah Vihear, Cambodia (B. Hok)

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were tested for IgM against flaviviruses (DENV, JEV, and Langat virus) and alphaviruses (CHIKV Ross C 347 strain and Sindbis virus). We used in-house IgM-capture ELISA as described by Vong et al. (*11*) with JEV, Langat, Sindbis, DENV, and CHIKV antigens; CHIKV was isolated by use of a mosquito cell line (clone C6/36 of *Aedes albopictus* cells) (*11*). Viral RNA was extracted from 140 μL of serum by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's recommendations. The presence of CHIKV RNA was determined by real-time RT-PCR selective for the *E1* gene, according to a protocol adapted from Pastorino et al. (*12*) for a different Taq polymerase kit (SuperScript III Platinum One-Step Quantitative RT-PCR kit; Invitrogen, Carlsbad, CA, USA). Each series of tests included a negative control.

Among 19 samples positive for CHIKV by real-time RT-PCR, 8 were selected for complete genome sequencing. A total of 22 overlapped PCR products were obtained by using primers published by Schuffenecker et al. (*13*) and sent to Macrogen (Seoul, South Korea) for sequencing.

Sequence assembly and alignment were performed by using the CLC Main Workbench 5.5 package (CLC bio A/S, Aarhus, Denmark). The complete coding region (11,319 nt) of 8 CHIKV isolates from Cambodia were aligned with 64 reference strains available in GenBank. Phylogenetic analysis was performed by using the maximum-likelihood approach incorporating the GTR+Γ4 model of nucleotide substitution with a bootstrap resampling process of 1,000 replications.

During this reemergence of CHIKV in 2011, a total of 24 patients with fever, sometimes associated with acute arthritis or encephalitis (suggesting that many classical infections were not reported because encephalitis is a rare complication of chikungunya), had positive RT-PCR and/or IgM-capture ELISA results for CHIKV. The first 2 cases were identified in May 2011 by the National Dengue Control Program in Battambang Province (eastern Cambodia) near the Thailand border (Figure 1). These 2 cases were in children hospitalized for suspected dengue. Subsequent cases were reported the same year in Siem Reap (2 cases, June and July), Kampong Thom (1 case, July), Kampong Cham (1 case, October), and Kandal (1 case, December) Provinces. Two other outbreaks were documented in villages in Preah Vihear Province (northern Cambodia) in August (9 cases) and December (8 cases) 2011 (Table). The sequence of outbreaks, in time and space, suggests that the virus was introduced to areas bordering Thailand and progressed through Cambodia, affecting city and villages

Table. Characteristics of 24 patients with positive results for chikungunya virus, Cambodia, 2011*

Patient no.	Project	Age, y/sex	Date of symptom onset	Date sampled	Province	Initial syndrome reported	IgM-capture ELISA		RT-PCR		Full-genome sequencing
							1st	2nd	Serum	SN	
V0603308	NDCP	2/M	May 26	May 28	BTB	Dengue†	Neg	Pos	Pos	Pos	Partial
V0603310	NDCP	4/F	May 26	May 28	BTB	Dengue	Neg	Pos	Pos	Pos	Complete
V0705309	CNS	5/M	Jun 23	Jun 30	SRP	Encephalitis‡	Neg	Pos	Neg	Neg	ND
V0719309	CNS	14/M	Jul 9	Jul 17	SRP	Encephalitis	Pos	Pos	Neg	Neg	ND
V0719310	CNS	7/M	Jul 10	Jul 13	KTH	Encephalitis	Pos	Pos	Pos	Neg	ND
V1005304	NDCP	15/M	Sep 30	Oct 4	KCH	Dengue	Neg	Pos	Pos	Pos	ND
V1024306	CDC	11/F	Aug 10	Aug 16	PVH	Measles§	Neg	NA	Pos	Neg	Complete
V1024307	CDC	11/F	Aug 15	Aug 16	PVH	Measles	Pos	NA	Neg	Neg	ND
V1024308	CDC	28/F	Aug 8	Aug 16	PVH	Measles	Neg	NA	Pos	Neg	Complete
V1024309	CDC	30/F	Aug 8	Aug 16	PVH	Measles	Pos	NA	Pos	Pos	ND
V1024310	CDC	17/M	Aug 13	Aug 16	PVH	Measles	Neg	NA	Pos	Pos	Complete
V1024311	CDC	2.5/F	Aug 16	Aug 16	PVH	Measles	Neg	NA	Pos	Pos	Complete
V1024312	CDC	13/F	Aug 16	Aug 16	PVH	Measles	Neg	NA	Pos	Pos	ND
V1024313	CDC	10/F	Aug 14	Aug 16	PVH	Measles	Neg	NA	Pos	Pos	Complete
V1024314	CDC	31/F	Aug 15	Aug 16	PVH	Measles	Neg	NA	Pos	Pos	Complete
V1214306	NMC	49/M	Dec 9	Dec 9	PVH	Dengue	Neg	NA	Pos	Neg	ND
V1214307	NMC	56/M	Dec 6	Dec 9	PVH	Dengue	Neg	NA	Pos	Neg	ND
V1214308	NMC	29/M	Dec 5	Dec 9	PVH	Dengue	Neg	NA	Pos	Pos	ND
V1214309	NMC	39/F	Dec 9	Dec 10	PVH	Dengue	Neg	NA	Pos	Pos	ND
V1214310	NMC	17/F	Dec 9	Dec 10	PVH	Dengue	Neg	NA	Pos	Pos	ND
V1214311	NMC	27/M	Dec 7	Dec 10	PVH	Dengue	Pos	NA	Neg	Neg	ND
V1214312	NMC	16/M	Dec 9	Dec 10	PVH	Dengue	Neg	NA	Pos	Pos	ND
V1214333	NMC	30/F	NA	Dec 10	PVH	Dengue	Neg	NA	Pos	Pos	ND
V1207304	NDCP	9/F	Dec 1	Dec 6	KAN	Dengue	Pos	Pos	Neg	Neg	ND

*All patients had negative IgM-capture ELISA results for flaviviruses (dengue 1–4, Japanese encephalitis, Langat) and other alphaviruses (Sindbis). RT-PCR, real-time reverse transcription PCR; 1st, acute-phase serum sample; 2nd, convalescent-phase serum sample; SN, supernatant; NDCP: National Dengue Control Program, National Malaria Center, Ministry of Health; BTB, Battambang; Neg, negative; Pos, positive; CNS, central nervous system infection (encephalitis) study; SRP: Siem Reap; ND, not done; KTH: Kampong Thom; KCH: Kampong Cham; CDC, Communicable Disease Control Department, Ministry of Health, Preah Vihear outbreak investigation in August 2011; PVH: Preah Vihear; NA, not available; NMC, National Malaria Center, Ministry of Health, Preah Vihear outbreak investigation in December 2011; KAN, Kandal.
†Dengue-like: fever with headache, retro-orbital pain, myalgia, joint pain, and/or hemorrhage.
‡Encephalitis: fever with convulsion, vomiting, and/or hemiplegia.
§Measle-like: fever with rash, cough, or join pain.

along major northwest to southeast routes (Figure 1). Average patient age was 20 years (range 2–56 years); cases were distributed equally among male and female patients.

Conclusions

The alignment of the *E1* gene sequence of CHIKV showed that all 8 strains carried the same amino acid substitution in the E1 protein (E1-A226V) as did the strains that were naturally selected by the mosquito vector a few months after the beginning of the Indian Ocean outbreak (*13*). Phylogenetic analysis of the complete genome sequence revealed that all viruses from Cambodia clustered with those isolated during the Indian Ocean outbreak and within the ECSA phylogenetic group (Figure 2). These isolates from Cambodia were closely related to the viruses isolated from southern Thailand during the 2008–2009 outbreak with bootstrap values <70 (data not shown) and to other isolates from the recent outbreaks in Asia (Singapore, Malaysia, Indonesia, and China). The pairwise nucleotide comparison of the complete coding region showed a high average percentage of similarity (>99.50%) with the recent isolates from Thailand, Malaysia, Singapore, and China. The identity between the strains from Cambodia ranged from 99.89% to 99.93% at the nucleotide level. Of note, the Cambodian strains can be separated into 2 groups supported by a bootstrap value of 100, suggesting that the viruses isolated in Battambang and Preah Vihea Provinces, which each border Thailand, could have been introduced separately, although we cannot exclude the possibility of introduction from other Asian countries as well.

As numbers of reported cases, numbers of provinces affected, and abundance of mosquito vectors (*Ae. aegypti* and *Ae. albopictus*) increase, the risk for local transmission will probably increase in the next few years, and levels of CHIKV infection could reach those of DENV infection. The ECSA genotype could then become endemic to Cambodia, which could face the same situation as in the 1960s, when a number of chikungunya cases were reported in Cambodia, although the 1960s epidemic did not last long (according to data available) and was not followed by continuous virus circulation leading to successive outbreaks. As CHIKV reemerges after 50 years of absence or low-

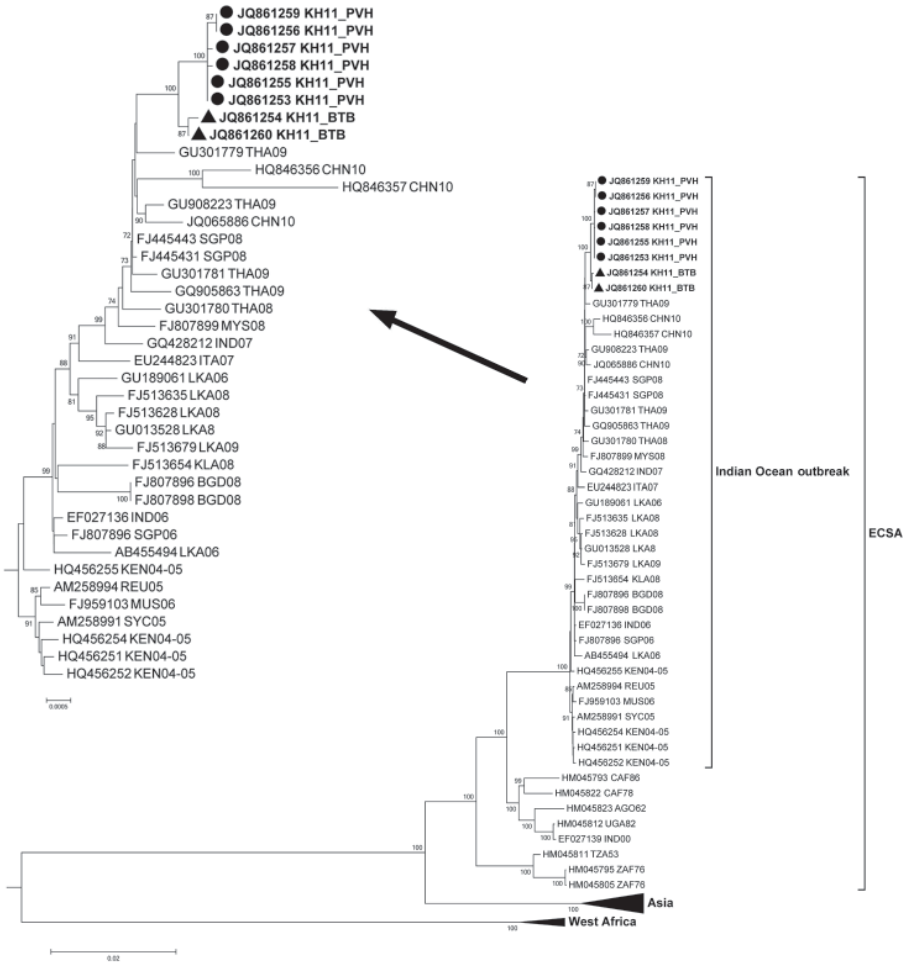

Figure 2. Phylogenetic tree based on the whole genome of chikungunya virus (CHIKV). Viruses were identified by using the GenBank accession number, country code, and year of isolation. Boldface indicates strains from Cambodia; circles indicate isolates from Preah Vihear Province; triangles indicate strains from Battambang Province. Arrow indicates enlarged Indian Ocean outbreak strains. All 8 strains from Cambodia carried the A226V mutation. Numbers represent the bootstrap support obtained for respective branches (>70). The tree was rooted by o'nyong-nyong virus (GenBank accession no. AF079456, UGA96-ONNV). ECSA, East Central South African genotype. Scale bars indicate nucleotide substitutions per site.

level transmission, cocirculation with DENV might cause substantial challenges for public health, especially hospital overloading and increased needs for case management. This outbreak of CHIKV ECSA genotype spread rapidly in Cambodia over a short 7-month period. The outbreak should serve as a warning for health authorities to prepare, not only in Cambodia, but also in other areas where, to our knowledge, this genotype has not been reported, such as Vietnam and Lao People's Democratic Republic.

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Dr Duong is a senior virologist at the Institut Pasteur in Cambodia. His research interests include arboviruses and emerging zoonotic viruses.

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Address for correspondence: Philippe Buchy, Institut Pasteur in Cambodia, Virology Unit, No. 5, PO Box 983, Monivong Blvd, Phnom Penh, Cambodia; email: pbuchy@pasteur-kh.org

Analysis of Complete Puumala Virus Genome, Finland

Angelina Plyusnina, Maria Razzauti, Tarja Sironen, Jukka Niemimaa, Olli Vapalahti, Antti Vaheri, Heikki Henttonen, and Alexander Plyusnin

Puumala virus causes nephropathia epidemica, a rodent-borne zoonosis that is endemic to Europe. We sequenced the complete Puumala virus genome that was directly recovered from a person who died and compared it with those of viruses from local bank voles. The virus strain involved was neither a unique nor rare genetic variant.

The outcome of a viral infection is determined by the agent's pathogenicity and by host factors, such as genetic predisposition. For RNA viruses, which are notorious for their swift evolution and adaptation, a pathogen's specific genotype usually is to blame for devastating effects (*1*). In many cases, however, the virus genome is not easy to search for particular mutations because recovery of complete viral sequences from clinical specimens remains extremely difficult. This is especially true for hantaviruses (family *Bunyaviridae*) that cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (*2*,*3*). Thus far, only a few complete hantavirus genomes originating from persons with clinical cases have been reported (*4*,*5*), and only 1 was recovered without passaging first in cell culture (*4*), which by itself can induce adaptive changes in the viral genome (*6*). We present the complete genome of PUUV directly recovered from a person with fatal infection.

Usually PUUV causes mild HFRS (also called nephropathia epidemica [NE]). In Finland, 1,000–3,000 NE cases are diagnosed annually, i.e., ≈60 cases/100,000 persons during years when the vole population peaks (*7*). Almost 100% of infected persons recover, and long-lasting complications are rare. The few fatal cases reported (*8,9*) showed no apparent geographic clustering. Thus, whether more severe illness could be connected to certain genetic variants of PUUV remains unknown.

Author affiliations: University of Helsinki and HUSLAB, Helsinki, Finland (A. Plyusnina, M. Razzauti, T. Sironen, O. Vapalahti, A. Vaheri, A. Plyusnin); and Finnish Forest Research Institute, Vantaa, Finland (J. Niemimaa, H. Henttonen)

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The Study

The patient was a previously healthy 37-year-old man with a history of smoking. He died in November 2008 of severe NE on day 4 after the onset of symptoms that started with high fever, vomiting and diarrhea, headache, and visual disturbances. His condition deteriorated quickly, and multiorgan failure developed, including respiratory distress, acute kidney failure, liver failure, and severe thrombocytopenia.

A standard autopsy was performed, and tissue samples were stored fresh at −70°C and fixed in formalin. PUUV infection was confirmed initially by IgM test and later by reverse transcription PCR (RT-PCR), followed by sequencing. Genetic analysis was performed from autopsy samples stored fresh at −70°C (the high quality of clinical samples was crucial for the downstream applications). Complete PUUV genomes were recovered in a set of nested and seminested PCR (sequencers of primers are available on request). Amplicons were gel-purified and sequenced directly by using ABI PRISM Dye Terminator sequencing kit (PerkinElmer/ABI, Foster City, CA, USA). Quantitative RT-PCR was used to measure PUUV load with DyNAmo Capillary SYBR Green kit (Finnzymes, Espoo, Finland). Copy numbers were calculated from a standard curve created by using in vitro transcribed PUUV small (S) segment RNA (T7 transcription kit, Fermentas, Vilnius, Lithuania).

Quantitative RT-PCR revealed the highest numbers of virus genome copies in lungs and kidneys: 1,881 and 1,136 per μg of total RNA, respectively. Copy numbers per μg of total RNA in other tissues were lower: 240 in the heart, 160 in the spleen, 50 in the liver, and 42 in the brain. In agreement with these findings, complete PUUV genome sequences (12,059 nt) were recovered from the lung and kidney and partial sequences of different lengths from heart, liver, and brain (Figure 1). Corresponding sequences recovered from different tissues were identical, i.e., no tissue-specific mutations were observed.

