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## Zoonoses

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# EMERGING INFECTIOUS DISEASES®





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# EMERGING INFECTIOUS DISEASES®

**Zoonoses** **June 2018**


## On the Cover

**Vincenzo Campi (1530/1535–1591), *Kitchen* (*Cucina*), 1580** (detail). Oil on canvas, 57 in x 87 in/145 cm x 220 cm). Pinacoteca di Brera, Milan, Italy.

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## Perspective



## Synopsis



## Research

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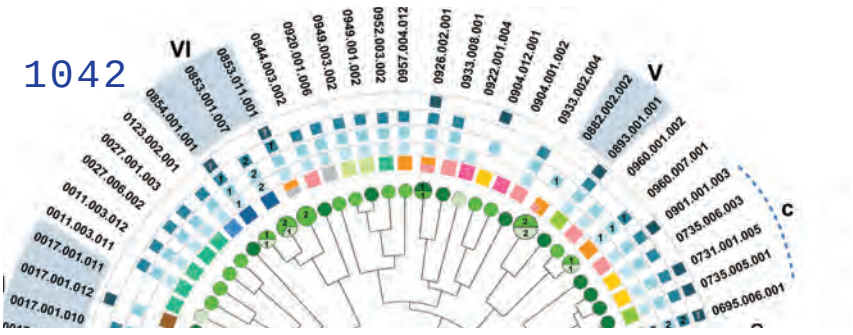


## Dispatches



## Research Letters

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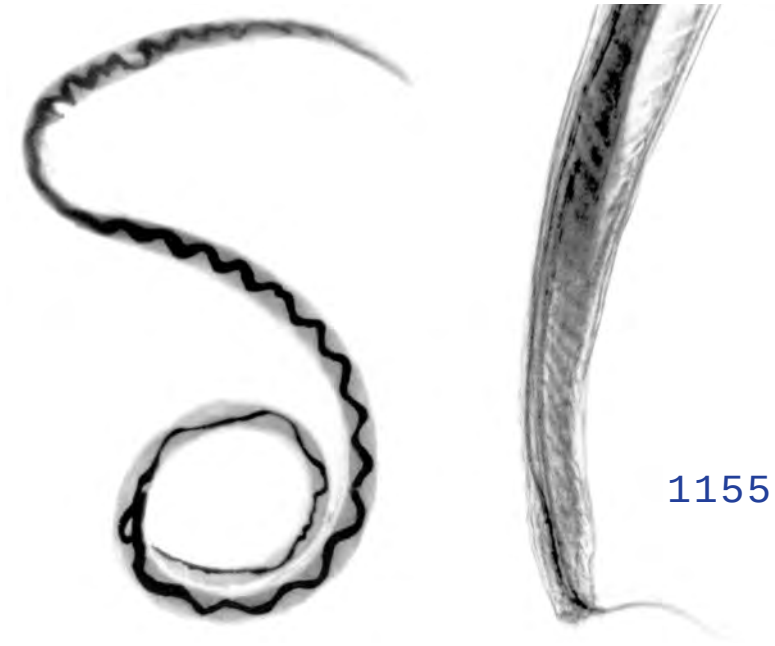
## Letter



## About the Cover







## Etymologia



## Corrections



**Vol. 23, No. 12**

The timing of detection of Crimean-Congo hemorrhagic fever virus in Crimea and the Democratic Republic of the Congo were unclear in Phylogenetic Characterization of Crimean-Congo Hemorrhagic Fever Virus, Spain (E. Ramírez de Arellano et al.).

Patient data were inaccurate and definition of contact categories unclear in Lack of Secondary Transmission of Ebola Virus from Healthcare Worker to 238 Contacts, United Kingdom, December 2014 (P. Crook et al.).

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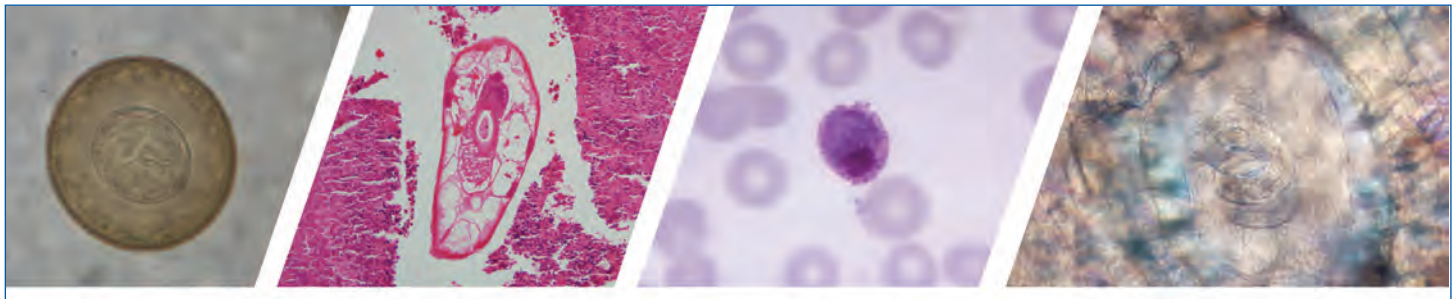
*Amblyomma mixtum* ticks were misidentified as *A. sculptum* in *Rickettsia africae* and Novel Rickettsial Strain in *Amblyomma* spp. Ticks, Nicaragua, 2013 (H. Vogel et al.).

The affiliation of author Pierre Zalloua was listed incorrectly in Containment of Highly Pathogenic Avian Influenza A(H5N1) Virus, Lebanon, 2016 (Z.E. Farah et al.).

Some data were inaccurate in the text and figures in Spread of Meropenem-Resistant *Streptococcus pneumoniae* Serotype 15A-ST63 Clone in Japan, 2012–2014 (S. Nakano et al.).



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# Ferrets as Models for Influenza Virus Transmission Studies and Pandemic Risk Assessments

**Jessica A. Belser, Wendy Barclay, Ian Barr, Ron A.M. Fouchier, Ryota Matsuyama, Hiroshi Nishiura, Malik Peiris, Charles J. Russell, Kanta Subbarao, Huachen Zhu, Hui-Ling Yen**

The ferret transmission model is extensively used to assess the pandemic potential of emerging influenza viruses, yet experimental conditions and reported results vary among laboratories. Such variation can be a critical consideration when contextualizing results from independent risk-assessment studies of novel and emerging influenza viruses. To streamline interpretation of data generated in different laboratories, we provide a consensus on experimental parameters that define risk-assessment experiments of influenza virus transmissibility, including disclosure of variables known or suspected to contribute to experimental variability in this model, and advocate adoption of more standardized practices. We also discuss current limitations of the ferret transmission model and highlight continued refinements and advances to this model ongoing in laboratories. Understanding, disclosing, and standardizing the critical parameters of ferret transmission studies will improve the comparability and reproducibility of pandemic influenza risk assessment and increase the statistical power and, perhaps, accuracy of this model.

The susceptibility of ferrets to influenza virus infection has been known for nearly a century. Ferrets and humans share similarities in lung physiology, cellular receptor distribution, and clinical signs of infection, making the ferret an attractive small mammalian model for laboratory study of influenza viruses (*1*). Influenza virus infection in ferrets emulates the severe disease elicited by highly pathogenic avian influenza viruses in humans and the transmissibility of seasonal human influenza viruses via respiratory droplets. Commonly reported experimental setups for the study of influenza virus transmission in ferrets include co-housing influenza virus–infected and uninfected ferrets (previously termed the direct contact model) or physically separating virus-infected and uninfected ferrets (previously termed the respiratory droplet model or the airborne transmission model) (*1*,*2*). In the co-housing design, transmission between ferrets can be mediated by any of the multiple routes that facilitate influenza virus transmission, including direct contact, indirect contact via fomites, or via respiratory droplets (airborne particles with >5 μm aerodynamic diameter) and droplet nuclei (airborne particles with <5 μm aerodynamic diameter). In the physical separation design, direct or indirect contact between donor and recipient ferrets is precluded by separating cages with a side panel or cage walls that permit air exchange between cages but prevent direct contact between ferrets. If the recipient ferret becomes infected with influenza virus, respiratory droplets and droplet nuclei expelled from the donor ferret represent the only possible source of transmission. Many influenza viruses that cause zoonotic infections in humans (e.g., most swine influenza viruses, avian influenza viruses of subtype H5N1 and other subtypes) are generally poorly transmitted between ferrets via respiratory droplets and droplet nuclei, whereas viruses associated with seasonal epidemics or pandemics in humans (e.g., influenza A[H1N1]pdm09 virus, the reconstructed 1918 virus) can be transmitted relatively efficiently (*3*–*7*). As such, the ferret model provides a useful tool for research on influenza virus transmission and pandemic risk assessment. Influenza transmissibility between ferrets is a parameter included in tools for assessing the potential pandemic risk for zoonotic influenza viruses: the Influenza Risk Assessment Tool (*8*) and the World Health Organization Tool for Influenza Pandemic Risk Assessment (*9*).

Recent experimental studies performed by using ferret transmission models have greatly expanded knowledge of influenza virus transmissibility. Among other findings, these studies have identified molecular correlates and determinants of airborne virus spread, differential innate host

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responses between viruses with distinct transmissible phenotypes, and the role of vaccination and antiviral administration in mitigating virus spread to susceptible contacts (*2*,*10*–*19*). Although the ferret model does not always recapitulate virus transmissibility observed among humans, possibly because of differences in prior exposure history and other unidentified host factors, these studies, in isolation and in tandem with additional laboratory experiments, have improved our ability to perform informative risk assessments of novel and emerging influenza viruses. For example, the limited transmissibility of influenza A(H7N9) viruses via airborne particles in ferrets, in the absence of sustained human-to-human transmission, indicates that the pandemic threat posed by this virus subtype is relatively higher than that of other studied avian influenza viruses; further refinements to the experimental model may help explain why this virus does not currently spread readily between humans.