To determine whether this fatal NE case was caused by an unusual or rare genetic variant of PUUV, we searched for identical or closely related genetic variants in bank voles trapped near the patient's house (storage buildings and surroundings within 500 m) in Pieksämäki, central Finland (62°18′N, 27°08′E). Travel history of the case-patient suggested that the infection had been acquired at his residence. In 2008, the vole population peaked in the southern half of Finland, including Pieksämäki, and 3,259 NE cases were diagnosed nationwide (*7*), the highest number ever registered in Finland. Sixty-three bank voles were snap-trapped during 3 consequent nights in December 2008.

Lung tissue samples from the bank voles were screened for PUUV N protein antigen by using immunoblotting,

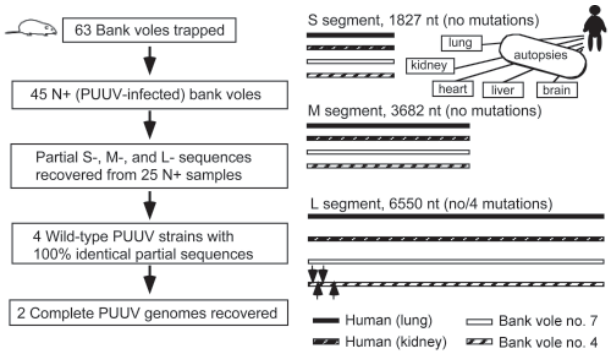

Figure 1. Comparison of PUUV-genome sequences recovered from human autopsies and rodent tissues. Locations of 4 silent mutations found in the L-segment sequences: G114A, U261C, A349G, and U378A are indicated by arrows (right column). PUUV, Puumala virus; S, small; M, medium; L, large; +, positive.

and 45 (71%) voles tested positive. Tissues from 25 virus-infected voles were taken for genetic analysis, and partial sequences of PUUV genome S, medium (M), and large (L) segments (≈12% of the total virus genome) were recovered from them.

In agreement with previously published data (*10*), the number of PUUV genome copies in bank vole tissues was within the range of 10^5–10^6/μg of total RNA, i.e., ≈100-fold higher than in tissues of the case-patient. Partial virus sequences from 4 voles were 100% identical to those from human tissues. Next, complete PUUV genome sequences were recovered from 2 of these voles; the sequences differed at only 4 positions in the L segment (all silent mutations; Figure 1, right column). One of the complete rodent-originated PUUV sequences was 100% identical to the sequence from the case-patient. PUUV sequences have been deposited in GenBank under accession nos. JN831943–JN831952. Phylogenetic analysis confirmed that the hantavirus involved belonged to *Puumala virus* species and was most closely related to the earlier described genetic variants from Finland, particularly to those circulating at Konnevesi (62°34′N, 26°24′E) and Puumala (61°52′N, 28°17′E) localities (Figure 2).

Conclusions

Our findings established an unequivocal genetic link between the fatal human NE case and local wild-type PUUV strains. These findings also revealed that no mutations had accumulated in the genome of PUUV during transmission of the virus to the patient and the fatal generalized infection that followed. Finally, we demonstrated that the wild-type PUUV strain that caused the fatal infection was neither a unique nor rare genetic variant; the exact sequence match to the complete human-originated PUUV sequence was found among the first 25 bank voles analyzed. Genetic links of the type have been reported for PUUV infections in Finland (*11*) and for Sin Nombre virus infection during the outbreak in the Four Corners area of the United States (*4*,*12*), but perfect sequence match was not observed.

In PUUV infections, renal insufficiency is a hallmark of the disease, but pulmonary, cardiac, central nervous system, ocular, and hepatic manifestations and, in severe cases, hypophyseal injury, also can occur (*13*). In the fatal case described here, death resulted from multiorgan failure

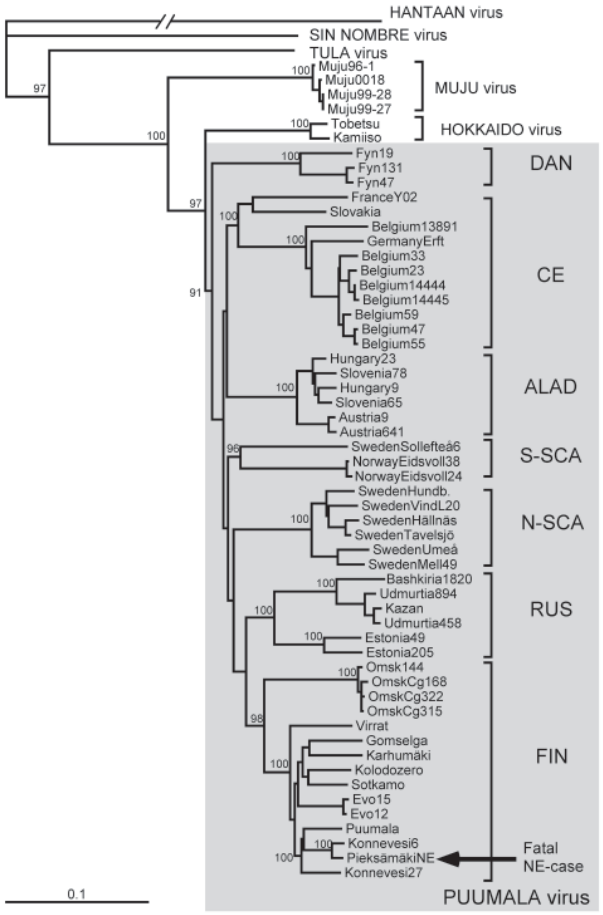

Figure 2. Phylogenetic tree of PUUV S segment sequences (coding region). Topologies of the M and L trees were similar (not shown). Calculations were performed by using the PHYLIP program package (distributed by J. Felsenstein, University of Washington, Seattle, WA, USA). Five hundred bootstrap replicates were generated by using the SeqBoot program and submitted to the distance matrix algorithm (DNAdist program), with the maximum-likelihood model for nucleotide substitutions). The resulting distance matrices were analyzed by using the neighbor-joining tree-fitting algorithm (Neighbor program). The bootstrap support values were calculated by using the Consense program. Hantavirus sequences used for comparison were recovered from GenBank. Gray shading indicates PUUV strains. PUUV, Puumala virus; S, small; M, medium; L, large; DAN, Danish; CE, Central European; ALAD, Alpe-Adrian; S-SCA, South Scandinavian; N-SCA, North Scandinavian; RUS, Russian; FIN, Finnish; NE, nephropathia epidemica. Scale bar indicates genetic distance.

when kidneys, lungs, heart, and liver were affected. The viral load was higher in the lungs and kidneys and lower in the heart, spleen, liver, and brain. Whether this load distribution is unique for fatal PUUV infections remains to be seen because corresponding data for other hantavirus infections are missing. Moreover, severe histopathologic changes were detected not only in lungs and heart but also in liver and hypophysis, whereas kidneys, in this respect, were almost normal. Thus, viral load does not seem to correlate with tissue pathology. A more detailed pathologic description of this and other lethal cases is under way.

Two more observations might be relevant to the case. First, human leukocyte antigen typing showed that the patient had the risk haplotype for severe NE, including a C4A null allele, i.e., a major antivirus defense system complement was impaired (T. Sironen et al., unpub. data). Second, the patient was a smoker and thus more likely to become infected with PUUV (*14*). These factors might have substantially affected the fatal outcome. We anticipate that our investigation will prompt further full-length genome analyses of the wild-type strains of bunyaviruses that cause infections in humans.

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Dr Plyusnina is an expert working with the Research Program Unit, University of Helsinki. Her research interests include genetics and evolution of hantaviruses.

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Address for correspondence: Alexander Plyusnin, Haartman Institute, University of Helsinki—Dept Virology, PO Box 21, University of Helsinki (Haartmaninkatu 3), Helsinki FIN-00014, Finland; email: alexander.plyusnin@helsinki.fi

Thelaziosis in Humans, a Zoonotic Infection, Spain, 2011

Isabel Fuentes, Isaías Montes, Jose M. Saugar, Stefania Latrofa, Teresa Gárate, and Domenico Otranto

After *Thelazia callipaeda* infection in dogs and cats were reported in Spain, a human case of thelaziosis in this country was reported, suggesting zoonotic transmission. The active reproductive status of this nematode in situ indicates that humans are competent hosts for this parasite.

Thelazia callipaeda (Spirurida, Thelaziidae) is a parasitic helminth transmitted by zoophilic insects of the order Diptera, family Drosophilidae, genus *Phortica* while feeding on ocular secretions of their hosts during summer (*1,2*). The parasitic first-stage larvae are ingested by the vectors along with the conjunctival secretions of infected animals; they mature into their third larval stage in 2–3 weeks; and they are released as third-stage infective larvae into the eye of a new host (*2*). Nematodes localize in the orbital cavity and associated tissues of canids, felids, rodents, and humans, causing mild (i.e., lacrimation, itching, exudative conjunctivitis) to severe (i.e., corneal ulceration and keratitis) signs and symptoms, if not properly treated (*3,4*).

T. callipaeda has long been called the oriental eyeworm, referring to its traditional distribution across eastern and southeastern Asia (i.e., China, North and South Korea, Japan, Indonesia, Thailand, and India) where infection is endemic in animals and humans (*5*), usually in poorer rural areas and mainly among children and the elderly. Since the first cases of canine thelaziosis identified in Europe, which were in northern Italy in 1988 (*6*), several studies have indicated that the disease is endemic throughout Italy (*7*). In recent years, thelaziosis in cats and dogs has also been reported in France, Germany, and Switzerland, highlighting the spread of the disease in Europe (*8*). Autochthonous cases of *T. callipaeda* infection among dogs have recently been reported in Spain near the western part of the country (La Vera, Cáceres); prevalence in some municipalities has reached 39.9% of dogs examined (*9*).

Author affiliations: Instituto de Salud Carlos III, Madrid, Spain (I. Fuentes, J.M. Saugar, T. Gárate); Hospital Ciudad de Coria, Cáceres, Spain (I. Montes); and Università degli Studi di Bari, Valenzano, Italy (S. Latrofa, D. Otranto)

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After the parasite spread among domestic and wild carnivores from Europe, the first human cases of this zoonotic disease in Italy and France were described (*10*). Although humans are competent hosts, they usually act as accidental-ending hosts in whom the third stage larvae can grow into adults but without epidemiologic effects on parasite transmission. This lacking of effect could be explained because humans, in contrast to animals, are likely to report symptoms and consequently have parasites removed, causing the interruption of transmission.

We report a case of human thelaziosis in Spain. This report highlights the emerging nature of this zoonotic disease and calls for attention to its possible public health consequences. In addition, the finding of a mature female parasite with developed larvae in the uterus suggests that humans may be proper hosts for *T. callipaeda* development in areas where thelaziosis is endemic in dogs or cats.

The Study

An adolescent girl, 17 years of age, from the village of Coria in the Province of Cáceres, Spain (40°N, 6°32′W), sought assistance at the regional hospital (Ciudad de Coria Hospital) in September 2011, describing the sensation of a foreign body in her left eye for 3 weeks. She reported having spent her holidays in the Cáceres countryside during July and August. Examination revealed lacrimation and conjunctival abnormalities or exudate; ophthalmologic examination revealed 2 filiform worms on the conjunctival fornix of the affected eye. Physical examination and laboratory analysis of blood showed results otherwise within reference values. After extraction of the slender worms with forceps, her symptoms resolved; an ophthalmic examination performed 2 weeks later yielded results within reference range.

The smaller of the 2 worms could not be retrieved for examination because it was destroyed during extraction, whereas the larger one was placed in physiologic saline solution and sent to the Centro Nacional de Microbiología, where it was fixed in 70% ethanol and identified on the basis of morphologic keys (*11*).

The retrieved worm was an adult female (17 mm long and 424 μm wide) and had a serrated cuticle with transverse striations (290/mm in the cephalic region, 200/mm in the midsection, and 240/mm in the tail section). The buccal capsule (28 μm wide, 26 μm deep) showed a hexagonal profile and 6 festoons (Figure). The vulva was anterior to the esophageal–intestinal junction; embryos were visible in the proximal area of the uterus, whereas larvae were visible in the distal area. The small size of the other worm (not recovered) and the active reproductive status of the female carrying young larvae suggested the unrecovered worm was most likely a male, who mated with the female in the patient's orbital socket.

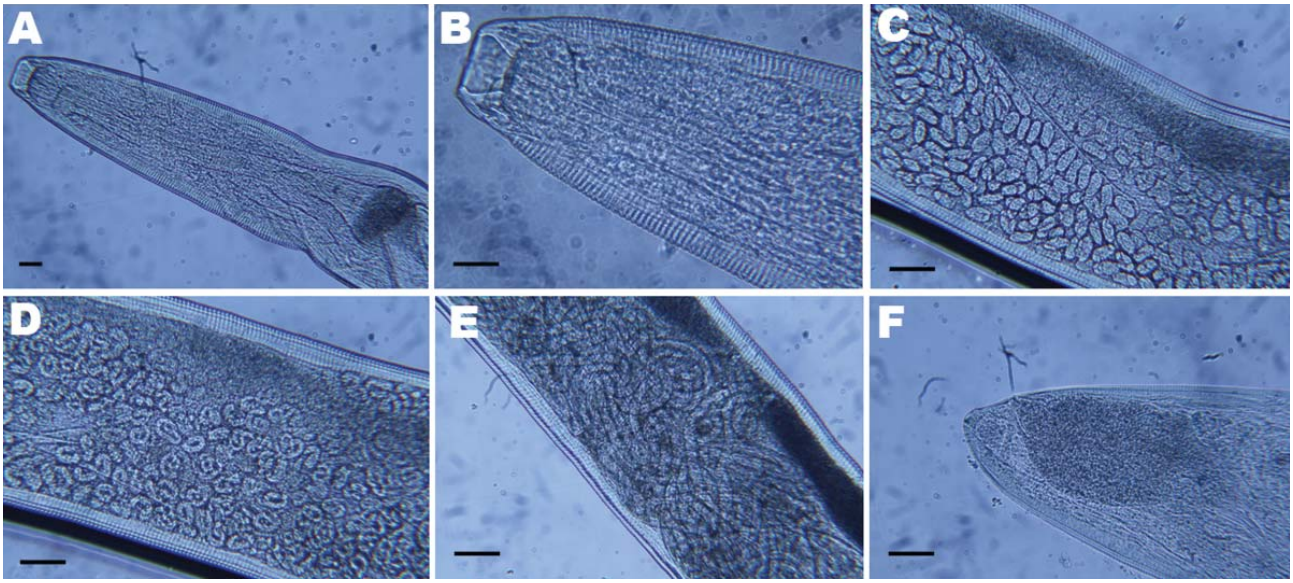

Figure. Light micrographs of *Thelazia callipaeda* showing A) posterior and B) anterior portion with cephalic end and buccal capsule; C) anterior portion containing embryonated eggs; D) middle portion containing rounded first-stage larvae; E) posterior portion containing first-stage larvae; F) caudal end. Scale bars = 25 µm.

The morphologic characteristics of the female worm led to its identification as *T. callipaeda*, which was molecularly confirmed by a specific PCR amplification (*12*). In brief, after we extracted genomic DNA from the female worm with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), a partial sequence of the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox*1, 689 bp) was amplified by PCR. Amplicons were purified by using Amicon Ultrafree-DA columns (Millipore, Bedford, MA, USA) and sequenced in an ABI-PRISM 377 by using the *Taq* DyeDeoxyTerminator Cycle Sequencing Kit (Applied Biosystems, Monza, Milan, Italy). Sequences were determined in both directions and aligned by using the ClustalX program (www.clustal.org). The alignments were verified and compared with the sequences available for the *cox*1 of *T. callipaeda* (GenBank accession nos. AM042549–556). Sequences obtained from the nematode were identical to the sequence representing haplotype 1 of *T. callipaeda* (GenBank accession no. AM042549) (*12*).

Conclusions

We described a case of human thelaziosis in Spain in a patient living in a geographic region where 182 (39.9%) of the 456 dogs examined were recently found positive for *T. callipaeda* by morphologic and molecular analyses (*9*). These data indicate that in an area where thelaziosis is endemic in animal populations, there is also a risk for parasitization in humans. According to what is known of the biology of *T. callipaeda* in dogs and vectors in Europe (*1,2*), the end of summer is the period of maximum activity of the vector (*1*). Summer is when the human patient reported ocular discomfort. The patient had signs and symptoms of a mild infection including eye discomfort and foreign body sensation; a proper diagnosis was made after 3 weeks.

Considering the high prevalence of infection in dogs reported in recent studies and the case of human thelaziosis here described, general physicians and ophthalmologists should take human thelaziosis into account in their differential diagnoses of conjunctivitis, ocular lacrimation, and corneal ulcers (*4*). Medical continuing education and awareness of this condition are needed to ensure that the infection does not go undiagnosed and that appropriate treatment for the primary problem and for complications such as allergic reaction or bacterial infection can be prescribed.

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Dr Fuentes is a staff researcher at National Center of Microbiology, Instituto de Salud Carlos III. Her primary research interest includes diagnosis, reference, and characterization of autochthonous, imported, and emerging parasitoses.

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Address for correspondence: Isabel Fuentes, Servicio de Parasitología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo Km2, 28220 Majadahonda, Madrid, Spain; email: ifuentes@isciii.es

Avian Influenza Vaccination of Poultry and Passive Case Reporting, Egypt

Timothée Vergne, Vladimir Grosbois, Yilma Jobre, Ahmed Saad, Amira Abd El Nabi, Shereen Galal, Mohamed Kalifa, Soheir Abd El Kader, Gwenaëlle Dauphin, François Roger, Juan Lubroth, and Marisa Peyre

We investigated the influence of a mass poultry vaccination campaign on passive surveillance of highly pathogenic avian influenza subtype (H5N1) outbreaks among poultry in Egypt. Passive reporting dropped during the campaign, although probability of infection remained unchanged. Future poultry vaccination campaigns should consider this negative impact on reporting for adapting surveillance strategies.

Egypt reported its first occurrence of highly pathogenic avian influenza (HPAI) virus subtype H5N1 in poultry on February 16, 2006 (*1*), and its first case in a human on March 20, 2006. As of June 2011, Egypt was the country most affected by HPAI (H5N1) outside of Asia (*2*). Vaccination of domestic (backyard) and commercial poultry, which began in March 2006, and other measures were implemented to control the disease, but outbreaks among poultry and humans continued to be regularly reported from various districts located mainly in the delta region of the country (*3*). In July 2009, vaccination of domestic poultry was stopped (*4*). The objective of this study was to assess the effect of vaccination of domestic poultry on the passive reporting of HPAI (H5N1) cases among poultry. The completeness of the passive surveillance of poultry cases at the district level during and after the mass vaccination campaign was estimated by using a 4-source capture-recapture method (*5*).

Author affiliations: Centre International de Recherche en Agriculture pour le Développement, Montpellier, France (T. Vergne, V. Grosbois, F. Roger, M. Peyre); French Agency for Food, Environmental and Occupational Health and Safety, Maisons-Alfort, France (T. Vergne); Food and Agriculture Organization of the United Nations, Cairo, Egypt (Y. Jobre, A. Saad, A. Abd El Nabi); Central Laboratory for Veterinary Quality Control of Poultry Production, Giza, Egypt (S. Galal, M, Kalifa); General Organisation for Veterinary Services, Cairo (S. Abd El Kader); and Food and Agriculture Organization of the United Nations, Rome, Italy (G. Dauphin, J. Lubroth)

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The Study

Two periods were selected for study: period 1 (December 2008–June 2009), during which mass vaccination of backyard poultry was ongoing, and period 2 (December 2009–June 2010), during which mass vaccination had ceased (Figure). Vaccination of commercial poultry in Egypt against avian influenza (AI) continued throughout the study period. In Egypt, the district level is the smallest administrative unit used for defining surveillance and control strategies related to HPAI (H5N1) among poultry. Thus, we used the district level to estimate the occurrence of HPAI (H5N1) during the 2 study periods.

HPAI (H5N1) circulation in poultry is recorded by the national surveillance of poultry coordinated by the General Organization for Veterinary Services (GOVS) in conjunction with the Central Laboratory for Quality Control of Poultry Production, based in Cairo. During the 2 study periods, poultry surveillance was structured into 3 distinct protocols: 1) passive surveillance reliant on disease reporting by farmers; 2) active surveillance of preslaughter poultry and high-risk areas (areas defined by GOVS as high risk based on specific criteria, such as the density of the poultry population and the detection of HPAI (H5N1) cases among humans and poultry in previous months); and 3) the community based animal health outreach program, a participatory surveillance network that relies on traditional information networks to track down and confirm HPAI (H5N1) events in areas where virus circulation is suspected.

The lists of cases collected through these complementary protocols by GOVS and the Central Laboratory for Quality Control of Poultry Production was provided by the Emergency Centre for Transboundary Animal Diseases Unit of the Food and Agriculture Organization in Egypt. Since 2006, the government has required the culling of poultry flocks in which cases of avian influenza were detected. As part of the initial program, government compensation for culled birds was also implemented, but because of misuse, the compensation program was stopped in 2007.

It is assumed that most human cases of influenza (H5N1) are linked to infections in poultry (*3*,*6*,*7*); thus, we postulated that a human case within a district revealed avian influenza (H5N1) virus circulation among poultry within that district. For methodological purposes, we hypothesized that a human could not get the infection from outside the district of residence. In Egypt, the protocol for surveillance of human influenza cases is based on reporting of suspected cases in district hospitals followed by confirmation of infection by the Central Public Health Laboratory in Cairo and the US Naval Medical Research

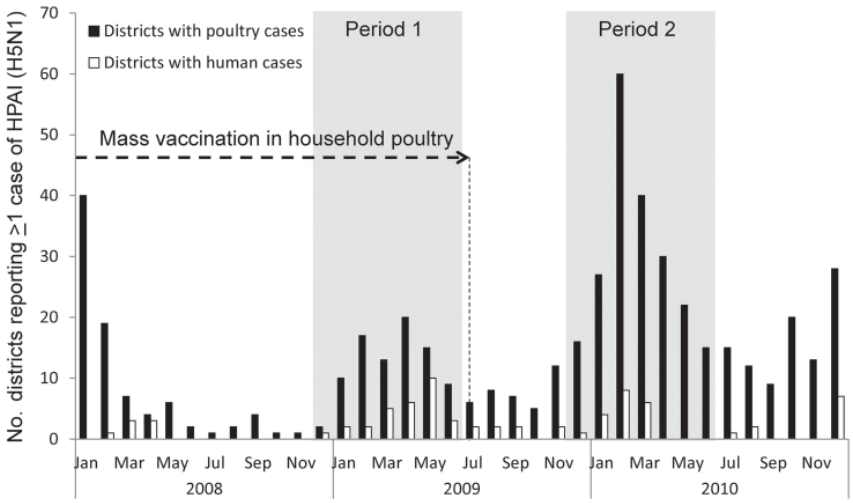

Figure. Number of districts in which avian influenza (H5N1) virus infection was detected among poultry and humans during and after a campaign of mass vaccination of backyard poultry, Egypt, January 2008–December 2010. Activity was identified by active, passive, or participatory surveillance at the district level. Cessation of the vaccination campaign appeared to cause a large increase in the number of infected districts that were detected during Period 2. Shading indicates periods of study. HPAI, highly pathogenic avian influenza.

Unit 3 (*8*). Data for human cases were obtained from the World Health Organization (www.who.int/csr/don/archive/country/egy/en/). From this list of cases, 1 case from period 2 was excluded because exposure to sick or dead poultry was not confirmed.

Capture-recapture methods were introduced in the field of ecology for estimating the size of wild populations and subsequently adapted to surveillance of infectious diseases in humans and animals (*5*,*9*,*10*). After accounting for the small sample sizes in our study (*11*), we used log-linear models to model cross-detection frequency data (Table 1) (*12*). The best model was selected by using the Akaike information criterion and projected onto the "no detection" history to estimate the frequency of districts where the virus was circulating but not reported (*5*). Details about our method can be found in the Online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/12-0616-Techapp.pdf).

For period 1, the best model was the independent model that assumed no interaction between any of the 4 detection sources. For period 2, a significant positive interaction ($p<0.05$) was detected between passive surveillance and participatory surveillance. The extent of the 2 outbreaks appeared similar at district level. The actual number of districts that reported infections in poultry was estimated to be ≈126 and 133, respectively, for period 1 and period 2 (Table 2). As a consequence, the Figure suggests that surveillance was affected heavily by underreporting during period 1. The completeness of poultry surveillance at the district level increased, rising from 46% to 69% between the 2 periods. Moreover, the sensitivity of the passive surveillance among poultry during period 2 was estimated to be more than twice as high as during period 1.

During period 2, participatory surveillance targeted zones where HPAI (H5N1) virus circulation had been informally reported in poultry, potentially overlapping spontaneous reports and resulting in a direct positive dependence on passive surveillance. No positive dependence between surveillance of humans and active or participatory surveillance of poultry was detected, even though such dependence could have been predicted because of possible investigations into poultry cases after human cases were reported.

The assumption of no indirect dependence between the 4 detection sources is the most critical aspect of this study. It is possible that all 4 sources had a higher probability of

Table 1. Avian influenza vaccination and disease detection history of districts reporting highly pathogenic avian influenza (H5N1) in poultry and humans, Egypt*

Detection history				No. districts reporting infection	
AS†	PS‡	Part S§	SH¶	Period 1	Period 2
1	0	0	0	13	6
0	1	0	0	22	38
0	0	1	0	4	5
0	0	0	1	15	4
1	1	0	0	3	7
1	0	1	0	1	1
1	0	0	1	7	1
0	1	1	0	1	20
0	1	0	1	2	3
0	0	1	1	1	1
1	1	1	0	1	2
1	1	0	1	2	1
1	0	1	1	0	0
0	1	1	1	0	5
1	1	1	1	1	2
0	0	0	0	NK#	NK

*Columns1–4 describe detection of infection by various protocols: 1 = infection reported using protocol; 0 = no infection reported using protocol, e.g., the first line corresponds to the districts in which infection was detected only by the active surveillance of poultry. Period 1, Dec 2008–Jun 2009; period 2, Dec 2009–Jun 2010.
†Active surveillance of poultry.
‡Passive surveillance of poultry.
§Participatory surveillance of poultry.
¶Surveillance in humans.
#NK, not known.

Table 2. Estimated surveillance parameters for avian influenza virus infection in Egypt*

Estimated parameters	Period 1		Period 2	
	Point estimates	95% CI	Point estimates	95% CI
Number of districts with infection among poultry	126	107–159	133	118–160
Completeness of the 4 detection sources	0.58	0.46–0.68	0.72	0.60–0.81
Completeness of surveillance among poultry	0.46	0.36–0.54	0.69	0.58–0.78
Completeness of passive surveillance	0.25	0.20–0.30	0.59	0.49–0.66

*Period 1, Dec 2008–Jun 2009; period 2, Dec 2009–Jun 2010.

detecting districts with a high incidence of disease than a low incidence of disease. As a consequence, for each period, this positive indirect dependence is likely to result in underestimation of the true number of districts where HPAI (H5N1) virus was circulating in poultry (*5*), causing some districts that had few outbreaks to go uncounted. The consequence of such an underestimation is a slight overestimation of the completeness of each detection source. However, because this potential bias occurred for both periods, it should not influence the overall trend estimated between the 2 periods.

Conclusions

Our findings support the hypothesis that mass avian influenza vaccination of domestic poultry may negatively affect passive surveillance for influenza infection among poultry (*4*). This phenomenon could be caused by changes in the clinical features of the disease linked to vaccination (e.g., lower mortality rate, fewer clinical signs). However, these changes should be considered negligible because of the limited efficacy of this vaccination strategy in the field (*4*). A disproportionate trust in the benefits of AI vaccination might be a more plausible explanation. This trust could lead to failure of the community to recognize the disease, based on the assumption that poultry vaccinated against HPAI (H5N1) could no longer be infected.

This study supports an updated interpretation of the evaluation of the HPAI (H5N1) control program in Egypt. We conclude that if reported outbreaks are the only data considered, the effectiveness of AI vaccination will be overestimated. Our findings stress the critical importance of the quality of data used in the evaluation of animal and public health control programs and the necessity to evaluate reporting rates.

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Mr Vergne is a PhD student at the Centre de Coopération Internationale en Recherche Agronomique pour la Développement. His primary research interests include the understanding of disease detection processes and the quantitative evaluation of disease surveillance systems.

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Address for correspondence: Timothée Vergne, TA C 22/E Campus International de Baillarguet, CIRAD, 34398 Montpellier Cedex 5, France; email: timothe.evergne@cirad.fr

Leptospirosis Diagnostic Challenges, American Samoa

To the Editor: Leptospirosis is common in the Pacific Islands (66.4 cases/100,000 population/year compared with 5 cases/100,000 population/year globally) (*1*) and is often misdiagnosed as dengue because of overlapping clinical features, poor awareness, and inadequate diagnostic facilities (*2*,*3*). Clinical manifestations range from asymptomatic to severe disease with pulmonary hemorrhage and renal and hepatic failure.

Global emergence of leptospirosis has been associated with environmental factors including rainfall, flooding, poverty, urbanization, and ecotourism (*1*–*4*), to which the Pacific Islands are vulnerable. Seroprevalence of leptospirosis in American Samoa is 15.5% (*5*), and recent reports confirm its emergence in the Pacific region (*6*). We report a case of severe leptospirosis in American Samoa (one of the world's wettest inhabited places) and illustrate diagnostic challenges and the need to improve laboratory capacity.

In January 2011 (wet season), a 15-year-old previously healthy Polynesian boy was examined for a 3-day history of fever, myalgia, fatigue, headache, sore throat, pleuritic chest pain, and vomiting. He spent much time outdoors, occasionally slept in the rainforest, and had recently waded through water.

Examination revealed lethargy, injected conjunctivae, mild periumbilical tenderness, fever (38.6°C), tachycardia (133 beats/minute), and hypotension (96/50 mm Hg). Lung sounds were clear, respiratory rate was 22 breaths/minute, and oxygen saturation was 99%. No rash or jaundice was noted. Laboratory investigations showed leukocytosis (9.35×10^9 cells/L), neutrophilia (85%), mild normocytic anemia (12.0 g/dL), and thrombocytopenia (42×10^9 platelets/L); chest radiographs showed mild infiltrate in the left lung.

Differential diagnosis included dengue, influenza, pneumonia, and leptospirosis. The patient was hospitalized for supportive treatment, but the next day he experienced shoulder pain, increased abdominal and chest pain, worsened thrombocytopenia, hypokalemia, hyperbilirubinemia, proteinuria, hematuria, and fecal occult blood. No abnormalities were found for the following: transaminase, alkaline phosphatase, blood urea nitrogen, and creatinine levels; blood culture; serologic test results for hepatitis; and abdominal ultrasonogram.

Intravenous penicillin was given for possible leptospirosis and/or pneumonia. Within 1 hour, the patient's condition deteriorated: temperature increased (40.2°C); and rigors, severe headache, and myalgia developed. Jarisch-Herxheimer reaction was considered (*7*), and intravenous penicillin was replaced with ceftriaxone. The patient deteriorated further and exhibited hypotension, tachycardia, tachypnea, jaundice, confusion, mucosal bleeding, and required intensive care treatment, including intravenous dopamine for shock. Repeat chest radiograph showed deterioration with bilateral infiltrates. The Figure shows progression of kidney and liver function and thrombocytopenia.

Serum collected on hospitalization day 2 was negative for IgM against *Leptospira* spp. according to the GenBio IgM ImmunoDOT test (San Diego, CA, USA) (*8*). Serum collected on day 3 was positive for IgM and IgG against dengue virus according to the TECO rapid diagnostic test (Anaheim, CA, USA) (*8*), suggesting dengue hemorrhagic fever. The patient continued to receive intravenous ceftriaxone because of worsening condition and clinical suspicion of leptospirosis. He improved with antimicrobial drug treatment and supportive care, and his serum on day 9 was positive for IgM against *Leptospira* spp.

Subsequent serologic testing in Brisbane, Australia, confirmed leptospirosis and excluded dengue. Microscopic agglutination test results confirmed acute infection with *L. interrogans* serovar Copenhageni; rising titers were found in serum collected on days 3 (<50), 8 (100), and 17 (400). All samples were negative for IgM and IgG against dengue virus according to the PanBio Dengue IgM and IgG Capture ELISA tests (Sinnamon Park, Queensland, Australia) (sensitivity 99.2%, specificity 96.2%) (*9*).