Despite the growing use of influenza virus transmission studies in the field, there is a wide and often underappreciated heterogeneity among these studies with regard to assessing influenza virus transmissibility via airborne particles in the ferret model. This heterogeneity includes host-specific, virus-specific, and environmental/laboratory-specific variables (Table 1) that are a help and a hindrance for understanding the relative risk for virus transmission with a particular virus strain or subtype (*1*). Robust data can be generated by several independent research groups performing parallel studies with genetically similar but not identical field viruses. Such an approach reduces potential strain-specific or method-specific biases. Conversely, each experimental variable may restrict or complicate direct comparisons of data from different research groups or institutions, depending on each variable's effect on the transmission outcome, making it impossible to combine data.

**Table 1.** Examples of heterogeneity in experimental designs among published risk-assessment studies using ferrets as models for influenza virus transmission studies and pandemic risk assessments*

| Parameter | Examples of variability |
|---|---|
| Virus (before ferret introduction) | Seed stock passage history, stock growth matrix, stock titer, wild-type vs. reverse genetics, plaque-purified vs. quasispecies, storage and propagation conditions |
| Ferret (before virus introduction) | Source/genetic lineage, serostatus, age, sex, weight, neutered or intact status, hormonal treatment (females), anesthetic used, housing conditions |
| Virus inoculation | Inoculation route, method, dose, and volume; buffer for dilution |
| Transmission experimental designs | Donor:recipient ratio, number of replicates per containment, caging size and setup, perforation size and exposure area between cages, distance between cages, directional airflow, air changes per hour, temperature and humidity, timing and duration of exposure, frequency and sites for sample collection |

*References for individual studies using these conditions are described in (*1*).

Current knowledge about viral, host, and environmental factors that may drive transmission is limited. To facilitate interpretation of data generated in different laboratories, efforts should be made to improve transparency in descriptions of methods and to better differentiate what constitutes a risk-assessment activity versus a research activity. After the Transmission of Respiratory Viruses conference, held June 19–21, 2017, in Hong Kong (*20*), an ancillary workshop was held on June 22, 2017, to discuss this topic.

Our article serves as a starting point for highlighting potential heterogeneity in ferret transmission experimental designs for future refinement. It is not intended as a policy statement for detailed recommendations on ferret experimental designs, when the effects of many of these variables are not fully understood. We discuss and summarize variables in the ferret transmission model.

## Identifying Variability

Major drivers of variability in studies of influenza virus transmissibility include differences in virus strain, dose, and inoculation route (*21*–*23*). Even when a common virus strain is used, results are potentially affected by the passage condition (e.g., multiplicity of infection), passage history (in embryonated chicken eggs or in mammalian cells), and storage condition of the virus. Risk-assessment studies are particularly challenging because the evaluations are conducted with recently isolated specimens that are not available from a central respository before animal inoculation; variation pertaining to input stock material is possible even when different laboratories have confirmed sequence identity of the same virus undergoing evaluation. Furthermore, several other factors have not been directly demonstrated but are likely to contribute to experimental variation in transmission studies: cage design, air flow direction, air changes per hour, facility-specific temperature and humidity levels, and others (Table 1) (*1*). Beyond these, additional variables, which are largely out of the control of researchers, include ferret suppliers (commercial or hobby and the quantity of ferrets available from each), country-specific animal welfare issues, institutional animal care and use committee guidelines, and pharmaceutical limitations (availability or restriction of anesthetics licensed for use).

Although some factors are considered to be controllable, much of the variability between groups cannot be easily overcome. For example, the absence of commercially available uniform caging for ferret transmission experiments (a reflection of country- and institution-specific size regulations and facilities constraints) represents a parameter that would be difficult to standardize. However,

a greater understanding of the relative role and contribution of different variables can improve our ability to better contextualize and interpret results among laboratories. For example, does the virus dose affect transmissibility or otherwise influence detection of transmissible quasispecies in contact ferrets? Gustin et al. previously demonstrated the potential effects of the dose and the route of inoculation (intranasal vs. aerosol) on transmission potential (*22*), suggesting the need to standardize these 2 parameters for risk-assessment studies. Similarly, the use of directional airflow or air changes per hour in ferret housing apparatuses is not always specified in reports of risk-assessment results. Anecdotally, these variables seem to play a role in modulation of virus transmissibility, and they should be examined systematically to ascertain which parameters (including but not limited to those shown in Table 1) would benefit from standardization, where possible, across laboratories in the field. This standardization and interpretation can take place only when all known major drivers of laboratory variability that influence virus transmissibility are described along with the results.

As discussed at the workshop, the participating laboratories analyzed the protocols used for evaluation of influenza virus transmissibility via airborne particles in the ferret model, which highlighted the breadth of experimental designs. It was also clear that all variables that are probable contributors to differential results are not routinely disclosed in peer-reviewed publications or other platforms where results are discussed. Lack of disclosure of all variables can complicate the ease of comparing findings between laboratories, warranting a closer look at the feasibility of using a more comparable study design between multiple laboratory groups for risk-assessment studies. As such, a push toward comprehensive description of specific experimental conditions would aid this effort and would probably be valuable when risk assessments performed in different laboratories are compared.

## Defining Risk Assessment

Beyond the experiment- and facility-based variability we describe, the lack of a standard protocol for the number of experimentally infected animals and the number of recipient ferrets (donor:recipient ratios) included in ferret transmission studies can affect the ability to interpret results among groups and represents a substantial controllable parameter. The ideal standard for risk assessment activities seems to be a 1:1 donor:recipient experimental setup to assess virus transmissibility via the airborne route, where each virus-infected ferret is exposed to only 1 recipient ferret. This design facilitates ease of interpretation of results and provides added rigor from a statistical perspective. This design also restricts exposure to virus-laden particles expelled from each donor ferret to its respective recipient, ensuring that any detected transmission event would have originated from exposure to separate donors. However, because of space limitations, these experiments are often conducted in replicates inside a single physical containment area with a shared ventilation system (i.e., housing multiple pairs of donors and respiratory droplet recipients in separate cages with shared air) while still maintaining a 1:1 donor:recipient ratio. If transmission is mediated by virus-laden particles expelled by donors, increasing the number of donors within a single containment area is likely to increase the concentration of virus-laden particles in the air, thereby increasing the observed transmissibility. Specifically, it is not known whether transmission kinetics would be comparable if 3 independent experiments were performed with 1 donor to 1 recipient (each ferret housed singly) versus 1 experiment with 3 donors and 3 recipients per containment area. Air sampling devices that allow monitoring of the quantities of virus-laden particles in the air throughout the experiment would help refine the experiment outcomes and are likely to become part of these experimental designs in the future.

Further impeding efforts to compare results among laboratories, many experiments include an additional contact ferret co-housed with an experimentally infected ferret to evaluate virus transmissibility in a direct contact setting while still assessing the respiratory droplet transmission to a recipient ferret housed in an adjacent cage. In this design, several ferrets may serve as donors (i.e., virus-inoculated ferrets, co-housed ferrets that became infected as a result of direct/indirect contact, or both). Moreover, the donor and direct contact–infected ferrets are likely to shed virus-laden particles at different times, further complicating the results of the transmission experiments. Ideally, the effects of different experimental designs should be investigated in systematic experiments, and researchers should strive to disclose this information as comprehensively as possible.

During workshop discussions, most researchers agreed that it would be helpful for the field to coalesce around a fixed 1:1 donor:recipient ratio (with or without several discrete pairs inside 1 physical containment area) for risk-assessment transmission experiments. Introduction of direct contact ferrets into the experimental setup would probably extend the amount of time that virus-laden particles can be released in the air. Virus amplification by direct contact ferrets may also lead to virus adaptation and emergence of variants with increased respiratory droplet transmission potential. Applying a 1:1 donor:recipient ratio would increase the consistency of the experimental design under which risk-assessment experiments are conducted across multiple laboratories, differentiating them from broader, more heterogeneous research-based assessments that would include more experimental designs and variables. However, individual laboratories have built up datasets and experience

over the years while performing risk-assessment studies with different strains of influenza viruses; thus, adopting a common protocol may be difficult to achieve within a short time.

Although viruses that are readily transmitted by the airborne route will exhibit robust transmission in a direct contact setting, some influenza viruses that are not transmitted efficiently via respiratory droplets are nonetheless transmitted between ferrets placed in direct contact, which facilitates transmission via multiple modes. Studies evaluating virus transmissibility between ferrets placed in direct contact may be influenced by many of the experimental drivers discussed here; when using this model, further contributions to variability are introduced by animal behavior and housing practices. Although scoring for the Centers for Disease Control and Prevention Influenza Risk Assessment Tool includes data derived from the direct contact transmission model in risk assessment, it is not fully clear how to interpret the relative pandemic risk resulting from viruses that transmit in a direct contact setting but not via respiratory droplets. As discussed above, a greater understanding of what confers virus transmissibility in both models will improve our ability to interpret results from more permissive direct contact models with the more stringent respiratory droplet transmissibility. This knowledge will improve our ability to appropriately include and aggregate results from both types of transmission studies in influenza virus risk assessments.

## Limitations of the Ferret Transmission Model

Although the ferret transmission model has greatly improved our understanding of influenza virus transmissibility, there are limits to what this model can contribute to risk assessment and how results are interpreted. In particular, inefficient virus transmission (e.g., when 1 of 3 recipient ferrets becomes productively infected) remains a difficult outcome to understand. It is often unclear whether this event results from genetic changes in the virus during the transmission event, reflects the transmitted infectious dose, or results from other contributing factors; concurrent contextualization of these results with other laboratory paramaters (inclusive of in vivo, in vitro, and aerobiology-based experimentation) can often provide additional insight. Similarly, interpretation of seroconversion in the absence of detectable virus in respiratory secretions or detectable virus in the absence of seroconversion can be difficult. Moving toward a consensus on the implications of inefficient transmission events would be helpful because, currently, efficient and inefficient transmission are not well defined.

Another major limitation of current ferret transmission studies is the small group size, which is driven by cost, size of the animals and their associated housing requirements, and ethical and practical constraints. For this reason, statistical analyses of data from transmission experiments are infrequently performed (*24*,*25*), and repetition of positive-control viruses is not uniformly feasible. Risk assessment studies are often performed with 3–4 replicates of transmission pairs. With this sample size, it is feasible to statistically infer virus transmissibility at the extremes of transmission potential (i.e., virus transmission to 4 of 4 ferrets versus 0 of 4 ferret pairs), but statistical power to compare viruses with intermediate transmissibility (transmissibility to 2 or 3 of 4 pairs) is limited. The opportunity for meta-analyses that combine results from different laboratories could be beneficial, especially for monitoring minor changes in transmission potential of a particular zoonotic virus as it evolves over time. However, meta-analyses can be performed only when experiments use comparable study designs, especially with regard to those parameters known to most dramatically influence virus transmissibility.

## Potential Refinements of the Ferret Transmission Model

Great efforts are being made to reduce the limitations discussed above by using novel and emerging technologies and research-based approaches. For identifying mutations that may have occurred during transmission events, Sanger sequencing has been frequently used. Recently developed technologies (e.g., use of neutral barcodes to individually track influenza viruses in a population) or deep-sequencing approaches have provided, and probably will continue to provide, additional information to aid in the interpretation of inefficient virus transmission events, elucidate transmission bottlenecks, and differentiate between within-group variability and larger differences in experimental setup and design (*26*–*28*). Although incorporating viral genome sequencing in all risk-assessment studies would be beneficial, the inclusion and standardization of these approaches represents a substantial challenge with regard to sample choice for testing (types of samples, dates of sample collection, titers of samples) and institutional restrictions on collection, interpretation, and dissemination of this information.

An additional avenue for improved understanding of virus transmissibility via respiratory droplets are aerobiology-based approaches. These approaches include analysis of the exhaled breath of infected ferrets and the amount and size distribution of virus-containing aerosols released by infected ferrets (*29*–*31*). Although it is unlikely that aerobiology-based information can be incorporated into standard risk-assessment ferret experiments, information gained from these experiments could improve our understanding and interpretation of influenza virus transmissibility in the ferret model, providing additional

data about the contributions of different variables to consistency between laboratories for experiments assessing virus transmission.

In vivo ferret transmission studies are not performed in isolation. The incorporation of these data into larger research efforts has greatly expanded our understanding of the complex determinants of influenza virus transmission in mammals. For example, hemagglutinin acid stability and the hemagglutinin–neuraminidase balance have been linked with virus transmissibility via the airborne route in ferrets, as have receptor binding preference, gene constellation, neuraminidase stalk length, and other parameters (*14*–*17*,*28*,*29*). In addition, studies examining the relative effects of environmental temperature and relative humidity on influenza virus stability and transmissibility (underscoring the need to report this information more specifically in published methods sections) will provide needed information pertaining to the seasonality of influenza virus spread in humans (*32*,*33*). Further refinement of the ferret model concurrent with studies using other modeling approaches, including but not limited to in vitro and ex vivo infection models, will continue to support in vivo transmission risk assessments in this species.

## Moving Forward

The plasticity of the ferret model permits a wide range of experimental approaches to assess influenza virus transmissibility. This plasticity represents a great advantage when designing research experiments to evaluate viral, host, environmental, and other factors that contribute to transmission between mammals. However, it might be beneficial for studies conducted primarily for risk-assessment purposes to be performed under conditions as uniform as possible. For example, moving toward a standardized 1:1 donor:recipient ratio in risk-assessment studies would probably enhance the comparability of results found by different research groups and would enable inclusion in meta-analyses (Table 2).

Current knowledge regarding the viral, host, and environmental parameters that drive transmission outcomes is limited. Understanding these parameters would be beneficial for infection control, and future studies should aim to validate these factors empirically. As data regarding the exact role of each of the potential parameters discussed in this article are developed, improved documentation of variables (Table 1) associated with risk assessments would facilitate comparison of data generated across different

**Table 2.** Features that may be conducive to uniform, reproducible risk-assessment transmission setups when using ferrets as models for influenza virus transmission studies and pandemic risk assessments*

| Property | Rationale | Sample phrasing | Perceived importance |
|---|---|---|---|
| Donor:recipient ratio of 1:1 | Improved statistical rigor, potential application for meta-analysis, and interpretation of results. The number of donor:recipient pairs housed inside containment with shared ventilation should be reported. | "Inoculated ferrets (n = 3) were each placed in a separate cage; 24 hours later, naïve ferrets (n = 3) were each placed in a different cage adjacent to an inoculated ferret." | High |
| Seronegative ferrets | Prior influenza virus exposure history can be difficult to quantify and control. The methods used for assessing prior exposure should be disclosed. | "Ferrets were serologically negative to currently circulating influenza A (H1N1 and H3N2) and B viruses before challenge, as confirmed by HI assay." | High |
| Harmonization of ventilation and environmental conditions | ACH, directional airflow, cage design, humidity/temperature information are reported concurrent with release of results. | "Ferrets were housed for the duration of the experiment in an environmental chamber with HEPA filtration operating at 20 ACH. Airflow velocity was found to be negligible between donor and recipient cages. Ambient temperature (20°–22°C) and relative humidity (40%) were monitored during the experiment." | High |
| Uniform definition of efficient transmissibility | Virus titers (with detection limit) and seroconversion are both required to demonstrate robust transmission event. | "Virus transmissibility was confirmed by detection of infectious virus and by seroconversion to homologous virus in recipient ferrets." | High |
| Dose, volume, and route of inoculation | Dose of inoculum may affect the transmission kinetics (*22*). A consensus within the risk-assessment group may be beneficial. | "Ferrets were inoculated by the intranasal route with $10^6$ PFU of virus in a volume of 500 μL" | High |
| Application of air sampling device to determine the size and quantity of virus-laden particles in air | The results may help correlate and refine the transmission phenotype. | "Variables were inclusive of vendor, duration of sampling, specification of collection matrices (buffers, gelatin[s], etc.), specification of virus confirmation via PCR and/or live virus detection, normalization correction of data (if applicable)." | Intermediate |

*Discussed at workshop held June 22, 2017, ancillary to Transmission of Respiratory Viruses conference held on June 19–21, 2017, in Hong Kong, China (*20*). ACH, air changes per hour; HEPA, high-efficiency particulate air.

laboratories. These should include, but are not limited to, stating the stock passage history and storage conditions; dose, volume, and route of inoculation; donor:recipient ratio (and the number of donor:recipient pairs present within a single containment area); and clarity in describing cage setup (coupled with illustrations when possible for easy understanding of the overall experimental conditions), specifying room and cage humidity/temperature, stating airflow directionality when present and air changes per hour, and specifying the condition of sample storage and processing procedures (e.g., whether samples are titered immediately for presence of infectious virus, or whether they are frozen and thawed before use).

Many unanswered questions are relevant for understanding the pandemic risk posed by novel and emerging influenza viruses that lie outside a standardized risk-assessment experimental setup. Some studies that would provide additional valuable information include modulation of distance between cages when assessing virus transmissibility via the airborne route, shortening the duration of exposure between inoculated donors and contact ferrets (*18*,*28*,*34*), and modifying the donor:recipient ferret ratio. Performing these types of experiments according to a standardized risk-assessment evaluation of virus transmissibility could provide valuable contextual information for a more nuanced risk assessment. Distinguishing between risk assessment and research activities will greatly facilitate interpretation and contextualization of data generated by different laboratories.

A useful exercise might be for several research groups to test and compare results of a transmission experiment by using 1 selected virus strain prepared by 1 laboratory. Many practical considerations would need to be discussed, including the particular strain to test and the anticipated transmission phenotype of this virus. Complete standardization of ferret transmission studies conducted worldwide is not possible; however, exercises that seek to examine the relative contributions of laboratory-specific drivers of experimental variability may identify critical parameters or conditions. Similarly, meta-analyses of published data, with included disclosure of the parameters listed in Table 1, could aid in our understanding of the relative contribution of variables involved in respiratory droplet transmission experimentation in ferrets.

Influenza viruses will continue to jump the species barrier and cause human infection and disease. Virus-transmissibility assessments in the ferret model represent one of numerous activities conducted to aid pandemic preparedness efforts in the event that a pandemic virus does emerge in the future. Continued refinement of the ferret model, concurrent with advances in identification of viral, host, and environmental factors that influence transmission risk (*35*), will facilitate assessments of novel and emerging influenza viruses and aid development of better infection control measures.