This case illustrates that leptospirosis in the Pacific Islands presents many clinical challenges. This patient experienced a life-threatening illness with multiple complications associated with severe leptospirosis, including possible Jarisch-Herxheimer reaction. Early diagnosis is crucial because appropriate treatment with antimicrobial drugs can reduce illness and death (*1*,*2*). Molecular techniques provide rapid diagnosis during the bacteremic phase but are expensive and often unavailable in developing countries (*1*–*3*). Rapid tests for dengue virus have limited sensitivity and specificity and can produce false-positive results in patients with leptospirosis and other conditions (*8*). Serologic testing for leptospirosis detects acute infections only after the second week of illness, so it was crucial that leptospirosis was not excluded early when results were positive for dengue virus but negative for *Leptospira* spp.

During January 2009–June 2011, incidence rates for dengue and leptospirosis among children ≤16 years of age in American Samoa were 517 and 159 cases per 100,000 population per year, respectively; incidence was highest in the wettest months. Incidence of each infection peaked in October 2009 (1,512 and 798 cases/100,000

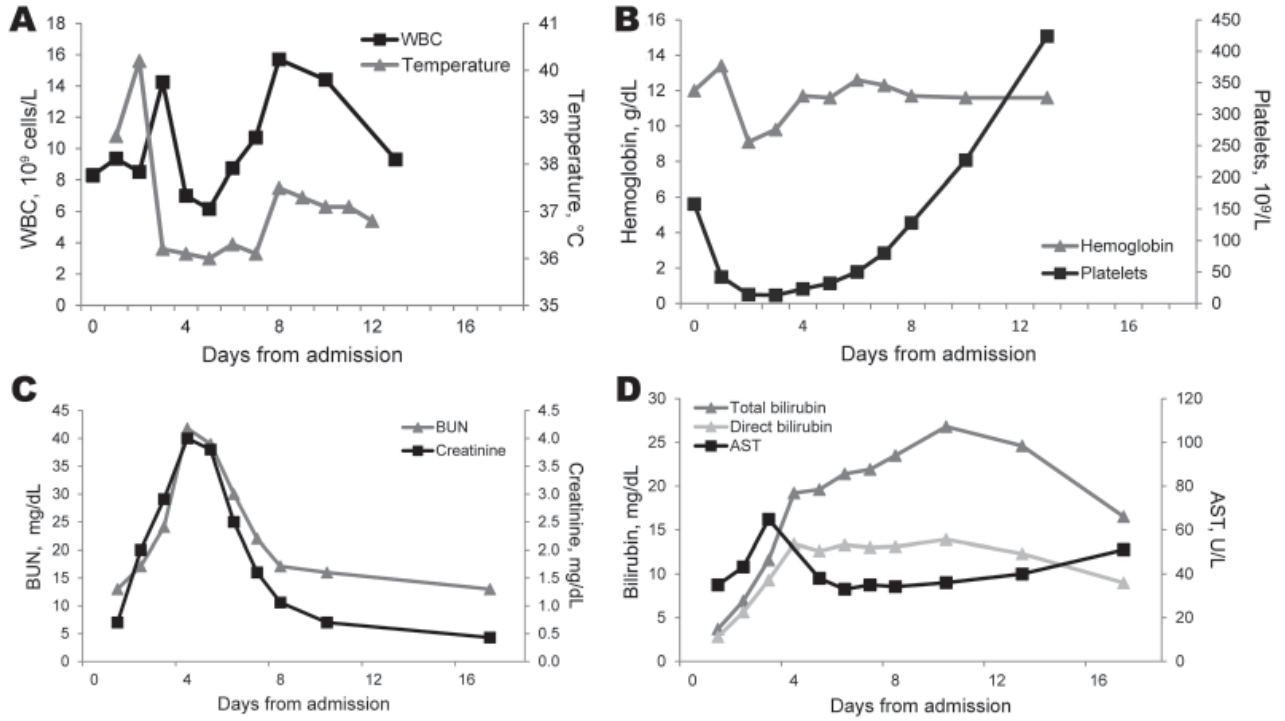

Figure. Clinical course of temperature and selected laboratory results in a patient with severe leptospirosis in American Samoa. A) Temperature and leukocyte (WBC) counts (reference range 5.0–9.1 × 10^9 cells/L). B) Hemoglobin (reference range 14.0–16.3 g/dL) and platelet counts (reference range 150–450 × 10^9 platelets/L). C) Blood urea nitrogen (BUN) (reference range 5–18 mg/dL) and creatinine (reference range 0.5–1.0 mg/dL) levels. D) Total bilirubin (reference range 0.3–1.2 mg/dL), direct bilirubin (reference range 0–0.2 mg/dL), and aspartate aminotransferase (AST) (reference range 15–45 U/L) levels.

population/year), possibly related to the late September 2009 tsunami (*10*).

Flooding increases risk for dengue infection (by providing mosquito breeding sites) and leptospirosis (by disseminating leptospires in the environment and increasing human–animal contact). Concurrent outbreaks and co-infections are not uncommon and can complicate diagnosis. Incidence rates for both infections will probably increase with climate change in the Pacific region (*4*). Cocirculation of dengue serotypes increases incidence of dengue hemorrhagic fever and dengue shock syndrome, which are difficult to clinically distinguish from severe leptospirosis. Jarisch-Herxheimer reactions in leptospirosis patients treated with antimicrobial drugs can further complicate diagnosis (*7*). To reduce leptospirosis in the Pacific Islands, awareness of the disease, understanding of limitations of rapid diagnostic tests, and more regional laboratory capacity are needed.

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Colleen L. Lau and John M. DePasquale

Author affiliations: The University of Queensland, Herston, Queensland, Australia (C.L. Lau); and Lyndon Baines Johnson Tropical Medical Center, Pago Pago, American Samoa (J.M. DePasquale)

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Address for correspondence: Colleen L. Lau, PO Box 12426, George St, Brisbane, Queensland 4003, Australia; email: colleen.lau@uqconnect.edu.au

African Swine Fever Virus, Tanzania, 2010–2012

To the Editor: African swine fever (ASF) is a highly contagious and deadly hemorrhagic disease of domestic pigs caused by African swine fever virus (ASFV), a double-strand DNA virus of the family *Asfarviridae* and genus *Asfivirus* (*1*). Twenty-two ASFV genotypes (I–XXII) have been identified on the basis of nucleotide sequencing of the variable 3′-end of the *B646L* gene encoding the major capsid protein p72 (*2*,*3*).

Historically, all ASFV p72 genotypes have been circulating in eastern and southern Africa, and genotype I has been circulating in Europe, South America, the Caribbean, and western Africa (*2*,*3*). Spread of ASFV beyond traditional geographic boundaries occurred with incursion of p72 genotype II into the Republic of Georgia and its subsequent spread into Armenia, Azerbaijan, and Russia (*4*,*5*) and incursion of genotype IX into western Africa (*6*). ASFV circulating in Tanzania has p72 genotypes X, XV, and XVI (*7–10*). We describe incursion and persistent circulation in Tanzania of a highly virulent p72 genotype II ASFV that is identical to the Georgia 2007/1 isolate in the 3′-end of the *B646L* gene.

An outbreak of ASF in domestic pigs occurred in November 2010 in the Kyela District of the Mbeya region in southwestern Tanzania, which coincided with another outbreak in a neighboring district of Karonga in northern Malawi (Figure, panel A). ASF continued to spread from Mbeya and ultimately reached the neighboring region of Iringa (Ludewa District) in February 2011 through feeding of pigs with swill from Mbeya. By March 2011, ASF had spread to Chunya, Ileje, Mbarali, Rungwe, and Tukuyu districts within Mbeya. The disease spread within the region because of the lack of zoosanitary measures and illegal movement of animals despite the quarantine in place. An outbreak on 1 farm in the Temeke District of the Dar es Salaam region in eastern Tanzania occurred in March 2011 after a farmer obtained pig stock from Mbeya. No further spread of the disease in Dar es Salaam was observed after early diagnosis, removal of affected pigs, and zoosanitary measures.

In October 2011, the disease spread to the Sumbawanga District of the Rukwa region through feeding of swill and illegal movement of animals. ASF was reported in February 2012 in Ifakara in the Kilombero District in the Morogoro region, and in July 2012 in the Kilosa District within this region. The disease spread into Kilombero District after 1 farmer purchased pigs for stock from the Iringa region. As of July 2012, ASF was reported again in the Mbeya and Iringa regions, from which it had been eliminated. This unique ASF outbreak in Tanzania persistently circulated for more than a year; previous outbreaks have been sporadic and resolved after shorter durations (*8–10*).

Mortality rates of 100% caused by ASF were recorded in domestic pigs of all ages in all outbreak areas. Affected pigs showed pyrexia and anorexia, dragged their hind legs, and then showed recumbence. In addition, affected animals had severe cutaneous hemorrhages, especially on medial and lateral sides of the pinna, forelimbs above the carpal joint, facial region, scrotum, and mammary glands (Figure, panels B and C). Postmortem lesions included darkening and enlargement of the spleen, severe hemorrhages of mesenteric and gastrohepatic lymph nodes, and hemorrhagic enteritis (Figure, panels D–G).

DNA was extracted from spleens of animals that either died of the disease or were killed at slaughterhouses during 2010–2012. The variable 3′-

end of the *B646L* (p72) gene was amplified by using p72U/p72D primers (*2*) and subjected to automated dideoxynucleotide cycle sequencing by using Big Dye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, Foster City, CA, USA) in a 24-capillary DNA sequencer (Genetic Analyzer 3500 xL; Applied Biosystems).

All obtained ASFV p72 nucleotide sequences (GenBank accession nos. JX391987 [TAN/10/Kyela], JX391988 [TAN/10/Tukuyu], JX391989 [TAN/11/Chunya], JX391990 [TAN/11/Ludewa], JX391991 [TAN/11/Temeke], JX391992 [TAN/12/Ifakara]) were 100% identical. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis of 2010–2012 ASFV p72 nucleotide sequences from Tanzania in GenBank showed 100% nucleotide identity with the Georgia 2007/1 ASFV isolate (GenBank accession no. FR682468) at nt positions 103,594–104,070.

The Georgia 2007/1 ASFV isolate was detected in Georgia in 2007 and has caused ASF outbreaks in Armenia, Azerbaijan, and Russia (*5*). This ASFV isolate belongs to p72 genotype II (*5*) and clusters with ASFV isolates from Mozambique, Zambia, Madagascar, Mauritius, and Georgia (*2*,*3*,*7*). Although the 2010–2012 outbreak in Tanzania coincided with the outbreak in Malawi in 2010, no ASFV belonging to p72 genotype II has been described in Malawi. ASFV isolates from the 2010 outbreak in Malawi should be sequenced to establish their relatedness to ASFV isolates from the 2010–2012 outbreak in Tanzania and determine an epidemiologic link between these outbreaks. Incursion and persistent circulation of a highly virulent p72 genotype II ASFV identical to the Georgia 2007/1 isolate has implications for transboundary spread of ASF.

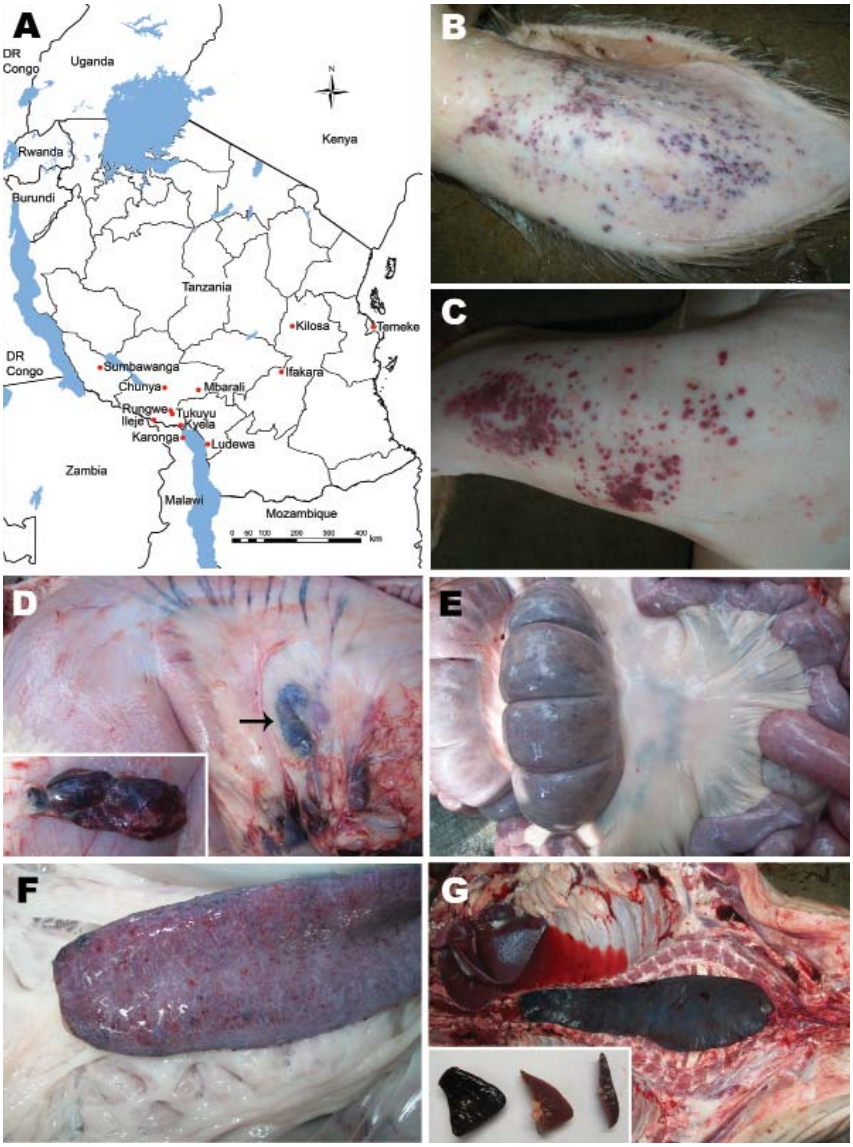

Figure. A) Locations in which African swine fever outbreaks occurred during 2010–2012. B–G) Postmortem lesions observed in slaughtered pigs at the Ifakara slaughterhouse of Kilombero District. Postmortem lesions include cutaneous hemorrhage on the medial side of the pinna (B) and forelimb above the carpal joint (C); hemorrhagic gastrohepatic lymph node (arrow and insert) (D), intestines (E) and spleen (F); and splenomegaly (G). Insert in panel G indicates portions of spleens obtained from different animals showing rounding of edges of the spleen caused by splenomegaly (spleen on the left and center) compared with normal edges of the spleen (spleen on the right). DR Congo, Democratic Republic of the Congo. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/12/12-1083-F1.htm).

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Gerald Misinzo, Christopher J. Kasanga, Chanasa Mpelumbe–Ngeleja, Joseph Masambu, Annette Kitambi, and Jan Van Doorsselaere

Author affiliations: Sokoine University of Agriculture, Morogoro, Tanzania (G. Misinzo, C.J. Kasanga); Central Veterinary Laboratory, Dar es Salaam, Tanzania (C. Mpelumbe-Ngeleja, J. Masambu); Kilombero District Council, Ifakara, Tanzania (A. Kitambi); and Higher Institute for Nursing and Biotechnology, Roeselare, Belgium (J. Van Doorsselaere)

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Address for correspondence: Gerald Misinzo, Department of Veterinary Microbiology and Parasitology, Faculty of Veterinary Medicine, Sokoine University of Agriculture, PO Box 3019, Morogoro, Tanzania; email: gmisinzo@gmail.com

Controlling Highly Pathogenic Avian Influenza, Bangladesh

To the Editor: Highly pathogenic avian influenza (HPAI) A(H5N1) virus is a deadly zoonotic pathogen. Since 2003, HPAI infections have been reported in millions of poultry and wild birds from 63 countries (*1*) and in 598 humans, among whom there have been 352 reported deaths in 15 countries (*2*). HPAI (H5N1) virus is endemic in Bangladesh, and the first outbreak occurred in March 2007. Since then, the virus has spread to 49 of 64 districts in Bangladesh, and samples from 536 farms have tested positive for the virus. Bangladesh now ranks among countries worldwide with the highest reported number of HPAI outbreaks (*1*). Intermittent outbreaks in Bangladesh and clusters of disease across the border in northeastern India are dramatic reminders that the emergence of new, mutant viruses in developing countries could lead to a pandemic among humans. Six cases of nonfatal HPAI (H5N1) infection have been reported in Bangladesh (*2*). Live bird markets that are in poor physical condition and that lack or have poor biosecurity are probable sources of HPAI transmission to humans and for bird-to-bird transmission (*3*–*5*).