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## About the Author

Dr. Belser is a microbiologist at the Centers for Disease Control and Prevention, Atlanta, Georgia. Her research interests include the pathogenicity, transmissibility, and tropism of influenza viruses, including H7 subtype viruses with pandemic potential.

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SYNOPSIS

# Absence of Nosocomial Transmission of Imported Lassa Fever during Use of Standard Barrier Nursing Methods

**Anna Grahn, Andreas Bråve, Thomas Tolfvenstam, Marie Studahl**

Nosocomial transmission of Lassa virus (LASV) is reported to be low when care for the index patient includes proper barrier nursing methods. We investigated whether asymptomatic LASV infection occurred in healthcare workers who used standard barrier nursing methods during the first 15 days of caring for a patient with Lassa fever in Sweden. Of 76 persons who were defined as having been potentially exposed to LASV, 53 provided blood samples for detection of LASV IgG. These persons also responded to a detailed questionnaire to evaluate exposure to different body fluids from the index patient. LASV-specific IgG was not detected in any of the 53 persons. Five of 53 persons had not been using proper barrier nursing methods. Our results strengthen the argument for a low risk of secondary transmission of LASV in humans when standard barrier nursing methods are used and the patient has only mild symptoms.

Lassa fever is a potentially severe viral hemorrhagic illness caused by Lassa virus (LASV). The reported mortality rate is 1% overall but can be up to 15%–20% for hospitalized patients (*1*,*2*). LASV is normally transmitted to humans by ingested or inhaled rodent (*Mastomyces natalensis*) excreta, mainly urine. However, person-to-person transmission can occur by contact with infected body fluids. Treatment with ribavirin has been shown to reduce mortality rates when administered early during infection (*3*) and has been used for postexposure prophylaxis.

The types of personal protective equipment (PPE) and medical facility to use when caring for Lassa fever patients have been discussed (*4*–*6*). In disease-endemic areas, such as Liberia, hospital staff has been reported to have higher prevalence of antibodies against LASV than the general village population (*7*,*8*). However, a prospective study conducted in Sierra Leone (*6*) showed no increased risk for nosocomial transmission when standard barrier nursing methods, including gloves, gowns, and masks with various rates of compliance, were used. In countries to which LASV is not endemic, risk for nosocomial transmission has been reported to be low in persons caring for the hospitalized index patient, even without more special precautions than barrier nursing methods (*9*–*12*). Serologic studies in these countries have not demonstrated nosocomial transmission resulting in infections in healthcare workers (*9*,*10*,*13*).

The recommendations of the US Centers for Disease Control and Prevention are to use barrier nursing methods, including gloves, gowns, masks, and goggles, and an isolation room when caring for a patient with Lassa fever (*14*). The World Health Organization (WHO) recommends gloves, long-sleeved gowns, and face shields or masks and goggles when caring for the patient and being within 1 m of the patient (*15*). In countries to which Lassa fever is not endemic, these patients are cared for mostly in high-level isolation units, at least after a diagnosis is made. However, these units are expensive, labor-intensive, and strenuous for the patient. In addition, the number of patients that can be treated in high-isolation facilities simultaneously is often limited within a country. We investigated whether LASV infection occurred in healthcare workers who used standard barrier nursing methods during the first 15 days of caring for a patient with Lassa fever in Sweden.

## Materials and Methods

### Index Patient

A 72-year-old woman was admitted to Sahlgrenska University Hospital in Gothenburg, Sweden, in March 2016, ten days after onset of fever, nausea, arthralgia, loose stools, and headache, and 2 days after onset of personality changes (*16*). The initial fever had resolved after 3 days and 7 days before hospitalization. The patient and her husband had visited Liberia for 6 weeks and returned to Sweden 5 days before onset of primary symptoms. After more common diagnoses had been ruled out and a hearing deficit developed in the patient, Lassa fever was suspected. Fourteen days after admission, the patient was given a diagnosis of Lassa

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fever after detection of LASV IgG and low titers of LASV IgM; LASV RNA was also detectable by PCR.

The patient had loose stools and vomited on 2 occasions during the first 14 days of hospitalization but had no fever (temperature ≥38°C). She needed help with hygiene issues, including toilet visits. Fifteen days after admission, the day when the diagnosis was confirmed by PCR, she was transferred to a high-level isolation unit. We detected LASV RNA by using PCR in samples from serum, whole blood, urine, and feces obtained during the first 15 days of hospitalization. The highest concentration of LASV RNA detected was $1.2 \times 10^5$ copies/mL. For other characteristics of this patient, see the Figure and Grahn et al. (*16*).

### Contacts

After making a diagnosis for the index patient, 15 days after admission, risk assessment and management of contacts were performed as reported (Table 1) (*11*). A high risk was defined as having unprotected exposure of damaged skin or mucous membranes (e.g., mucosal exposure to splashes, needlestick injury) to potentially infectious blood or body fluids, or unprotected handling of clinical/laboratory specimens. A low risk was defined as having close direct contact with the patient (e.g., routine medical/nursing care, handling of clinical/laboratory specimens) and using barrier nursing methods when handling body fluids. Barrier nursing methods used before diagnosis were basic hygiene procedures and PPE, including gloves and plastic apron without sleeves when at risk for direct contact with body fluids from the patient. No face masks were used. After diagnosis (15 days after admission), facial shield (without face mask) was added to the PPE.

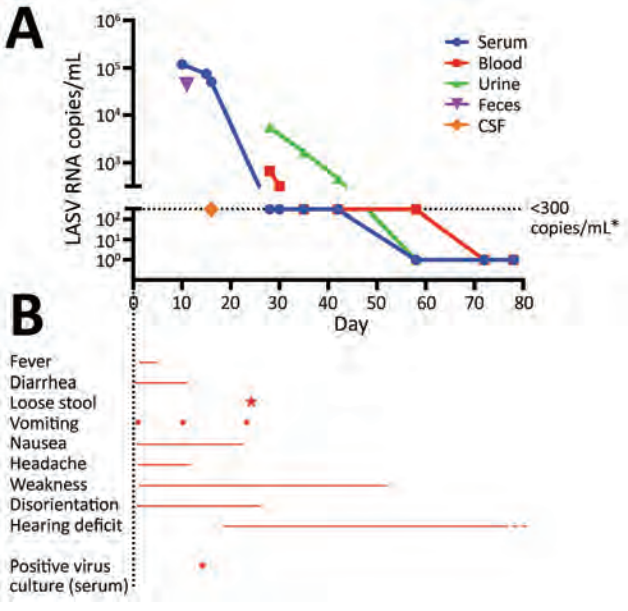


**Figure.** Characteristics of index patient (72-year-old woman) with imported Lassa fever, Gothenburg, Sweden. A) Results of virus PCR. B) Signs and symptoms and positive serum culture result. Symbols indicate days when signs/symptoms occurred and day of positive serum culture result, and lines indicate continuing signs/symptoms. Dashed line indicates a decrease in this symptom. *The limit of quantitation of LASV was 300 copies/mL, and detectable but not quantifiable levels of LASV were defined as <300 copies/mL. Day 10 is the day of hospital admission. CSF, cerebrospinal fluid; LASV, Lassa virus.

Contacts at possible risk (low or high risk) were monitored 21 days postexposure. This monitoring included measuring body temperature twice a day and awareness of any new symptoms. If fever or any symptoms appeared, these contacts were informed to contact an established safety officer. A total of 80 contacts were identified as at possible risk. Seventy-six contacts were personnel or students at the Sahlgrenska University Hospital who had close contact with the index patient or handled her body fluids. Four contacts were family members. All 80 contacts were identified as at possible risk and were categorized into low-risk exposures at the time when the diagnosis of the index patient was made. In addition, 45 personnel at the high-level isolation unit, all of them having used enhanced protective equipment (including powered air purifying respirator) when caring for the patient, were also monitored as a safety routine.

As part of this study, all 76 healthcare workers at possible risk were again assessed through interviews conducted by 2 study doctors according to a more detailed questionnaire. Questions were asked to evaluate the contact with the index patient or her body fluids and timing of the contacts. Contacts were asked to participate in the study and provide serum samples after >2 incubation periods (2 × 21 days).

This study was approved by the Medical Ethics Committee at Gothenburg University, and written informed consent was obtained for inclusion in the study. The study was performed in accordance with ethics standards in the Declaration of Helsinki and its later amendments.

### Analyses for LASV IgG

We stored serum samples at –70°C. We performed serologic analysis by using an immunofluorescence test with LASV strain SL-NL (002v EVA880)–infected confluent Vero cells. Serum samples were analyzed for LASV-specific IgG in 2-fold serial dilutions starting at 1:10. Serum from the index patient obtained 3 months after onset of disease was used as positive control (IgG titer >1:2,560).

## Results

Of the 76 personnel who were defined as being at possible risk, we included 53 in the study. The remaining 23 personnel were not included because we were unable to contact them despite several telephone calls or because they were unable to report for blood sampling. We obtained demographic characteristics for the 53 personnel (Table 2). The included personnel provided blood samples for a median

**Table 1.** Level of risk related to exposure to a patient with Lassa fever and action, by category*

| Risk category | Description | Action |
|---|---|---|
| No risk (category 1) | No contact with the case-patient; casual contact with the case-patient (e.g., sharing room without direct contact with any potentially infectious material) | Inform of absence of risk; give category 1 (general) fact sheet |
| Low risk (category 2) | Close direct contact with the case-patient (e.g., general routine medical/nursing care, handling of clinical/laboratory specimens), but did not handle body fluids or wore personal protective equipment appropriately | Self monitor† for fever and other symptoms compatible with Lassa fever; report to the safety officer nurse if fever ≥38°C or new somatic symptoms, with further evaluation as necessary; give category 2 fact sheet |
| High risk† (category 3) | Unprotected exposure of damaged skin or mucous membranes (e.g., mucosal exposure to splashes, needlestick injury) to potentially infectious blood or body fluids; or unprotected handling of clinical laboratory specimens | Report own temperature daily‡ and report this temperature and any new somatic symptoms to the safety officer nurse every day, with further evaluation as necessary; give category 3 fact sheet |

*Adapted from Kitching et al. (*11*).
†Consideration for ribavirin prophylaxis within this group.
‡Contacts to be monitored for 21 d from last possible exposure to the case-patient.

of 77 days (range 69–110 days), and samples were analyzed for LASV IgG. Antibodies were not detected in any samples from the 53 personnel.

Of the 53 personnel in the study, 15 had different symptoms of illness during the incubation period (influenza-like symptoms, including sore throat, cough, and rhinitis, n = 12; stomachache, nausea, or vomiting, n = 3; and symptoms of urinary infection, n = 1). Five of these 15 personnel had a temperature >38°C. The 5 personnel who reported fever in conjunction with symptoms possibly suggestive of Lassa fever also provided blood samples during the incubation period. Test results for LASV RNA were negative.

Twelve of the 53 personnel had obtained blood (n = 11), cerebrospinal fluid (n = 1), urine (n = 1), or feces (n = 1) samples from the index patient. Twenty personnel had assisted the patient during lavatory visits and dealt with stool, urine, or both from the patient as a result. Seventeen laboratory personnel had handled blood, cerebrospinal, stool, and urine samples from the patient.

Five personnel and 1 medical student reported that they were not wearing gloves when in close contact with or handling specimens from the patient. Of these 6 persons, 2 doctors, 1 physiotherapist, 1 occupational therapist, and 1 medical student performed physical examinations, and 1 of the laboratory staff did not wear gloves when handling agar plates containing blood from the patient. Two of these 6 persons reported influenza-like symptoms, and 1 of these 2 persons reported a temperature >38°C during the incubation period.

In addition, 5 personnel who were wearing gloves and plastic aprons were exposed to body fluids from the index patient on unprotected undamaged skin or mucous membranes. Of these 5 personnel, 1 was possibly exposed to profuse vomitus on mucous membranes, 1 was possibly exposed to droplets of saliva on her face after the index patient coughed, and 3 were exposed to urine on undamaged unprotected skin. Four of these 5 personnel reported influenza-like symptoms (n = 3) or stomach ache (n = 1); 3 of the 4 reported a temperature >38°C during the incubation period.

Of the 53 personnel, 2 were categorized as being at high risk; all others were categorized as being at low risk after interviews had been conducted. These findings were in contrast to categorization during the acute phase, when all personnel were categorized as being at low risk. During the acute phase, information regarding high-risk exposures was misjudged or inappropriate probably because of less structured questions during the acute phase and a stressful situation. The 2 nurses who had been categorized as being at high risk after the study interview was conducted were the 1 possibly exposed to profuse vomitus on mucous membranes and the 1 possibly exposed to droplets of saliva on her face after the index patient coughed.

## Discussion

The lack of LASV IgG in blood samples from all included personnel who had been exposed to the index patient supports the suggestion that risk is probably low for hospital transmission of LASV when standard barrier nursing methods are used, at least when the symptoms are mild with few occasions of vomiting and diarrhea of the index patient. These results are consistent with those of previous studies, which showed a low risk for person-to-person transmission in hospital settings (*6*,*9*–*11*,*13*,*17*). In countries to which LASV is not endemic, ≈40 cases of imported Lassa fever have been reported since LASV was first identified in 1969, and only 1 case of secondary transmission in this type of country has been reported (*18*). The secondary case was a funeral home employee who had been handling a body before a diagnosis of Lassa fever was made at postmortem analyses.

In addition, 1 case of probable nosocomial transmission was reported in Germany in 2000 (*5*). A physician who had been examining a patient with Lassa fever and not using any barrier nursing methods was found to be reactive for LASV IgG when high-risk and medium-risk

**Table 2.** Characteristics of 53 of 76 persons at Sahlgrenska University Hospital, Gothenburg, Sweden, who had contact with case-patient with Lassa fever*

| Contact classification | Total | Sex, F:M | Mean age, y (range) | Low risk | High risk |
|---|---|---|---|---|---|
| Doctor | 3 | 1:2 | 38 (28–49) | 3 | 0 |
| Nursing/AHP | 28 | 26:3 | 35 (22–58) | 27 | 2 |
| Laboratory staff | 18 | 17:1 | 45 (22–62) | 18 | 0 |
| Radiology | 2 | 2:0 | NA | 2 | 0 |
| Medical students | 1 | 0:1 | NA | 1 | 0 |
| Total | 53 | 46:7 | 39 (22–62) | 51 | 2 |

*AHP, allied health professionals (e.g., physiotherapist and occupational therapist); NA, not analyzed because of confidentiality reasons.

contacts were screened for LASV IgG. These antibodies were specific for the LASV strain isolated from the index patient. However, no specific increase in antibody titer was observed, and secondary transmission could not be confirmed. In the index patient in this study, virus RNA concentration in serum increased concomitant with disease progression. Other than the report from Germany (*5*), 3 studies have been conducted in countries to which LASV is not endemic in which serologic testing showed no asymptomatic infections in contact persons, including medium-risk and high-risk contacts (*9*,*10*,*13*). Our study strengthens the argument that the risk for asymptomatic infections in nosocomial settings is modest, at least if the disease manifests with relatively mild symptoms.

In our study, the index patient was provided care for 15 days with standard barrier nursing methods. A delay in diagnosis is not uncommon for imported cases (*4*,*11*,*19*) and is probably at least partly dependent on often nonspecific symptoms for Lassa fever. During these 15 days, 5 personnel and 1 medical student were in close contact with the index patient or with specimens from the patient, without use of barrier methods. In addition, 5 other personnel were exposed to body fluids from the index patient on unprotected skin, 1 of them possibly on mucous membranes. Indeed, risk for nosocomial transmission can be even higher before diagnosis when use of barrier nursing methods might be inappropriate, as in the case in Germany (*5*). Other situations with high risk for nosocomial transmission include profuse excretion of body fluids, such as vomitus, watery stool, or blood with high amounts of virus shedding, or invasive care in an intensive care unit. To avoid nosocomial transmission, barrier nursing methods must always be used by healthcare workers when caring for patients with potentially contagious diseases, according to national recommendations.

Moreover, it is not evident how to categorize the contacts, and there are different suggestions in the literature (*11*,*18*,*20*,*21*). It is also useful to ask structured questions and to follow criteria strictly when categorizing contacts to avoid incorrect categorization, as was seen in our study. In addition, it is not evident that all viral hemorrhagic fevers should be categorized the same way because there are considerable differences in illness and mortality rates for various viral hemorrhagic fevers (e.g., between Lassa fever and Ebola). In our study, all contacts except for 2 were categorized as being at low risk despite exposure of unprotected skin to body fluids or close physical contact with the index patient. This type of categorization is in contrast to that of the study in Germany, in which these types of contacts were categorized as high risk and prophylaxis with oral ribavirin was administered (*5*). However, ribavirin is associated with side effects, including pancreatitis and liver injury (*22*). In addition, prophylactic efficacy has not been demonstrated for humans. Because no secondary transmission of LASV has been proven in contacts with exposure of body fluids to unprotected skin or close physical contact, including in our study, we regard the categorization of Kitching et al. (*11*) as reasonable. The criteria for high-risk contacts in that study included exposure of body fluids to mucous membranes or damaged skin, such as by needle injury. Of the exposed personnel in our study, no one received ribavirin after risk classification and categorization. Thus, we agree with Kitching et al. that prophylaxis with ribavirin should only be considered in the instance of confirmed, extensive exposure to potentially infected body fluids.

Another aspect of transmission is the degree of illness. A patient with severe symptoms, including profuse vomiting, diarrhea, and bleeding, implies a high risk for virus transmission, and one could also presume that the degree of illness reflects the viremia (*23*), although data on the association between degree of illness and amount of infectious virus in different body fluids are scarce (*5*,*16*,*24*). However, despite the absence of fever 7 days before admission, the index patient in the present study had moderately high concentrations of LASV RNA in serum at admission and traces of viral RNA (<300 copies/mL) up to 32 days after admission. In addition, she had detectable virus RNA in feces and urine, and virus might be shed from the urine for a long time after recovery (*25*).

Whether detectable LASV RNA in different body fluids represents living virus or only the viral genome incorporated into dead and dying cells from necrotic tissue that might gain direct access to the circulation is unknown. It is also not clear how transmissible LASV is at different concentrations in different body fluids. Only virus cultivation can determine whether body fluids contain replication-competent virus. In a recent study, results for LASV cultivation in blood were positive for up to 11 days

but were negative after fever resolution, although LASV RNA was detectable for a longer period in different body fluids (*24*). In our study, results of virus cultivation in serum were positive for up to 16 days, even after fever resolution. However, cultivation is difficult to perform, especially from materials such as urine or feces, and is not sufficiently sensitive. Thus, there is no useful method available for distinguishing virus RNA/DNA in different body fluids from living virus or dead and dying cells from necrotic tissue, a prerequisite for evaluating the extent of contagiousness.