In 2008, a global project of the United States Agency for International Development, Stamping Out Pandemic and Avian Influenza (STOP AI), was initiated in Bangladesh. The project began with biosecurity training for veterinarians and livestock science graduates on some large-scale commercial farms. The local STOP AI office was established in Dhaka, the capital of Bangladesh, in February 2009, and the organization managed the project through its completion in September 2010 (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/pdfs/12-0635-Techapp.pdf). STOP AI initially organized 7 highly successful live bird market biosecurity training programs in 5 geographic divisions of Bangladesh; later, STOP AI piloted cleaning and disinfection activities in 2 live bird markets, Mohammadpur and Kaptan Bazaar, in Dhaka by working closely with the United Nations' Food and Agriculture Organization. The Food and Agriculture Organization subsequently conducted cleaning and disinfection activities in 24 other markets within Dhaka and other districts in Bangladesh.

We focused on understanding the inter-relationships among household poultry producers, commercial farmers, suppliers, transporters, processors, and consumers that facilitate the process of producing and moving poultry, i.e., the entire poultry value chain (PVC). We describe how improved biosecurity on poultry farms and hygienic standards in live bird markets can reduce HPAI outbreaks. In resource-limited countries, like Bangladesh, these improvements can be made through training, technical support, financial assistance for infrastructure renovations, and incentive-driven trust-building between service

providers and key PVC stakeholders. To determine whether interventions would reduce the number of HPAI infections, we implemented changes during 2009–2010 in 2 districts in Bangladesh, Gazipur and Dinajpur, that had a high number of cases (online Technical Appendix Figure 2).

Using field-tested questionnaires, we conducted a baseline survey during in-person interviews with 1,372 poultry stakeholders (Table). Stakeholder workshops were held in each district to share survey findings and design biosecurity improvement programs. STOP AI implemented biosecurity training for 1,319 people in 53 subsector-specific 1-day sessions in Gazipur and Dinajpur (online Technical Appendix Table). We created biosecurity improvement models (e.g., farm boundary, footbath) in 12 commercial farms in Gazipur and selected 2 live bird markets in each district for infrastructure improvements, including biogas and compost plants, that were needed for the cleaning and disinfection activities (online Technical Appendix Figure 3). We provided technical support and ≤25% ($750) of the cost for each farm and ≤50% ($10,000) for each market on a cost-sharing basis.

After completion of all interventions, we conducted a final survey of 514 poultry stakeholders, including 70% of the original trainees from both districts (Table). We analyzed pre- and post-intervention survey data by using GraphPad Software (www.graphpad.com/quickcalcs/index.cfm). The results indicated that awareness of the proper disposal of birds that were culled or died because of HPAI had increased in both districts ($p<0.0001$); awareness of human HPAI cases rose substantially ($p<0.0001$); an understanding of how HPAI is spread (e.g., through sick or wild birds) changed ($p<0.001$); use of personal protective equipment (masks, gloves) and other precautionary measures (washing hands) increased ($p<0.0001$); awareness of protecting birds from HPAI (e.g., separately housing chickens and ducks) increased ($p<0.05$); and a preference for purchasing slaughtered birds instead of live birds at the markets increased ($p<0.0001$).

Substantially fewer HPAI outbreaks were reported and no clusters of infection were found during our intervention, 2009–2010 (online Technical Appendix Figure 1), probably indicating that control measures were effective. The challenge now is to sustain the progress that has been made. Several months after completion of the STOP AI interventions, their effect on the incidence of disease in Bangladesh was limited. However, STOP AI could not be expected in the short

Table. Analysis of pre- and postintervention survey data for biosecurity practices for HPAI (H5N1) virus in Gazipur and Dinajpur districts, Bangladesh, 2009–10*

	No. persons surveyed (% aware of practice; 95% CI)			
	Gazipur		Dinajpur	
Biosecurity practice	Baseline survey, n = 821	Final survey, n = 300†	Baseline survey, n = 525	Final survey, n = 209†
Awareness of bird deaths caused by HPAI	191 (23; 21–26)	219 (73; 68–78)	116 (22; 19–26)	88 (42; 36–49)
Awareness of bird culling resulting from HPAI	163 (20; 17–23)	186 (62; 56–67)	56 (11; 8–14)	47 (22; 17–29)
Awareness of HPAI cases among humans	138 (17; 14–20)	172 (57; 52–63)	21 (4; 3–6)	113 (54; 47–61)
Understand how HPAI is spread				
Do not know how HPAI is spread	209 (25; 23–29)	42 (14; 11–18)	286 (54; 50–59)	21 (10; 7–15)
Perceive that wild birds are the cause	466 (57; 53–60)	207 (69; 64–74)‡	134 (26; 22–29)	154 (74; 67–80)
Recognize sick poultry as a vector	43 (5; 4–7)	140 (47; 41–52)	53 (10; 8–13)	61 (29; 23–36)
Awareness of how to protect people				
Wear masks	190 (23; 20–26)	204 (68; 63–73)	44 (8; 6–11)	94 (45; 38–52)
Wear gloves	122 (15; 13–17)	136 (45; 40–50)	54 (10; 8–13)	75 (36; 30–43)
Wash hands	207(25; 23–29)	166(55; 50–60)	58 (11; 9–14)	139 (67; 60–73)
Kids should not handle birds	3 (0; 0.1–1)	203 (68; 62–73)	1 (0; 0.01–1)	54 (26; 20–32)
No need to protect	243 (30; 27–33)	0 (0)	294 (56; 52–60)	4 (2; 0.6–5)
Awareness of how to protect birds				
Separate chickens and ducks	28 (3; 3–4)	92 (31; 26–36)	18 (3; 2–5)	36 (17; 13–23)
Clean and disinfect poultry cages	288 (35; 32–38)	182 (61; 55–66)	39 (7; 5–10)	99 (47; 41–54)
Restrict entry to farms	226 (28; 25–31)	131(44; 38–49)	56 (11; 8–14)	56 (27; 21–33)
Vaccinate against Newcastle disease	12 (1; 0.8–2)	67 (22; 18–27)	7 (1; 0.6–3)	47 (22; 17–29)
Properly dispose of feces	158 (19; 17–22)	79 (26; 22–32)§	16 (3; 2–5)	117 (56; 49–63)
Wear proper clothing	38 (5; 3–6)	67 (22; 18–27)	29 (6; 4–9)	43 (21; 16–27)
Clean and disinfect transport vehicles	35 (4; 3–6)	105 (35; 30–40)	10 (2; 1–4)	20 (10; 6–14)
Keep dogs and cats away from farms	92 (11; 9–14)	99 (33; 28–39)	22 (4; 3–6)	33 (16; 11–21)
Do not know	170 (21; 18–24)	46 (15; 12–20)¶	267 (51; 47–55)	2 (1; 0.04–4)
Bird purchase preference				
Dead bird (slaughtered at market)	152 (19; 16–21)	136 (45; 40–51)	50 (10; 7–12)	70 (33; 27–40)
Live bird (slaughtered at home)	652 (79; 77–82)	167 (56; 50–61)	484 (92; 90–94)	136 (65; 58–71)

*HPAI, highly pathogenic avian influenza.
†Two-sided χ^2 test of significance compared with baseline data had p value of <0.0001, except as noted.
‡p = 0.0002.
§p = 0.013.
¶p = 0.049.

term to dramatically reduce the high incidence of HPAI in Bangladesh. We have progressively and dramatically increased the scope and benefits of our pilot PVC implementation program, but additional work is needed. To help spread PVC approaches throughout the country, community leaders, imams of local mosques, and school teachers can be trained to implement awareness programs on safe practices for raising poultry and regular cleaning and disinfection of live bird markets. The strengthening of biosecurity measures will help control the spread of HPAI virus and other zoonotic diseases.

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Shankar P. Mondal, David Tardif-Douglin, Robert Ryan-Silva, and Rich Magnani

Author affiliations: Development Alternatives Inc., Bethesda, Maryland, USA (S.P. Mondal, D. Tardif-Douglin, R. Ryan-Silva, R. Magnani); and University of Minnesota, St. Paul, Minnesota, USA (S.P. Mondal)

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Address for correspondence: Shankar P. Mondal, Department of Veterinary and Biomedical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN 55108, USA; email: shankarpm@yahoo.com

Hepatitis E Virus Genotype 3 in Shellfish, United Kingdom

To the Editor: Bivalve mollusks (shellfish), such as mussels and oysters, are filter feeders; they concentrate microorganisms of human and animal origin (up to 100×) from the surrounding environment. Several recent reports have linked the incidence of human infection with hepatitis E virus (HEV) to consumption of undercooked pork, game products, and shellfish (*1*,*2*). Infectious HEV has been found in swine manure and wastewater (*3*); therefore, application of manure to land and subsequent runoff could contaminate coastal water, leading to contamination of shellfish and, subsequently, possible human infection. Because they are filter feeders, bivalve mollusks are biologically relevant sentinels and can indicate potential pathogens that are contaminating the environment. It is essential to ensure that this sustainable resource of coastal areas, where mussels and oysters are farmed or collected wild, is not subjected to environmental contamination that could lead to public health risks.

Risk management for bivalve mollusks, aimed at control of fecal pollution, relies heavily on the use of *Escherichia coli* as an indicator of fecal (sewage) contamination and is enacted under European food regulations (Regulation 854/2004, www.cefas.co.uk/media/455777/extract_reg_no_854_2004.pdf). However, although these regulations probably reduce the number of infections, especially bacterial infections, they are not viewed as adequately controlling the risk for viral infections. Specific risks are posed by the robustness of viruses in the environment and the different behavior of viruses within bivalve mollusks compared with behavior within bacterial fecal indicators.

HEV is deemed to be inactivated during processing procedures used to prepare mussels for consumption; however, HEV is only 50% inactivated at 56°C and 96% at 60°C for 1 hour, it is stable when exposed to trifluorotrichloroethane, and it is resistant to inactivation by acidic and alkaline conditions (*4*). Most shellfish are usually eaten raw, but viable virus can also pose a risk to public health in shellfish that are lightly steamed or preserved by smoking and/or in acetic acid. Indeed, a recent study by the Food Standards Agency, in which >800 oyster samples from 39 growing beds in the United Kingdom were collected and screened during 2009–2011, found norovirus at low levels in at least 76% of oysters (*5*). Other studies identified hepatitis A virus and norovirus in shellfish production areas and in ready-to-eat products in the United Kingdom (*1*,*6*). In fact,

depuration experiments demonstrated no decrease in titers against hepatitis A virus over a 23-hour cleansing period (*7*). In addition, acute HEV infection attributed to consumption of shellfish was diagnosed for 33 passengers who recently returned from a cruise (*2*). However, data have been restricted to questionnaires implicating consumption of shellfish as a source of transmission; no follow-up analyses of the contaminated foodstuff have been conducted. Thus, possible transmission routes for HEV remain poorly studied in the United Kingdom (*2*).

To determine whether HEV is present in mussels collected locally for human consumption, we examined 48 mussels from 5 tidal locations in Scotland. We collected closed mussels from the west coast of Scotland (11 at Lunderston Bay and 28 at Ardrossan) and the east coast of Scotland (9 at Stannergate, Dundee; Ferryden, Montrose; and the Ythan Estuary at Newburgh).

The site at Ardrossan was near a slaughterhouse and a meat preparation purification plant that processes pigs. The plant was considered a potential source of contamination, and mussels were collected in a 10-m^2 area around an outfall (drain/sewage pipe) directly in line with the processing plant.

A total of 36 (92%) of the 39 mussels from the west coast were positive by PCR for HEV, and 5 (55%) of the 9 from the east coast were positive. The mean value of HEV RNA detected in the samples was 4.25 $\log_{10}$ IU/mL (range 3.73–5.2 $\log_{10}$ IU/mL), and the assay was validated by using the current candidate HEV World Health Organization standard (http://whqlibdoc.who.int/hq/2011/WHO_BS_2011.2175_eng.pdf). Phylogenetic analysis showed that most bivalve mollusk sequences clustered with HEV genotype 3 from humans and swine (Figure; online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0924-Techapp.pdf). Also, HEV sequences isolated specifically from a UK human source corresponded with sequences isolated from the bivalve mollusks. The presence of a swine-like HEV genotype 3 in freshwater bivalve mollusks has also been reported in Japan and South Korea (*1*,*9*).

Worldwide, an estimated 40,000 persons die and another 40,000 experience long-term disability as a result of consuming raw or undercooked shellfish (*10*). This study, demonstrating the presence of HEV in mussels collected locally in Scotland for human consumption, raises concern as to whether these shellfish are a potential source of infection, as reported (*2*). The association between environmental contamination with HEV and possible transmission by eating shellfish warrants investigation.

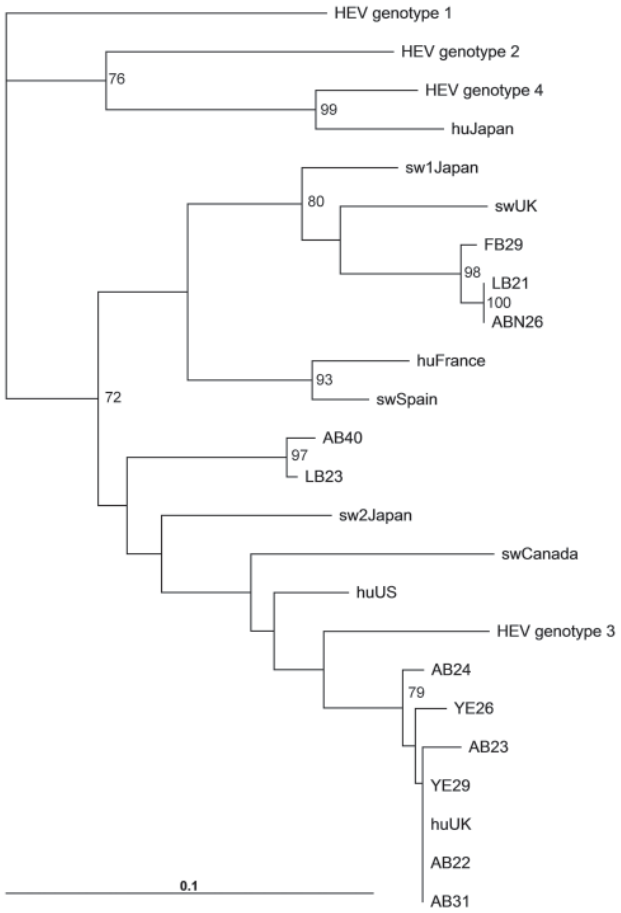

Figure. Phylogenetic analysis of HEV open reading frame 2 sequences isolated from *Mytilus* spp. RNA was isolated from 50–100 mg of digestive gland or gill. Tissue was homogenized in 300 μL phosphate-buffered saline, and viral RNA was isolated by using a viral RNA kit (QIAGEN, Crawley, UK), and PCR was conducted by amplifying nucleotides 6332–6476 as described (*8*). The nucleotide sequences were aligned and bootstrapped, and phylogenetic neighbor-joining trees were constructed by using the ClustalW software (www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic trees were visualized by using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Bootstrap values >70% are indicated. Scale bar indicates nucleotide substitutions per site. Sample site codes: AB, Ardrossan Beach; LB, Lunderston Bay; ABN, Aberdeen; FB, Ferrybridge; YE, Ythan Estuary. Sequences: Sw, swine; hu, human (followed by country of origin). GenBank accession numbers for reference sequences: HEV genotype 1, B73218; HEV genotype 2, M74506; HEV genotype 3, CO31008; HEV genotype 4, C272108; huUK (KernowC1), HQ389543; HuUS, JN837481; swUK AF503512; huFrance, JN906974; swCanada, AY115488; swSpain, JQ522948; sw2Japan AB248521, huJapan AB161719.

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Claire Crossan, Paul J. Baker, John Craft, Yasu Takeuchi, Harry R. Dalton, and Linda Scobie

Author affiliations: Glasgow Caledonian University, Glasgow, Scotland, UK (C. Crossan, P.J. Baker, J. Craft, L. Scobie); University College London, London, UK (Y. Takeuchi); and European Centre for Environment and Human Health, University of Exeter Medical School, Truro, UK (H.R. Dalton)

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Address for correspondence: Linda Scobie, Department of Life Sciences, Glasgow Caledonian University, Cowcaddens Rd, Glasgow, G4 0BA, UK; email: linda.scobie@gcu.ac.uk

Autochthonous Gnathostomiasis, Brazil

To the Editor: Gnathostomiasis is an infestation by nematodes of the genus *Gnathostoma*; the main source of infection is raw freshwater fish. In the past, gnathostomiasis was regarded as restricted to certain Asian and Central American countries, but increase of migratory flux and changes in alimentary habits have contributed to importing cases into areas where the disease is not endemic (*1*,*2*). We report a case of autochthonous gnasthostomiasis in Brazil.

A 37-year-old man from Rio de Janeiro sought medical attention in 2005 because of low fever, cough, abdominal tenderness, and pain in the left shoulder. The symptoms started 15 days after a recreational trip to Tocantins, where he practiced sport fishing and ate sashimi-style freshwater raw fish (*Cichla* sp.) that had just been caught. He reported no history of traveling to a gnathostomiasis-endemic area. Initial work-up depicted eosinophilia (43%), and a computed tomographic scan of the chest revealed left pleural effusion. Two weeks later, winding, linear, reddish lesions appeared on his back, which lasted 3 days (Figure, panel A). Serologic testing for *Schistosoma mansoni* was weakly positive. Acute schistosomiasis was diagnosed, and treatment with praziquantel was begun. In 4 weeks, all symptoms faded.

In 2009, the patient took albendazol for helminthic prophylaxis, and 3 weeks later, deep migratory, swelling, reddish nodules occurred on the thorax; each lesion lasted ≈6 days, and new lesions appeared at intervals of 1–5 days in a somewhat linear array (Figure, panel B). By this time, hemograms displayed eosinophilia of 25%, but a computed tomographic scan of the chest showed no abnormalities. Results of a complete ophthalmologic examination were unremarkable, and a fecal examination was negative for parasites. Gnathostomiasis was highly suspected on the basis of the clinical and epidemiologic findings and results of skin biopsies. Histopathologic examination revealed a dense superficial and deep dermal infiltrate of eosinophils and neutrophils but did not show the parasite. Two samples of plasma were sent to Thailand for immunoblot in search of the diagnostic band (24-kDa antigen) of *Gnathostoma spinigerum*, resulting in high titers. Albendazol, 800 mg/day for 21 days, and a single dose of ivermectin, 0.2 mg/kg, were administered and, despite initial improvement, the disease relapsed, requiring a second cycle of the medications. No signs of disease occurred during 2 years of follow-up.

Gnathostomiasis is found mostly in Japan and Thailand. In the Americas, most cases occur in Mexico

(*3*). Gnathostomiasis was previously reported in Brazil, but the patient was infected in Peru (*4*).

Four species are known to cause disease in humans, and *G. spinigerum* is the most frequent cause. Adult parasites live in the stomach of definitive hosts (dogs, cats, and other fish-eating mammals), and eggs are eliminated in feces. These hatch and release the first-stage larvae in fresh water; larvae are ingested by the first intermediate host, a copepod, and develop into second-stage larvae. Copepods are ingested by the second intermediate hosts (fish, eels, frogs, birds, and reptiles), and larvae mature to the third stage. When eaten by an appropriate definitive host, third-stage larvae evolve to adults and finally reach the stomach of their host.

Third-stage larvae cannot mature in humans and keep migrating in skin, subcutaneous tissue, or other organs. Initial signs and symptoms are fever, anorexia, nausea, vomiting, diarrhea, malaise, urticaria, and epigastric pain. Eosinophilia is frequent. After 2–4 weeks, larvae migrate to skin or subcutaneous tissue, causing winding linear erythematous lesions or migratory swelling nodules. Cutaneous gnathostomiasis is the most common form of disease. The larvae also can migrate to lungs; genitourinary tract; digestive tract; ears; eyes; and rarely, the central nervous system, which may result in death (*5*). If left untreated, gnathostomiasis may remit and recur several times until death of the larvae ≈12 years after infection.

The rate of detection of larvae in skin biopsy specimens varies from 24% to 34%, and the diagnosis frequently needs confirmation by serologic testing (*3*). Immunoblot is highly sensitive and specific and is regarded as the most valuable ancillary technique (*1*,*6*). The diagnosis in the patient reported here had a 4-year delay, despite investigation in several renowned institutions.

The treatment of choice is albendazol, 800 mg/day for 21 days, but ivermectin, 0.2 mg/kg in a single dose or for 2 subsequent days, is an alternative (*7*). More than 1 treatment cycle might be required (*8*). Albendazole promotes outward migration of the larvae to the dermis, and we believe that the low doses used for helminths by the patient reported here might have activated quiescent larvae and triggered new lesions (*9*).

A high index of suspicion is necessary to diagnose this disease in areas where it is not endemic. Gnathostomiasis must be suspected in a patient who has a history of eating raw freshwater fish, persistent eosinophilia, and larva migrans–like lesions and/or migratory deep nodules in a linear array. History of traveling to gnathostomiasis-endemic areas is not strictly necessary, considering recent reports of gnathostomiasis acquisition in previously unaffected regions (*10*).

Thiago Jeunon de Sousa Vargas, Sabrina Kahler, Cassio Dib, Marcio Barroso Cavaliere, and Maria Auxiliadora Jeunon-Sousa

Author affiliations: ID–Investigação em Dermatologia, Rio de Janeiro, Brazil (T. Jeunon de Sousa Vargas, M.A. Jeunon-Sousa); Hospital Federal de Bonsucesso, Rio de Janeiro (T. Jeunon de Sousa Vargas, S. Kahler, C. Dib); and Colégio Brasileiro de Cirurgiões, Rio de Janeiro (M. Barroso Cavaliere)

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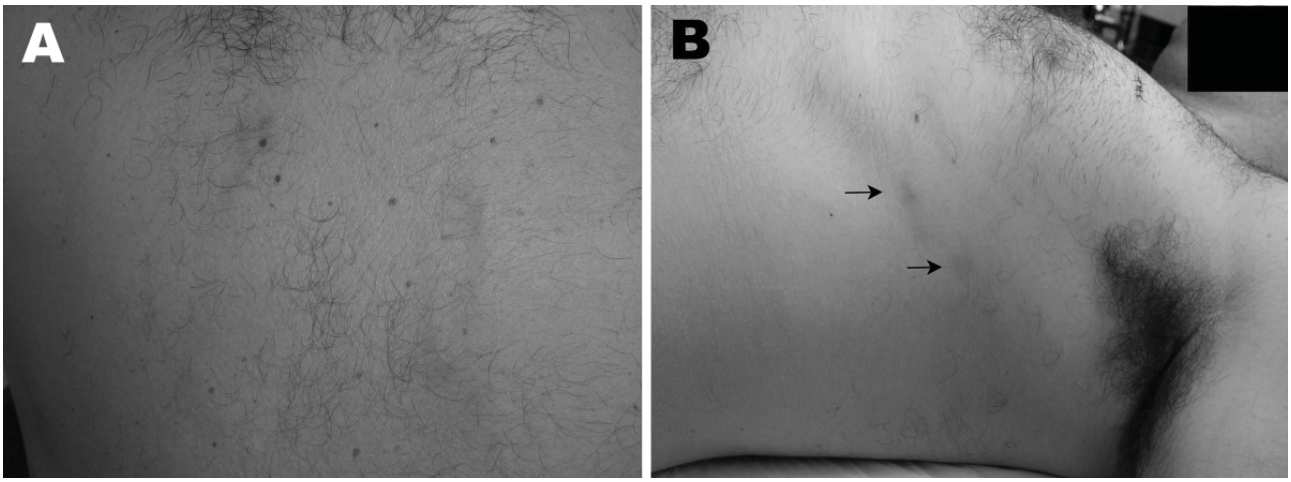

Figure. Gnathostomiasis in a 37-year-old man, Brazil. A) Evanescent winding, linear, reddish lesions on the back in 2005. B) Deep migratory reddish nodules (arrows) on the lateral thorax, occurring in 2009 after treatment with albendazol for helmintic prophylaxis.

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Address for correspondence: Thiago Jeunon de Sousa Vargas, Hospital Federal de Bonsucesso—Dermatology and Anatomical Pathology, Av. Londres 616, Bonsucesso Rio de Janeiro, Rio de Janeiro CEP:21041-030, Brazil; email: thiago.jeunon@gmail.com

Zoonotic Transmission of Pathogens by *Ixodes ricinus* Ticks, Romania

To the Editor: The *Ixodes ricinus* tick is a predominant vector of a large variety of pathogens of veterinary and medical consequence in Europe (*1*). The most prevalent *I. ricinus*–borne infection of persons in Europe is Lyme borreliosis, a multisystemic disorder caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex (*2*). Persons bitten by ticks can also become infected with many other pathogens, such as bacteria (*Anaplasma* spp., *Francisella* spp., *Coxiella burnetii*, *Bartonella* spp., *Rickettsiae* spp., and *Neoerhlichia mikurensis*); parasites (*Babesia* spp., *Theileria* spp.); and arboviruses (tick-borne encephalitis virus, Crimean-Congo hemorrhagic fever virus, and Eyach virus) (*1*). Symptoms induced by such pathogens are often diverse and nonspecific, complicating accurate diagnosis of the disease.

In Romania, cases of Lyme disease and tick-borne encephalitis (caused by tick-borne encephalitis virus) have been identified (*3*). However, little is known about the public health impact of these diseases, and none of the other tick-borne pathogens present in Europe have been reported as causes of infection in Romania. Although *I. ricinus* is the most abundant and widespread tick in Romania (*3*), the public health impact of *I. ricinus*–borne disease is likely to be underestimated. Therefore, the first step in evaluating the distribution of these potential pathogens is to establish their presence in ticks from previously unexplored areas.

We conducted a study to identify the main tick-borne bacterial and parasitic human pathogens known to be present in Europe but not previously detected in Romania. We tested for the presence of DNA from spotted fever group *Rickettsia* spp., *Anaplasma* spp., *Francisella tularensis*, and *Babesia* spp. in 147 *I. ricinus* ticks collected from roe deer and goats at 2 sites in eastern Romania: Bacau (46°35′0″N, 26°55′0″E) and Galati (45°26′22″N, 28°2′4″E). Specimens were tested by PCR, using specific primers for each pathogen or group of pathogens, as described (*4*). Sequences obtained from Eurofins MWG Operon (Ebersberg, Germany) were identified by using BLAST (www.ncbi.nlm.nih.gov/BLAST) and compared with sequences available in GenBank.

DNA from *Rickettsia* spp. was detected in 20 (13.6%) ticks. Sequence analyses revealed that 9 (6.1%) sequences were related to the *R. monacensis* strain IRd/Serbia *glta* gene (99%–100% nt similarity) (GenBank accession no. GQ925820) and 11 (7.48%) were related to *R. helvetica gltA* gene (99%–100% nt similarity) (GenBank accession no. AM418450). DNA from *Anaplasma* spp. was identified in 33 (22.4%) ticks. Analysis revealed that 30 of the 33 amplified fragments showed 100% identity to the 16S rDNA gene of a symbiont in the family *Anaplasmataceae*, *Candidatus* Midichloria mitochondrii (GenBank accession no. EU780455), and the remaining 3 were related to known pathogenic species identified in Romania: 2 (1.4%) exhibited 100% identity to *Anaplasma phagocytophilum* (GenBank accession no. EU982548), and 1 (0.7%) showed 99% similarity to *Ehrlichia muris* (GenBank accession no. GU358691). *Francisella tularensis*–specific DNA was amplified from 4 DNA extracts (2.7%). All 4 sequences were identical and shared 99% similarity with the *F. tularensis* peptidyl-propyl *cis-trans* isomerase gene (GenBank accession no. CP003048). *Babesia* spp.–specific DNA was amplified in 1 DNA extract (0.7%), and it shared 99% sequence identity with the *Babesia* sp. EU1 18S

rDNA gene (GenBank accession no. HQ830266).

This is a report on the identification of the human pathogens *R. monacensis*, *R. helvetica*, *A. phagocytophilum*, *E. muris,* and *Babesia* EU1 in Romania. *R. monacensis* (also known as the Cadiz agent or *Rickettsia* IRS3 and IRS4) was first identified in *I. ricinus* tick samples from Germany (*5*) and was recently recognized as the cause of Mediterranean spotted fever-like rickettsiosis in Spain (*6*). *R. helvetica* was isolated from *I. ricinus* ticks in Switzerland in 1979, and, since then, it has been isolated in many other European countries (*7*). R. helvetica was associated with human infections in the late 1990s (*8*).

The role of animals as reservoirs for these pathogens is unknown. *A. phagocytophilum* is the causative agent of granulocytic anaplasmosis in humans, cattle, horses, and dogs, and is widespread throughout Europe (*8*). *E. muris* has been detected in ticks from Finland and European Russia and from ticks and rodents from Slovakia. In 2009, E. muris was also detected in patients with febrile illness in the United States (*9*). *Babesia* sp. EU1 has been detected in roe deer and ticks in several countries, and in 2003 was associated with human disease in Italy and Austria (*4*,*10*). Tularemia is known to be present in Romania and is thought to be exclusively and directly transmitted by hares. However, detection of *F. tularensis* DNA in *I. ricinus* ticks suggests that this bacterium might also be tick-borne.

In conclusion, the detection of DNA of various human pathogens in ticks in Romania strongly suggests that these microorganisms circulate in the country. Because all of these pathogens affect humans, our study highlights the urgent requirement for further research to assess their impact on public health in Romania.

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Oana Alina Paduraru, Jean Philippe Buffet, Martine Cote, Sarah Bonnet, Sarah Moutailler, Vlad Paduraru, Françoise Femenia, Marc Eloit, Gheorghe Savuta, and Muriel Vayssier-Taussat

Author affiliations: USC Bipar, Bartonella et Tiques, Maisons-Alfort, France (O.A. Paduraru, J.P. Buffet, M. Cote, S. Bonnet, S. Moutailler, F. Femenia, M. Vayssier-Taussat); Universitatea de Ştiinţe Agricole şi Medicină Veterinară, Iasi, Romania (O.A. Paduraru, V. Paduraru, G. Savuta); Pathoquest, Paris, France (O.A. Paduraru, M. Eloit); Ecole Nationale Vétérinaire d'Alfort, Maisons-Alfort (M. Eloit); and Institut Pasteur, Paris (M. Eloit)

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Address for correspondence: Muriel Vayssier-Tassat, AFSSA-UMR BIPAR, 23 Rue du General de Gaulle, Maisons-Alfort 94700, France; email: mvayssier@vet-alfort.fr

Prion in Saliva of Bovine Spongiform Encephalopathy–Infected Cattle

To the Editor: A definitive diagnosis of bovine spongiform encephalopathy (BSE) in cattle usually relies on Western blot and immunohistochemical testing of samples from the obex region of the brainstem. These conventional diagnostic tests can detect the presence of the abnormal (disease-associated) form of the prion protein (PrP^{Sc}) in brain samples several months before the onset of clinical signs; however, there is no appropriate, universal tool for early preclinical and antemortem diagnosis of BSE. Furthermore, confirmation of the disease is currently only possible by postmortem examination of brain tissues. In this study, we used the serial protein misfolding cyclic amplification (sPMCA) technique to determine the presence of PrP^{Sc} in saliva samples collected from BSE-infected cows before and after the onset of disease (*1*).

In a previous study (*2*), we analyzed the tissue distribution of PrP^{Sc} in cattle up to 66 months after they were orally inoculated with a relatively low dose (5 g) of homogenized brainstem from animals with naturally occurring BSE in England. In 2011, after publication of that study and 83.3 months after the cows were inoculated, clinical signs of BSE developed in 1 cow (no. 5444); necropsy was performed 84.7 months after inoculation. In addition, we used saliva samples from 2 BSE-affected cows (nos. 5413 and 5437) (*2*) to determine the presence of PrP^{Sc}.

We collected saliva samples from animals at 4 monthly intervals, beginning in 2009, 56 months after inoculation. Samples were stored at -80°C until analysis. Using the sodium phosphotungstic acid precipitation method, we concentrated (100-fold) individual 1-mL saliva samples from each time point. We then diluted the concentrated samples 1:10 with the normal isoform of prion protein substrate containing 0.5% potassium dextran sulfate. Using the sPMCA technique as described (*1*), we amplified the samples in 3–8 tubes, and we used Western blot to analyze the proteinase K–treated sPMCA products (*2*).

Using Western blot and immunohistochemical tests, we detected the accumulation of PrP^{Sc} in brains collected at necropsy from the 3 cows examined. In addition, using the sPMCA technique, we detected PrP^{Sc} signal in 1) saliva samples that were concentrated from samples collected from the same 3 cows at necropsy and in 2) concentrated saliva samples that were collected from 2 of the cows (nos. 5413 and 5444) at the early clinical stages of disease.

After saliva samples underwent 3 rounds of amplification, we detected PrP^{Sc} in a saliva sample that was collected from cow number 5437 two months before the clinical onset of clinical symptoms (Figure). For 2 of the cows (nos. 5413 and 5437), the positive ratio of salivary PrP^{Sc} at round 4 of amplification increased as the disease progressed (Figure). Because PrP^{Sc} signal could be detected in BSE-infected brain homogenates diluted up to 10^{-10} after 2 rounds of amplification (*1*), we estimated PrP^{Sc} levels in the nonconcentrated original saliva samples to be lower than those in BSE-infected brain homogenate diluted to 10^{-12}. No PrP^{Sc} signal was detected in samples collected from the 3 cows 3–5 months before the onset of clinical symptoms or from age-matched noninfected controls, even after 4 rounds of amplification.