Last, we have verified that barrier nursing methods are not consistently defined. In our study, we used basic hygiene procedures, including use of gloves and plastic aprons when persons were at risk for direct contact with body fluids, before diagnosis. Definition of barrier nursing methods of WHO and the Centers for Disease Control and Prevention when caring for a patient with suspected or confirmed Lassa fever includes gloves, gowns, and facial shields or masks and goggles, although WHO emphasizes that these precautions are most necessary when being in close contact (≤1 m) with the patient.

In summary, our study strengthens the argument for low risk of secondary transmission of LASV in humans when proper basic nursing methods are used and the disease manifests with relatively mild symptoms. The adequate safety level when caring for patients with suspected or confirmed Lassa fever in countries to which LASV is not endemic should be discussed. Further studies of how infectivity varies depending on severity of symptoms and route of transmission are essential.

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# Occupation-Associated Fatal Limbic Encephalitis Caused by Variegated Squirrel Bornavirus 1, Germany, 2013

**Dennis Tappe, Kore Schlottau, Daniel Cadar, Bernd Hoffmann, Lorenz Balke, Burkhard Bewig, Donata Hoffmann, Philip Eisermann, Helmut Fickenscher, Andi Krumbholz, Helmut Laufs, Monika Huhndorf, Maria Rosenthal, Walter Schulz-Schaeffer, Gabriele Ismer, Sven-Kevin Hotop, Mark Brönstrup, Anthonina Ott, Jonas Schmidt-Chanasit,[1] Martin Beer[1]**

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**Release date: May 16, 2018; Expiration date: May 16, 2019**

**Learning Objectives**

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

1. Identify clinical features of limbic encephalitis (LE) in a zookeeper infected with variegated squirrel bornavirus-1 (VSBV-1), based on a case report
2. Identify immunologic and pathologic features of LE in a zookeeper infected with VSBV-1
3. Assess management of LE and of VSBV-1 infection, and preventive measures for VSBV-1 infection, based on a case report of a zookeeper infected with VSBV-1.

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Limbic encephalitis is commonly regarded as an autoimmune-mediated disease. However, after the recent detection of zoonotic variegated squirrel bornavirus 1 in a Prevost's squirrel (Callosciurus prevostii) in a zoo in northern Germany, we retrospectively investigated a fatal case in an autoantibody-seronegative animal caretaker who had worked at that zoo. The virus had been discovered in 2015 as the cause of a cluster of cases of fatal encephalitis among breeders of variegated squirrels (Sciurus variegatoides) in eastern Germany. Molecular assays and immunohistochemistry detected a limbic distribution of the virus in brain tissue of the animal caretaker. Phylogenetic analyses demonstrated a spillover infection from the Prevost's squirrel. Antibodies against bornaviruses were detected in the patient's cerebrospinal fluid by immunofluorescence and newly developed ELISAs and immunoblot. The putative antigenic epitope was identified on the viral nucleoprotein. Other zoo workers were not infected; however, avoidance of direct contact with exotic squirrels and screening of squirrels are recommended.

Limbic encephalitis, a term coined in 1960 as a clinical/anatomic description (*1*), is a rare regional inflammation of the brain involving mainly the limbic system but also other anatomic structures. Clinically, limbic encephalitis onset is subacute, and the disease is characterized by short-term memory deficits, seizures, and psychiatric symptoms (*2*). The disease is commonly regarded as an autoimmune-mediated condition associated with autoantibodies directed against various intracellular or neuronal cell surface/synaptic antigens induced by underlying neoplasia, such as thymoma or small cell lung cancer (*2–4*). However, several cases of unknown etiology in patients seronegative for autoantibodies have been reported (*5–8*).

We retrospectively investigated a case of unexplained fatal limbic encephalitis in a seronegative animal caretaker at a zoological garden in northern Germany in 2013. Our investigation was triggered by the recent detection of the novel zoonotic variegated squirrel bornavirus 1 (VSBV-1) by real-time reverse transcription PCR (rRT-PCR) in an exotic Southeast Asian Prevost's squirrel (*Callosciurus prevostii*) from the zoo where the caretaker had worked (*9*). VSBV-1 had been discovered in 2015 as the cause of a cluster of fatal cases of encephalitis in eastern Germany among private breeders of another exotic squirrel species, the Central American variegated squirrel (*Sciurus variegatoides*) (*10*). In addition to investigating the fatal encephalitis case, we used newly developed tests for VSBV-1 to serologically screen all animal caretakers at the zoo who had contact with squirrels.

## Patient, Materials, and Methods

### The Case

In July 2013, a 45-year-old female zoo animal caretaker from the federal state of Schleswig-Holstein, northern Germany, experienced fever, dysphonia, cough, pharyngitis, vertigo, and paresthesia (below her eye), followed by ataxia, coma, and pituitary gland insufficiency. The patient had had no previous medical conditions and no history of immunosuppression, and her HIV serologic results had been negative. Cerebrospinal fluid (CSF) analysis showed lymphocytic pleocytosis. Peripheral blood inflammatory parameters were elevated, with relative neutrophilia and lymphopenia. Initial cranial magnetic resonance images (MRIs) showed no abnormalities. Follow-up MRIs taken 3 weeks later demonstrated lesions in a bilateral limbic distribution (medial temporal lobes, anterior cingulum, insula, hippocampus, hypothalamus, periventricular tectum), in the basal ganglia (Figure 1), and in the upper myelon. Limbic encephalitis was diagnosed on the basis of morphologic appearance and progressed within 1 week. Extended laboratory workup results for central nervous system (CNS) infection and autoimmune disease were within reference limits (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/6/17-2027-Techapp1.pdf). No underlying neoplasia was detected. Repeated electroencephalography showed generalized slow activity and evidence of a current nonconvulsive epileptic seizure. Histopathologic examination of a brain biopsy sample had demonstrated glial activation and lymphocyte infiltration. No neurotropic bacteria, fungi, parasites, or viruses had been detected by microscopy, culture, or PCR. Creutzfeldt-Jakob disease was excluded by protein aggregate filtration and paraffin-embedded tissue blotting (*11*). The patient had required mechanical ventilation because of bilateral pneumonia and received broad antiinfective chemotherapy (including acyclovir throughout), anticonvulsants, and steroids later in the course of the disease (online Technical Appendix Table 1). Within 3 months after symptom onset, she died of myeloencephalitis of undetermined etiology. Postmortem examination of the brain demonstrated edema, necrosis, and perivascular lymphocyte cuffing in limbic structures and in the basal ganglia.

### Molecular Investigations

Patient samples available for analysis for this study were archived frozen CSF and formalin-fixed paraffin-embedded brain tissue, myocardium, lungs, kidney, liver, spleen,