We demonstrated the presence of PrP^{Sc} in saliva of BSE-affected cows

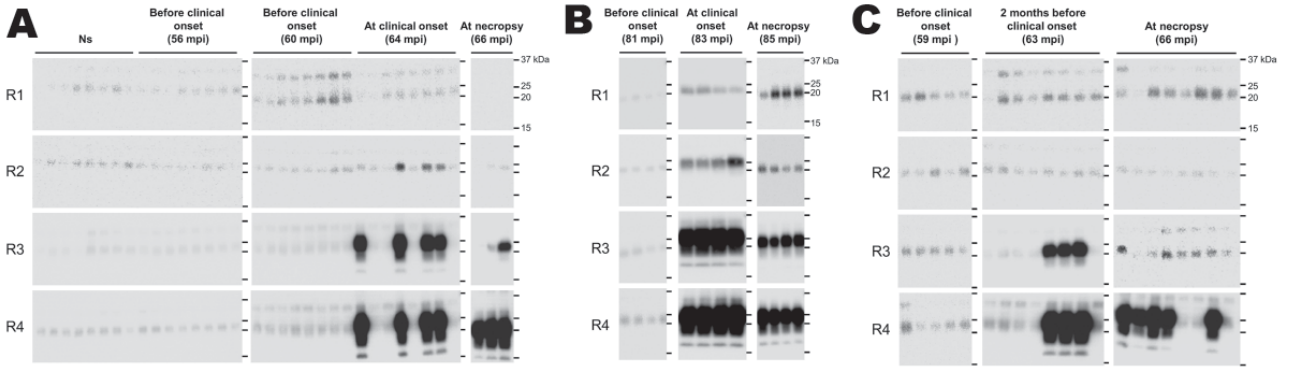

Figure. Western blot detection, using the serial protein misfolding cyclic amplification technique, of the abnormal (disease-associated) form of the prion protein (PrP^{Sc}) in concentrated saliva samples from 3 cows experimentally infected by inoculation with the agent of bovine spongiform encephalopathy: cows 5413 (A), 5444 (B), and 5437 (C). PrP^{Sc} was detected in saliva samples at the initial clinical and terminal stages of the disease (A, B). PrP^{Sc} was also detected in a saliva sample, after 3 rounds of amplification, obtained 2 months before the onset of clinical symptoms in 1 of the 3 cows (C). All saliva samples were concentrated by using the sodium phosphotungstic acid precipitation method. After protein misfolding cyclic amplification, extra bands with a molecular weight higher than that for PrP^{Sc} were occasionally observed, likely corresponding to prion protein aggregates or to residue of the normal isoform of prion protein resulting from incomplete proteinase K digestion. Molecular mass markers (in kDa) are shown on the right. R1–R4, rounds 1–4 of amplification; Ns, no seed control; mpi, months postinoculation.

during the clinical stage of the disease, and in 1 case, at the preclinical or asymptomatic stage. Our findings suggest that PrP^{Sc} is likely to be detected in the saliva of BSE-affected cattle during the clinical stage of disease, after accumulation of PrP^{Sc} in the brain. PrP^{Sc} was found in the salivary glands of BSE-affected cattle at the terminal stage of infection (*1*). Therefore, once the infectious agent reaches the central nervous system, it may spread centrifugally from the brain to the salivary glands through the autonomic nervous system.

Infectivity of saliva and the presence of PrP^{Sc} in saliva have been reported in other ruminants affected with transmissible spongiform encephalopathy. Infectivity of saliva was demonstrated in deer with chronic wasting disease (*3*) and in scrapie-affected sheep (*4*); the immunolabeled PrP^{Sc} accumulated in the salivary glands of scrapie-affected sheep (*5*). A low level of PrP^{Sc} was detected in concentrated buccal swab samples of preclinical scrapie-infected sheep by using sPMCA (*6*,*7*). These results suggest that small amounts of PrP^{Sc} may accumulate in the salivary glands and are then secreted into saliva.

The presence of infectious prions in saliva may explain the facile horizontal transmission of scrapie in sheep (*4*–*6*) and chronic wasting disease in deer (*4*,*8*). There has been no epidemiologic evidence, however, that saliva, milk, blood, and cerebrospinal fluid from BSE-infected cattle are infectious (*9*). Nonetheless, the potential risk for BSE transmission by body fluids or excretions from BSE-infected cattle is cannot be ruled out by the current data.

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Hiroyuki Okada, Yuichi Murayama, Noriko Shimozaki, Miyako Yoshioka, Kentaro Masujin, Morikazu Imamura, Yoshifumi Iwamaru, Yuichi Matsuura, Kohtaro Miyazawa, Shigeo Fukuda, Takashi Yokoyama, and Shirou Mohri

Author affiliations: National Agriculture and Food Research Organization, Tsukuba, Japan (H. Okada, Y. Murayama, N. Shimozaki, M. Yoshioka, K. Masujin, M. Imamura, Y. Iwamaru, Y. Matsuura, K. Miyazawa, T. Yokoyama, S. Mohri); and Hokkaido Research Organization, Shintoku, Japan (S. Fukuda)

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Address for correspondence: Yuichi Murayama, Prion Disease Research Center, National Institute of Animal Health, National Agriculture and Food Research Organization, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan; email: ymura@affrc.go.jp

Reptile- and Amphibian-associated Salmonellosis in Childcare Centers, United States

To the Editor: *Salmonella* spp. infection represents a major public health problem in the United States; nearly 1.4 million human cases and 600 associated deaths are reported each year (*1*). Reptile and amphibian exposures might cause >70,000 of these cases annually (*2*). Furthermore, children are at increased risk of acquir-

ing *Salmonella* spp. and experiencing severe manifestations of disease (*3*,*4*). Given the increasing popularity of reptiles and amphibians as pets, reptile- and amphibian-associated salmonellosis is a substantial public health concern (*5*).

The public has a generally low level of awareness that *Salmonella* spp. can be acquired from reptiles and amphibians (*6*); a poll conducted by the US Centers for Disease Control and Prevention (CDC) during 2003 showed that as few as 4 of 49 states require pet stores to provide information about salmonellosis to persons purchasing reptiles (*4*). A Food and Drug Administration ban, activated in 1975, on the sale of small turtles subsequently prevented an estimated 100,000 cases of salmonellosis in children each year (*7*). To further reduce the risk of reptile- and amphibian-associated salmonellosis, the CDC has issued recommendations advising that children <5 years of age avoid contact with reptiles and amphibians and that these animals not be kept in childcare centers. The CDC also recommends that all persons wash their hands after handling reptiles and amphibians (*8*).

We reviewed the regulations as of December 2011 for childcare centers in all US states aimed at preventing reptile- and amphibian-associated salmonellosis (Table). To gather these data, we searched the websites for each state's public health department or the state's equivalent of an early childhood learning agency. When searches on the Internet did not yield the desired information, the appropriate state agencies were contacted by phone or email. In some instances, we corresponded with the designated State Public Health Veterinarian.

Overall, only 50% of states had regulations that required staff and/or children to wash their hands after touching any animals in childcare centers. Twelve states banned reptiles from childcare centers; 3 of these 12 states also banned amphibians, and these were the only states we found to have banned amphibians from childcare centers. While some states did not allow potentially dangerous or harmful animals in childcare centers, a minority of these states went further to expressly ban reptiles as well (of the 23 states that banned potentially dangerous or harmful animals, 8 states also banned reptiles). One state (Colorado) explicitly banned reptiles, amphibians, and potentially dangerous or harmful animals from childcare centers and also required staff and children in the center to wash their hands after touching animals.

This survey has several limitations. Given the ambiguity in the language used in some regulations and that the language was not standardized between states, we might have misinterpreted some of the documents we reviewed. Furthermore, we might have unintentionally overlooked regulations that were already in place during our investigation, and hence our findings might underestimate the true number of states that have such policies. In some cases, cities and counties have regulations that provide increased protection beyond those implemented at the state level.

In summary, we found great variation between state regulations for childcare centers aimed at reducing transmission of *Salmonella* spp. from reptiles and amphibians to humans. The discrepancy in the regulations of states that banned potentially dangerous or harmful animals from childcare centers but that did not also specifically ban reptiles and amphibians was paradoxical, considering the well-recognized risk that these animals pose for transmitting *Salmonella* spp. We do not know how many childcare centers across the United States currently house reptiles or amphibians. However, our data suggest that there is room for revision of the regulations in many states which could in turn augment efforts to prevent *Salmonella* spp. transmission from reptiles and amphibians. We believe that the recommendations issued by the CDC for the prevention of salmonellosis from reptiles and amphibians (*4*) could serve as a practical guide as state regulations are updated. Our own experience has indicated that greater collaboration between public health organizations and the agencies responsible for setting regulations for childcare centers can be informative and productive. Similarly, state agencies can work with the pet industry and childcare centers to develop approaches that are mutually beneficial.

Although pets provide many benefits to humans, particularly during the early years of life *(9)*, any exposure that children have to animals must pose minimal risk to the children's health. Ultimately, keeping reptiles and amphibians out of childcare centers and requiring that staff and children wash their hands after touching animals offers a simple way to better safeguard the health of children while having a minimal effect on practices of childcare centers.

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Table. State regulations for contact between children and animals in childcare centers, United States, 2011

Description of state regulation	No.(%) states
Bans all animals that show evidence of disease from childcare centers	22 (44)
Bans all potentially dangerous or harmful animals from childcare centers	23 (46)
Bans all reptiles from childcare centers	12 (24)
Bans all amphibians from childcare centers	3 (6)
Requires staff and/or children to wash hands after handling animals	25 (50)

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Neil M. Vora, Kristine M. Smith, Catherine C. Machalaba, and William B. Karesh

Author affiliations: Columbia University, New York, New York, USA (N.M. Vora); and EcoHealth Alliance, New York (N.M. Vora, K.M. Smith, C.C. Machalaba, W.B. Karesh)

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Address for correspondence: Neil M. Vora, 460 W 34th St, 17th Floor, New York, NY 10001-2320, USA; email: neilvora@gmail.com

Wild Boars as Hosts of Human-Pathogenic *Anaplasma phagocytophilum* Variants

To the Editor: Michalik et al. (*1*) reported a 12% prevalence of *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis and tick-borne fever of ruminants, in wild boars in Poland. *A. phagocytophilum* has been reported with low prevalence among wild boar in the Czech Republic, Slovenia (*2*), and Japan (*3*). In Spain and Mississippi, United States, *A. phagocytophilum* in wild boars or feral pigs, respectively, has not been reported (*4*,*5*). Furthermore, in Slovenia and Poland, the *A. phagocytophilum* gene sequences found in samples from wild boars were identical to those found in samples from humans and the tick vector *Ixodes ricinus* (*1*). These results suggested, as pointed out by Michalik et al. (*1*), that wild boar might play a role in the epizootiology of *A. phagocytophilum* by serving as a natural reservoir host, at least in some regions.

To test this hypothesis, we conducted transcriptomics studies to characterize host response to *A. phagocytophilum* infection in naturally and experimentally infected boars (*6*,*7*). The results suggested that boars are susceptible to *A. phagocytophilum*, but are able to control infection, mainly through activation of innate immune responses and cytoskeleton rearrangement to promote phagocytosis and autophagy. Control of *A. phagocytophilum* infection in boars might result in infection levels below PCR detection or infection clearance, contributing to the low percentage of infection prevalence detected for this species in most regions.

The low detection levels suggest that boars have a low or no impact as a reservoir host for *A. phagocytophilum*. Even if boars remain persistently infected with *A. phagocytophilum* at low levels by downregulating some adaptive immune genes and delaying the apoptotic death of neutrophils through activation of the Jak-STAT pathway, among other mechanisms (*6*), their role as a source of infection for ticks remains to be demonstrated.

José de la Fuente and Christian Gortazar

Author affiliations: Instituto de Investigación en Recursos Cinegéticos, Ciudad Real, Spain (J. de la Fuente, C. Gortazar); and Oklahoma State University, Stillwater, Oklahoma, USA (J. de la Fuente)

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Address for correspondence: Jose de la Fuente, Department of Veterinary Pathobiology, College of Veterinary Medicine, 250 McElroy Hall, Oklahoma State University, Stillwater, OK 74078, USA; email: jose.de_la_fuente@okstate.edu

Migratory Birds, Ticks, and Crimean-Congo Hemorrhagic Fever Virus

To the Editor: In a recently published study, Estrada-Peña et al. reported the finding of Crimean-Congo hemorrhagic fever virus (CCHFV) in adult *Hyalomma lusitanicum* ticks from red deer (*Cervus elaphus*) in Spain during 2010 (*1*). Phylogenetic analysis showed that the virus was most likely of African origin. Here, we present a model for the transfer of CCHFV-infected ticks by migratory birds from Africa to Europe.

CCHFV is an RNA virus in the genus *Nairovirus*, family *Bunyaviridae*. It is transmitted to humans through tick bites or by contact with blood or tissues from infected ticks, livestock, or humans. Manifestations of severe cases are internal and external hemorrhages and multiorgan failure; the case-fatality rate is ≈30% (*2*,*3*). CCHFV has the widest geographic distribution of any tick-borne virus, encompassing ≈30 countries from eastern China through Asia, the Middle East, and southeastern Europe to Africa (*3*,*4*). During the past decade, the virus has emerged in new areas of Europe, Africa, the Middle East, and Asia and has increased in disease-endemic areas (*5*) (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0718-Techapp.pdf).

In response to the emergence of CCHFV in Europe, during spring 2009 and 2010, we screened migratory birds for ticks as they traveled from Africa to Europe. At 2 bird observatories on the Mediterranean Sea (Capri, Italy, and Antikythira, Greece), 14,824 birds of 78 different species were caught and examined for ticks. Most (88%) of the 747 collected ticks were identified as members of the *Hyalomma marginatum* complex, most probably *H. rufipes* and *H. marginatum* sensu stricto (s.s.), i.e., the principal vectors of CCHFV (*2*). Of 10 morphologically representative ticks, 9 were identified by molecular methods as *H. rufipes* and 1 as *H. marginatum* s.s. (*6*).

Ticks belonging to the *H. marginatum* complex are common in large parts of the African and Eurasian continents. The immature ticks feed mainly on birds and, to a lesser extent, on small mammals, whereas the adults actively seek larger mammals, including hares, wild and domesticated ungulates, or humans (*4*). In accordance with this pattern, 99% of the collected ticks in our study were larvae and nymphs.

On April 23, 2009, a woodchat shrike (*Lanius senator senator*) was caught at the Antikythira Bird Observatory in the Greek archipelago. The bird was a female in her second calendar year and harbored 19 *H. marginatum* complex ticks (3 larvae and 16 nymphs, most likely *H. rufipes*). Three of the nymphs, 1 half-fed and 2 fully engorged, were found positive by real-time PCR for the CCHFV small (S) segment by using methods previously described (*7*), amplifying a 127-bp product. The 3 positive samples were sequenced and found to be identical. Previous studies, based on the S segments, have identified 7 phylogenetically distinct genotypes: Africa 1–3, Asia 1–2, and Europe 1–2 (*8*). Europe 1 has been reported from Russia, Turkey, Greece, Bulgaria, and the Balkans, and Europe 2 is the nonpathogenic strain AP92 found in Greece. Alignment of the Antikythira strain with CCHFV S segment sequences deposited in GenBank showed that it had the greatest similarity with strains belonging to the genotype Africa 3 (*8*). In addition, a phylogenetic tree clearly places the Antikythira sequence within the Africa 3 clade (Figure).

The woodchat shrike winters in a belt from Senegal to Somalia and breeds in southern Europe and northern Africa (*9*). The Antikythira

bird was caught during its rapid northward migration on a small island where birds normally stop over briefly just after crossing the Sahara Desert and the Mediterranean Sea. Also, the infected ticks were either half fed or fully engorged nymphs that, in the case of *H. marginatum* complex ticks, normally attach to the bird as larvae; this finding indicates that these ticks had attached before the bird began migration. Furthermore, 9/10 morphologically representative ticks were identified by molecular methods as *H. rufipes*, a species within the *H. marginatum* complex most common on the African continent (*4*,*6*). On the basis of these findings, we propose that this bird was infested somewhere in sub-Saharan Africa.

Migratory birds acting as long-distance transporters of ticks containing various human pathogens have been reported (*10*). Pre-adult ticks can stay attached to avian hosts during migration, thereafter detaching at breeding or stopover sites, where mammalian hosts can potentially establish new foci (*4*). Regarding the finding in Spain (*1*), one could speculate that new cycles of CCHFV transmission could be initiated through viremic or nonviremic (cofeeding) mechanisms involving, for example, transstadially infected adult *H. rufipes* ticks and susceptible *H. lusitanicum* ticks that are feeding on the same mammalian host.

Further research is needed on the interaction between birds and ticks in relation to the geographic distribution of CCHFV. Monitoring the influx of migratory birds carrying CCHFV-infected ticks might give disease-prevention authorities a useful tool for predicting the potential emergence of new disease foci in Europe.

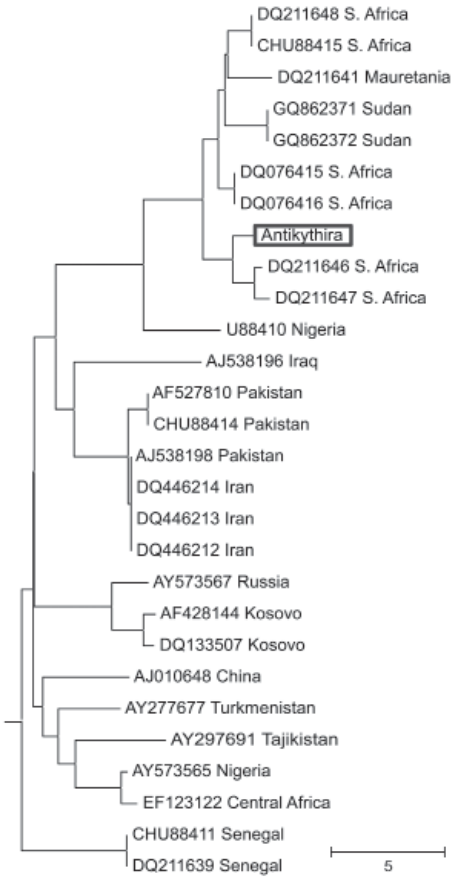

Figure. Neighbor-joining tree of Crimean-Congo hemorrhagic fever virus small segment sequences retrieved from GenBank and the novel 127-bp sequence isolated in this study (boxed). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. A total of 127 positions were in the final dataset. Trees generated with maximum-likelihood and maximum-parsimony methods (not shown) exhibited nearly identical topology to this tree. The corresponding part of the Nairobi sheep disease virus small segment was used as an outgroup. The analyses were conducted in MEGA5 software (www.megasoftware.net) using a ClustalW alignment. Accession numbers and geographic origins of the sequences are shown. Scale bar indicates number of base differences per sequence. S. Africa, South Africa.

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Mats Lindeborg, Christos Barboutis, Christian Ehrenborg, Thord Fransson, Thomas G.T. Jaenson, Per-Eric Lindgren, Åke Lundkvist, Fredrik Nyström, Erik Salaneck, Jonas Waldenström, and Björn Olsen

Author affiliations: Uppsala University, Uppsala, Sweden (M. Lindeborg, C. Ehrenborg, T.G.T. Jaenson, E. Salaneck, B. Olsen); Hellenic Ornithological Society and Natural History Museum of Crete, Crete, Greece (C. Barboutis); Swedish Museum of Natural History, Stockholm, Sweden (T. Fransson); Linköping University, Linköping, Sweden (P.-E. Lindgren, F. Nyström); Swedish Institute for Infectious Disease Control, Solna, Sweden (A. Lundkvist); and Linnaeus University, Kalmar, Sweden (J. Waldenström)

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Address for correspondence: Erik Salaneck, Department of Medical Sciences, Section of Infectious Diseases, Uppsala University, Ing 33 75185, Uppsala, Sweden; email: erik.salaneck@medsci.uu.se

Lucas Cranach the Elder (1472–1553) *Cardinal Albrecht of Brandenburg as St. Jerome* (1526) Oil on wood panel (114.9 cm × 78.9 cm) (detail) John and Mable Ringling Museum of Art, The State Art Museum of Florida, a division of Florida State University, USA

Why Are We by All Creatures Waited on?

Polyxeni Potter

Painter, engraver, designer of woodcuts, Lucas Cranach the Elder lived and worked during the last part of the Renaissance and embodied the integrative qualities valued by his age. In addition to accomplished artist, he was a successful entrepreneur, civic leader, and brilliant inventor. Among other innovations, he is credited with influencing the Danube school, a circle of painters along the Danube Valley, known for their advanced painterly style, printmaking, and etching; for early printing of woodcuts in color; for the full length portrait as an independent art form; and for various techniques intended to speed up painting and standardize technical processes.

Cranach's early years and travels are sketchy. He was born in Germany's Upper Franconia, the small town of Kronach, from which he took his name. He probably received the first art training from his father, the painter Hans Maler. A contemporary of two major artists from Germany, Matthias Grünewald and Albrecht Dürer, Cranach competed with them on local projects and patronage and often turned especially to Dürer's work for inspiration. At age 30, he moved to Vienna, where he made influential associations with local humanists and painted two of his finest portraits. Some religious works showing appreciation of the beauty of nature, a characteristic of the Danube School, also date from this period. Soon he moved to Wittenberg to serve as court painter to Frederick the Wise, elector of Saxony, a position he retained for life under various electors.

Cranach became "one of the wealthiest burghers in Wittenberg." He was elected city councilman several times and mayor twice, while fully engaged in painting, engraving, and directing all aesthetic needs of the court. He established and ran a prosperous studio that produced copies of his best works and all manner of decorative arts, from coin designs for the electorate to furniture. His sons, Hans and Lucas Cranach the Younger, both artists, worked in this studio and used his style so successfully that it is still difficult to fully authenticate what was done by his hand alone. The enterprise continued for decades after his death. His business acumen was such that he also ran a publishing press and had licenses to sell wine and own an apothecary.

Cranach's greatest artistic contribution was his landscapes, which included elaborately detailed animals. He added to period art and Dürer's naturalism an element of fantasy through the ornate treatment of forms. He also painted female nudes, whimsical mythologic scenes, and religious images containing contemporary everyday features. He is well remembered for his portraits, now a repository of the major figures of his age. He left behind perhaps the best portraits of Martin Luther. Other commemorated notables included Luther's family and, despite Cranach's own commitment to Protestantism, many Catholic clergy despised by Luther.

Cardinal Albrecht of Brandenburg as St. Jerome, on this month's cover, is a culmination of many elements in Cranach's work. This type of portrait, borrowing the image of a respected person to elevate that of another, was not unusual at this time. Dürer and others practiced it successfully, and many a churchman honored it. Invoking the virtues and protection of the saint in this portrait was Cardinal Albrecht, elector and archbishop of Mainz (1490–1545), a patron of artists and intellectuals as well as defender of the faith during the Counter-Reformation. Accused of extravagance and worldliness, he commissioned this portrait, in the guise of a religious icon, to proclaim his own beliefs and values. An admirer of St. Jerome, he wished to be likened to him. This revered saint, the most learned man of his age, was known not so much for his asceticism, which was without blemish, but for his knowledge—translating the Bible from Greek and Hebrew into Latin, a crowning literary achievement even by today's standards.

At the time of St. Jerome (342–420), there were no cardinals in the Catholic Church. The iconography that would come to define the saint "… sitting in a chair, beside him that hat which cardinals wear nowadays and at his feet the tame lion," was put forth by Giovanni d'Andrea, a canonical lawyer at the University of Bologna, in a biography of

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

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the saint and was perpetuated in later accounts. The lion legend behind the imagery goes all the way back to Aesop, but with the saint as the figure removing a thorn from the lion's paw and forever gaining the beast's loyalty and affection.

Jerome was a popular subject in art. Cranach alone painted at least eight images of the saint. The one on this month's cover was patterned after Dürer's effort on the same theme, a mirror image. Same small space, a cabinet or private room serving as a study or retreat—its mathematical perspective focusing on the main figure and the positioning of objects making the space appear larger; same ample window, allowing light and shadow effects; same antler-laden chandelier. Cranach and Dürer were colleagues, though Jerome is Cranach's patron, the all-powerful cardinal. Somber, dour even, he sits ambiguously at the lectern, on a contemplative break from reading. His hat is shown in the foreground.

The painting is a treasure trove of symbolism, from the religious artifacts on the wall and in the cupboard behind the saint to the books, cups, fruit, hourglass, slippers, and other objects on and around the desk; and finally the animals, an unlikely menagerie, intended to symbolize characteristics laudable to the patron: monogamy, industriousness, frugality, loyalty, rejection of earthly desires. Thrown in with the other objects, the animals seem posed and indifferent to each other and their surroundings.

Despite the naturalism so valued in the art of Cranach's time and his own interest in painting them, the animals in this portrait of St. Jerome remain just icons of human values. Stylized and scattered at the feet of the saint, they show none of their individuality or wildness. John Donne (1572–1631), a cleric as well as poet of the ages, aware of this two-dimensional treatment of animals, and not only in art, addressed what he saw as the inherent unfairness in perceiving them as strictly serving the interests of humans. In one of his holy sonnets, he asked outright, "Why are we by all creatures waited on?" and why do these creatures that are "more pure than I," since they "have not sinned," "provide food to me?"

Images of animals in art have changed, becoming more accurate and refined as humans were able to travel the world, see them in their natural habitat and study their anatomy and physiology. And the way animals are perceived by humans has changed science. Donne's questions may not be entirely resolved but are eased by current understanding of animal–human phylogenetic closeness and knowledge of the zoonoses. Less an adversarial "them or us" issue, this current relationship is one of connectivity and sharing, on the physiologic as well as the emotional level. Many animals have gone from simply being domesticated to becoming members of human families and valued companions as pets. Others have come closer to humans as urbanization closed in on their habitat, and yet others have traveled far from their original nests. They all, too, on the zoonotic level, in the home and in the wild, share their infections freely and interact with humans outside traditional areas of exposure.

In this issue of the journal, human Hendra virus infection was acquired by close contact with horses infected by spillover from fruit bats, the natural reservoir for these viruses. MRSA organisms harboring a novel variant were detected in cats and dogs, which suggests that the variant is not restricted to human hosts. In Bangladesh, where HPAI H5N1 is endemic in poultry, live bird markets are a factor in human exposure. In the United States, agricultural fairs have been associated with bidirectional, influenza virus transmission between swine and humans. Fairgoers without routine occupational exposure to swine not only may be more susceptible to swine influenza viruses than those routinely exposed, they may also expose swine to a broader range of influenza A viruses for additional mixing. Identifying risk factors for transmission in both these venues would represent a step toward effective control.

Like Cranach's animals, these human–animal interactions are symbolic of other, larger values, not moral and religious but biologic. Because, despite their surface unrelatedness, the interactions described in reports from around the world tie into one important common denominator, their zoonotic potential.

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Address for correspondence: Polyxeni Potter, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D61, Atlanta, GA 30333, USA; email: pmp1@cdc.gov

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To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to **www.medscape.org/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Farm Animal Contact as Risk Factor for Transmission of Bovine-associated *Salmonella* Subtypes

CME Questions

1. You are seeing a 40-year-old woman with a 2-day history of diarrhea, fever, and abdominal pain. Initial results from a stool culture demonstrate *Salmonella* species. What should you consider regarding the epidemiology of *Salmonella* infections?

A. The incidence of *Salmonella* infections has gradually declined over the past 15 years
B. Approximately half of *Salmonella* infections are due to contaminated food
C. Fecal contamination of beef carcasses at the time of slaughter may result in foodborne transmission of *Salmonella*
D. Milk is an even more important means of transmission of *Salmonella* than is meat

2. Which of the following statements regarding characteristics of cases and controls in the current study is most accurate?

A. The average age of patients was slightly over 30 years
B. There were as many cases with bovine-associated *Salmonella* as there were controls with non–bovine-associated *Salmonella*
C. The most common serovar among cases was Enteritidis
D. The most common serovar among controls was Typhimurium

3. You take this patient's history for possible exposure to *Salmonella*. Which of the following factors was most significantly associated with bovine-associated *Salmonella*?

A. Consuming undercooked ground beef
B. Contact with farm animals
C. Drinking unpasteurized milk
D. International travel

4. The patient confirms that she has had some exposure to animals. Which of the following factors was most important in promoting an increased risk for bovine-associated *Salmonella* in the current study?

A. Attendance at a petting zoo or animal fair
B. Contact with animal manure
C. Working in veterinarian's office
D. Exposure to cows specifically during the past 5 days

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to **www.medscape.org/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for *AMA PRA Category 1 Credits*™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Enterovirus 71–associated Hand, Foot, and Mouth Disease, Southern Vietnam, 2011

CME Questions

1. You are seeing a 3-year-old girl with a two-day history of fever. She developed oral ulcers this morning, and you suspect that she has hand, foot, and mouth disease (HFMD). In the current study, what virus type was associated with the majority of cases of HFMD requiring hospitalization?

A. Enterovirus 71
B. Coxsackievirus A16
C. Echovirus 13
D. Poliovirus 2

2. The patient is treated with supportive care but returns to your clinic the next day when her mother notes myoclonus in her fingers. While the patient still has fever and a rash, there are no other symptoms or physical signs. According to the clinical grading system used in the current study, what grade of HFMD does this patient now have?

A. Grade 1
B. Grade 2
C. Grade 3
D. Grade 4

3. What should you consider regarding the clinical presentation of cases of HFMD in the current study?

A. Most children were over 5 years old
B. The mortality rate exceeded 10%
C. The average time for progression to most severe illness was 5 days
D. Children with caregiver-reported myoclonus had a low rate of clinical progression

4. The patient goes on to develop multiple complications of HFMD. What treatments were used for the associated complications in the current study?

A. Myoclonus — diazepam
B. Seizures — carbamazepine
C. Fever unresponsive to antipyretics — IVIg
D. Hypertension — beta blockers

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Listeriosis Outbreaks and Associated Food Vehicles, United States, 1998–2008

Influenza A(H1N1)pdm2009 in the Southern Hemisphere, Winter 2009

Novel Framework to Assess Epidemiologic Effects of Influenza Epidemics and Pandemics

20 Years of Automated Biosurveillance Data, England and Wales

Changing Incidence and Clinical Spectrum of Staphylococcal Infections in Children, California, 1985–2009

Microevolution of Highly Pathogenic Avian Influenza Virus A (H5N1) in Humans, Egypt, 2007–2011

Pneumocystis jirovecii and Increased Death Rate in HIV-infected Patients with Pneumonia

Novel *Listeria monocytogenes* Strains from Cantaloupe-related Listeriosis Outbreak, United States, 2011

Routine Pneumococcal Conjugate Vaccination and Invasive Pneumococcal Disease in Children, England and Wales

Novel Polyomavirus associated with Brain Tumors in Free-Ranging Raccoons, Western United States

Risk Factors for Nipah Virus Infection among Pteropid Bats, Peninsular Malaysia

Full-Genome Characterization of Rat Hepatitis E Virus Strain Isolated in Vietnam

Sheep-to-Human Transmission of Orf Virus during Eid al-Adha Religious Practices, France

Fatal Adenovirus 14 Infection Associated with Other Severe Illnesses, Canada

Puumala Virus Infections and Cardiovascular Causes of Death

Rinderpest Virus Sequestration and Usage in the Post eradication Era

Hepatitis E Virus Genotype 4 Outbreak, Italy, 2011

Complete list of articles in the January issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

December 1–7, 2012
ASLM's First International Conference
Cape Town, South Africa
http://www.aslm.org

2013

February 15–18, 2013
4th International Meeting on Emerging Diseases and Surveillance (IMED)
Vienna, Austria
http://imed.isid.org

February 25–28, 2013
2013 ASM Biodefense and Emerging Diseases Research Meeting
Washington, DC
http://www.asmbiodefense.org

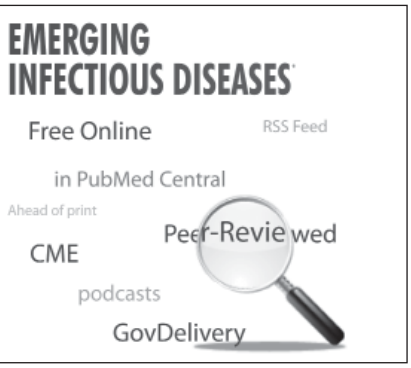
March 3–7, 2013
The Conference on Retroviruses and Opportunistic Infections (CROI) 2013
Georgia World Congress Center
Atlanta, GA, USA
http://www.retroconference.org

September 5–10, 2013
Options for the Control of Influenza VIII
Cape Town, South Africa
http://www.isirv.org

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.



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The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit figures in black and white. If you wish to have color figures online, submit both in black and white and in color with corresponding legends. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpeg or .tif files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.