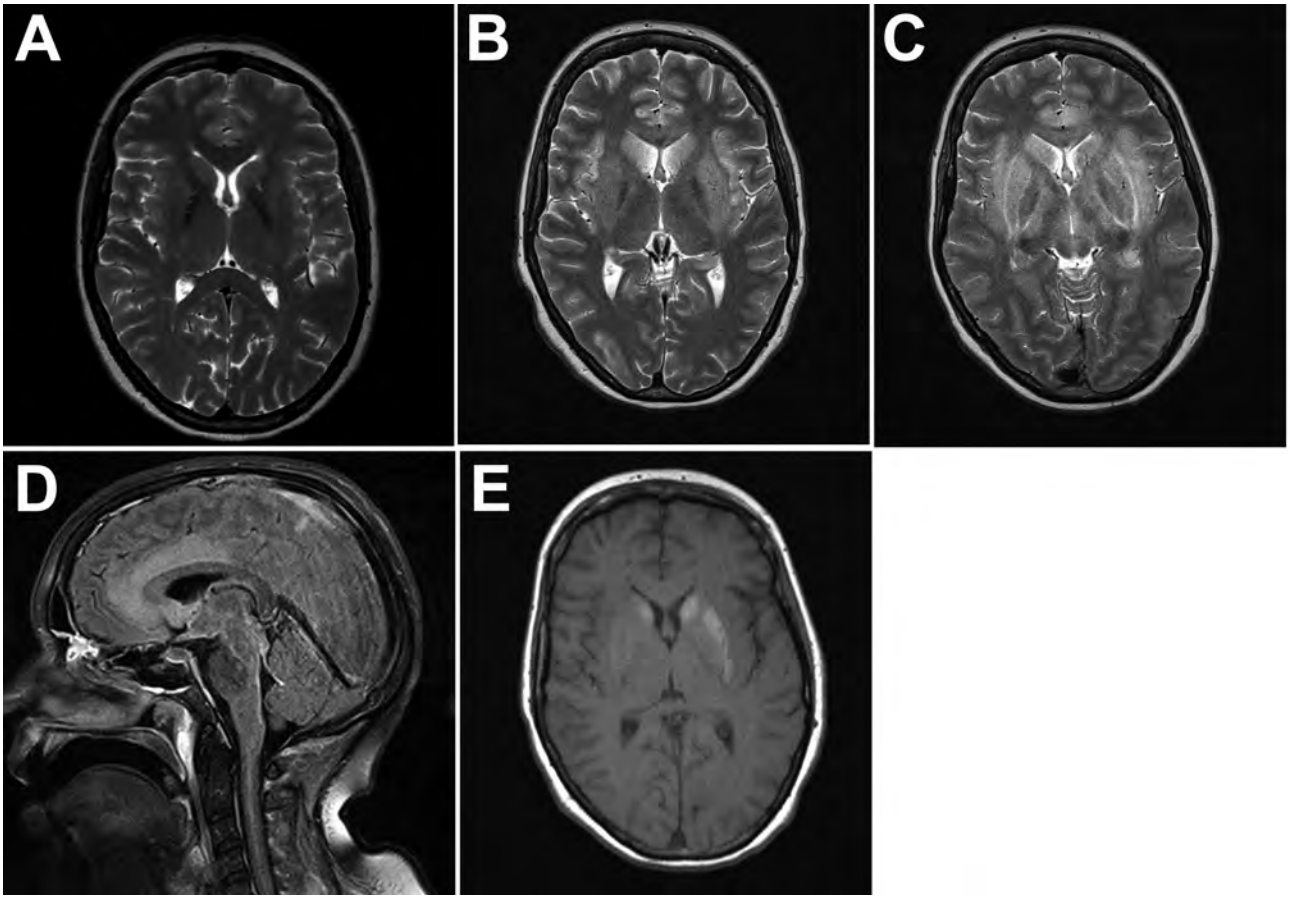


**Figure 1.** Magnetic resonance imaging of the brain throughout the course of the disease in patient who died of limbic encephalitis caused by variegated squirrel bornavirus 1 (VSBV-1), Germany, 2013. A) T2-weighted transversal image at admission showing no pathologic changes. B) T2-weighted image 3 weeks after admission showing edema in limbic structures (insula, hippocampus, anterior cingulate) and in the basal ganglia. C) T2-weighted image 4 weeks after admission showing progressive edema. Additional myelopathy extended from the medulla down into the thoracic segments (not shown). D) FLAIR image 4 weeks after admission showing edema in the anterior cingulate cortex. E) T1-weighted image 4 weeks after admission without contrast showing slight hemorrhage in the basal ganglia.

pancreas, bone marrow, and intestine. We performed VSBV-1–specific rRT-PCR on all samples as described (*10*), using a detection limit of 10 genome equivalents/reaction.

Metagenomic analysis of the brain samples followed a standard workflow as described (*12*,*13*). We used a MiSeq instrument (Illumina, San Diego, CA, USA) for sequencing, or we performed Sanger sequencing as described (*9*). VSBV-1 sequences from the animal caretaker and 4 species of squirrel (*Callosciurus prevostii*, *C. finlaysonii*, *Sciurus variegatoides*, and *Tamiops swinhoei*) from different zoos and other holdings were compared with previously described VSBV-1 sequences. Comparisons were performed by the Bayesian Monte Carlo Markov Chain sampling method implemented in BEAST (*14*) and in parallel a maximum-likelihood inference by using PhyML version 3.1 (*15*) with the complete genome and complete major protein N (p40) gene sequences. Using the Akaike information criterion in jModelTest 2 (*16*), we found that the best nucleotide substitution model that fit the data was the general time-reversible plus gamma plus invariate sites model.

We aligned sequences by using the MAFFT (multiple alignment using fast Fourier transform) algorithm and compared and analyzed the VSBV-1 genomes in Geneious version 9.1.4 (https://www.geneious.com/). All sequences were confirmed as nonrecombinant by the various methods for recombination detection implemented in RDP4 (*17*).

### Immunohistochemistry

We obtained polyclonal antiserum against VSBV-1 and Borna disease virus (BoDV) N and P proteins from rabbits immunized with the respective recombinant antigens and purified by protein A ion exchange (Davids Biotechnologie, Regensburg, Germany). We tested reactivity of

the rabbit antiserum and preimmune serum by immunofluorescence antibody test (IFAT) and on a newly developed bornavirus immunoblot. We used 10 unrelated human brain tissue samples as negative controls. After pretreatment with proteinase K and endogenous peroxidase blocking, we incubated the formalin-fixed paraffin-embedded sections with the antiserum (1:1,000–1:5,000 in phosphate-buffered saline at room temperature overnight), followed by a goat anti-rabbit biotinylated polymer antibody, a streptavidin-horseradish peroxidase complex, and the 3-amino-9-ethylcarbazole substrate (DCS, Hamburg, Germany).

### Serologic Assays and Antibody Epitope Mapping

To detect bornavirus-specific IgG in serum and CSF, we used a persistently BoDV-infected cell line in a standard indirect IFAT (*10*); we also developed an ELISA and an immunoblot. We screened protein A–purified antibodies from the patient's CSF by using peptide microarrays as described (*18*). For the VSBV-1 IgG ELISA, sequences of VSBV-1 N and P genes were cloned and expressed as maltose-binding protein (MBP) fusion proteins in *Escherichia coli* strain BL21 Gold (DE3) (Novagen-Merck, Darmstadt, Germany). The protein was purified by amylose affinity chromatography and eluted. The N terminal MBP tag was cleaved by a 3C protease at 4°C overnight. After the protease was removed, we further purified the protein sample by size exclusion chromatography (Superdex 200, Sigma-Aldrich, Munich, Germany). We coated polystyrene microtiter plates (Polysorp; Nunc, Roskilde, Denmark) with 2 μg/mL VSBV-1 N or P protein and incubated them overnight at 4°C. After blocking with 6% bovine serum albumin, we added 1:400 diluted human serum in 1% bovine serum albumin diluent. After incubation for 2 h at 37°C, we added 100 μL anti-human IgG (Dako Cytomation, Hamburg, Germany; diluted 1:6,000) and incubated the plates at 37°C for 1 h. The reaction was stopped after 5 min incubation at room temperature with 3,3′,5,5′-tetramethylbenzidine. Last, we measured the optical density (OD) at 450 nm (reference 620 nm) and calculated the final OD for each serum sample as the difference between the OD measured in VSBV-1 N- or P-containing and MBP-containing wells. The final ODs for serum dilutions of 1:400 were regarded as positive if the mean OD exceeded the mean OD + 3 SD obtained with 200 control samples from healthy blood donors. For the bornavirus IgG immunoblot (line immunoassay system; EUROIMMUN AG, Lübeck, Germany), we coated recombinantly expressed VSBV-1 and BoDV N and P proteins on a nitrocellulose membrane as narrow lines. We incubated the strips at room temperature with serum (30 min, 1:50) or CSF (3 h, 1:4), followed by alkaline phosphatase-conjugate (serum 30 min, CSF 1 h; 1:10 each) and nitro-blue-tetrazoliumchloride as substrate (serum 10 min, CSF 20 min). Intensities of the detected antibodies were automatically evaluated by using the EUROLineScan software (EUROIMMUN AG). For validation, we tested 150 samples from healthy blood donors from northern Germany. For the antibody epitope mapping, we synthesized 360 15-mer peptides covering the whole p40 sequence of VSBV-1 (GenBank accession no. CEK41887) with an offset of 1 aa by using the synthetic peptide arrays on membrane support technique (*19*) and printed them onto glass slides by using the SC2 method (*20*). We also performed an alanine scan for highest antibody binding. Slides were washed with absolute ethanol for 3 min and then 3 times with Tris-buffered saline (TBS) for 3 min. Next, we blocked slides overnight at room temperature with 2% (wt/vol) casein in TBS-Tween 20. The CSF sample was diluted 1:120 in blocking buffer. We incubated the slides with the sample overnight in a humidified chamber at 4°C. After washing 3 times (5 min) in TBS-Tween 20, we incubated the slides with anti-human IgG coupled to Cy5 for visualization of bound antibodies at a dilution of 1:240 in blocking buffer for 1.5 h at room temperature. After washing slides twice in TBS-Tween 20 followed by 3 times in distilled water (5 min) and dried in a nitrogen stream, we immediately scanned slides by using an Agilent DNA Microarray scanner (Agilent Technologies, Waldbronn, Germany). The resulting pictures were obtained by using Agilent Feature Extraction Software version 10.7.3. We used the program Chimera (*21*) for 3D illustrations and distance measurements.

## Results

### rRT-PCR Detection, Whole-Genome Sequence, and Phylogeny of VSBV-1

We detected VSBV-1 RNA in the patient's CSF, plexus, paraventricular brain areas, striatum, substantia nigra, and cerebellum (quantitation cycle values 24.1–35.2), whereas PCR of 8 cortical areas was negative (online Technical Appendix Table 2). PCR of myocardium, lung, liver, kidney, spleen, bone marrow, pancreas, and intestine samples also yielded negative results. Phylogenetic analysis of the complete genome (Figure 2) and the N protein gene datasets (online Technical Appendix Figure 1) demonstrated that the newly sequenced VSBV-1 from the deceased animal caretaker and previously described VSBV-1 strains from the exotic squirrels formed a distinct and highly supported monophyletic group (novel species *Mammalian 2 bornavirus*) within the bornavirus phylogeny. Phylogenetic analysis performed only on the VSBV-1 dataset revealed that the VSBV-1 strain from the animal caretaker (BH55/16) clustered with the virus sequence from the single infected contact *C. prevostii* squirrel (BH12/16) from the zoo (Figure 2,

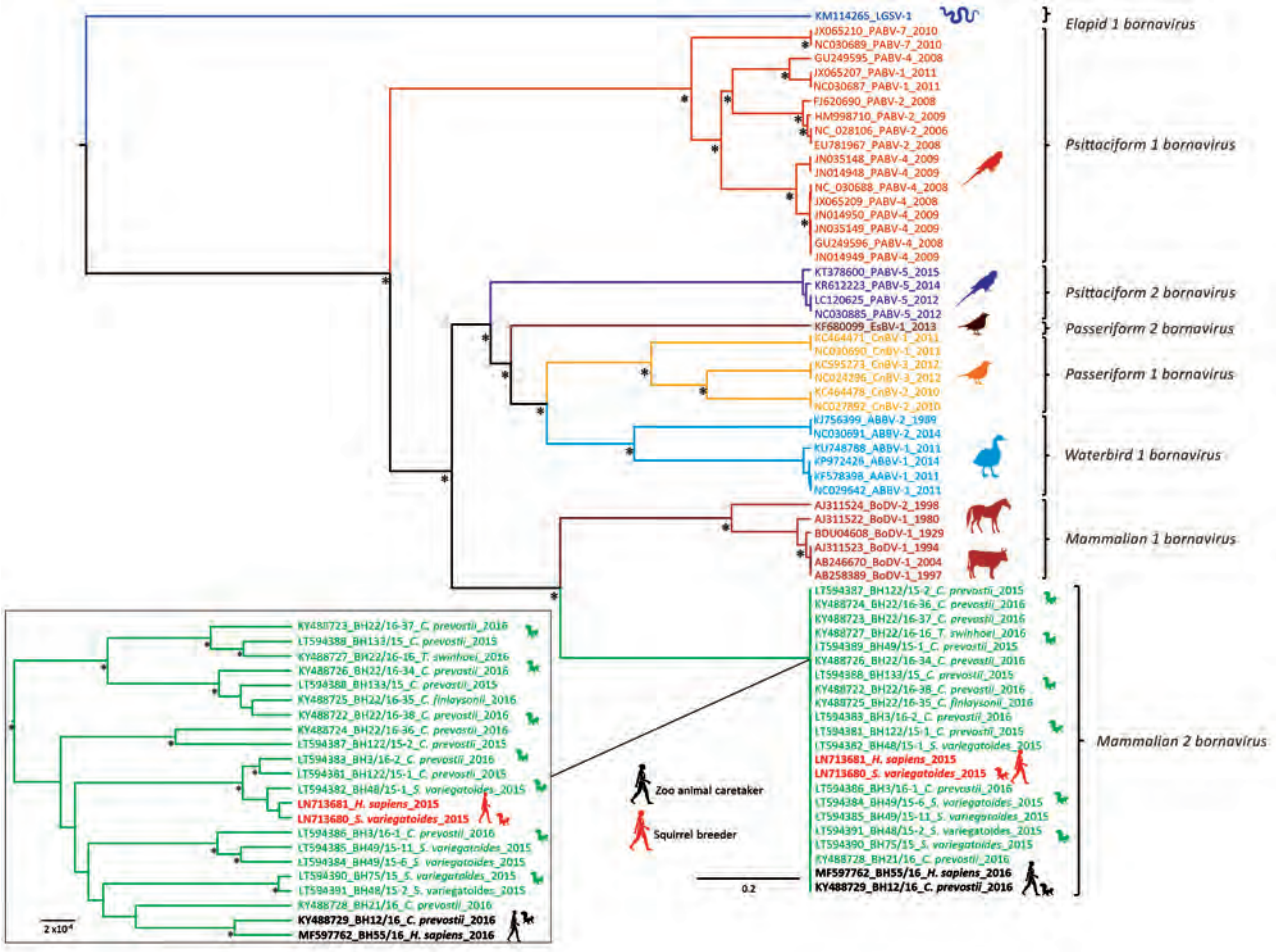


**Figure 2.** Phylogenetic analysis of complete coding sequences from variegated squirrel bornavirus 1 (VSBV-1) and other members of *Bornaviridae*. The phylogenetic trees were inferred by using the Bayesian Markov Chain Monte Carlo method and in parallel a maximum-likelihood method (tree not shown). Statistical support of grouping from Bayesian posterior probabilities (clade credibilities ≥90%) and maximum-likelihood bootstrap replicates (≥70%) are indicated with an asterisk. Taxon information includes GenBank accession number and virus abbreviation. Branch colors are based on bornavirus species. The VSBV-1 sequence from the patient generated during this study and the highly similar VSBV-1 sequence from the zoo squirrel are shown in bold. Inset shows detail of mammalian 2 bornavirus section. Scale bars represent nucleotide substitutions per site.

inset; online Technical Appendix Figure 1, inset). Identity matrix analysis showed 99.90% nt and 99.92% aa identity between the BH55/16 and BH12/16 VSBV-1 strains; identities with other VSBV-1 strains were 99.69%–99.80% nt for BH55/16 and 98.41%–99.40% aa for BH12/16. A single unique amino acid substitution ($N_{390}T$) was found in the G gene of both strains (Table 1), and 3 unique synonymous nucleotide substitutions were detected in the N, M, and G genes.

### Immunohistochemical Findings

When we applied the VSBV-1 and BoDV N protein antiserum, brain tissue sections of the subcortical areas showed nuclear and neuropil immunostaining of brain cells (Figure 3). All sections of uninfected human control brain were negative. The rabbit antiserum showed a nuclear pattern by IFAT (endpoint titer for BoDV N 10,240) and strong reactivity with respective homologous and heterologous antigens on the immunoblot. The respective preimmune serum samples were negative by IFAT, immunoblot, and immunohistochemistry. In addition, intranuclear eosinophilic inclusion bodies, resembling bornavirus-like Joest-Degen bodies, were visible during histopathologic examination of the basal ganglia (Figure 3).

### Serology and Antibody Epitope Mapping Findings

We detected bornavirus-specific IgG in high concentrations in the patient's CSF by IFAT in a nuclear pattern (IgG endpoint titer 2,560 [online Technical Appendix Figure 2]) and by ELISA (OD 1.41 against N protein and 0.30 against VSBV-1 P protein). IgG reactivity on the

**Table 1**. Comparison of amino acid substitutions of human-derived variegated squirrel bornavirus 1 strain from a deceased zoo animal caretaker with those detected in exotic squirrels of 3 species and private squirrel breeders on the basis of protein coding region sequences*

| Protein | Human (*Homo sapiens*) BH55/16 | Squirrel | | | Unique substitutions |
|---|---|---|---|---|---|
| | | *Sciurus variegatoides* | *Callosciurus prevostii* | *Tamiops swinhoei* | |
| N | No | No | No | No | 0/0 |
| X | No | No | **$T_{120}$I** | No | 1/1 |
| P | No | $L_{93}$I | **$P_{21}$S**, $L_{93}$I | $L_{93}$I | 1/2 |
| M | No | No | **$S_{71}$F** | No | 1/1 |
| G | **$S_{175}$N**, **$E_{185}$D**,$V_{228}$M, **$\underline{N_{390}T}$** | $S_{59}$P, **$E_{73}$K**, **$S_{165}$G**, $T_{170}$A, **$N_{188}$D**, $V_{228}$M, $S_{237}$P, **$R_{244}$K**, **$V_{313}$I**, **$N_{390}$S** | **$S_{19}$L**, **$E_{60}$K**, **$A_{106}$V**, **$N_{111}$D**, $T_{170}$A, **$S_{175}$N/I**, **$N_{188}$S**, **$G_{196}$D**, $V_{228}$M, $S_{237}$P, $S_{238}$L, **$S_{254}$P**, **$S_{332}$P**, **$Y_{335}$S**, **$\underline{N_{390}T}$**, **$I_{480}$M** | $S_{59}$P, $S_{237}$P, $S_{238}$L | 17/24 |
| L | $I_{112}$V, $L_{779}$F, $T_{832}$I, $G_{1365}$R, $G_{1388}$S | $I_{112}$V, **$E_{143}$K**, $M_{166}$V, $S_{360}$L, $A_{756}$V, $L_{779}$F, $T_{832}$I, $G_{1365}$R, $G_{1388}$S | **$K_{63}$R**, $I_{112}$V, **$N_{136}$S**, **$A_{144}$T**, $M_{166}$V, **$V_{539}$I**, $A_{756}$V, $L_{779}$F, $T_{832}$I, **$I_{1053}$V**, **$K_{1170}$L/R**, **$Q_{1286}$H**, $G_{1365}$R, $G_{1388}$S, **$P_{1423}$A**, **$S_{1474}$L** | $M_{166}$V, **$I_{194}$V**, $S_{360}$L | 11/19 |

*Boldface indicates the unique amino acid substitutions detected in each host. Boldface and underlining indicate the mutation detected only in the variegated squirrel bornavirus 1 strain from the deceased animal caretaker (BH55/16; GenBank accession no. MF597762) and her contact squirrel (*Callosciurus prevosti*).

immunoblot against VSBV-1 N was strong and against VSBV-1 P and BoDV P antigens was less (Table 2). Patient serum was no longer available for testing. Epitope mapping of the protein A–purified CSF antibodies revealed a single spot signal on the peptide microarray corresponding to the sequence 116-FVKVSRFYGERTASR-130 (Figure 4). An alanine scan array of the detected peptide showed that 8 aa of the 15-mer peptide were found to be essential for antibody binding at the N-terminus and particularly at the C-terminus of the peptide sequence. Mapping of the epitope to the 3D-structure 1N93 of the viral p40 nucleoprotein showed that the peptide is part of the N terminal accessible surface. The measured distances of <25Å of the covered area suggest that the antibody target sequences form a discontinuous epitope or are located on 2 adjacent monomers because the N terminal part of the epitope FVXV and the C-terminal part TASR form a binding pocket with a distance of 12Å.

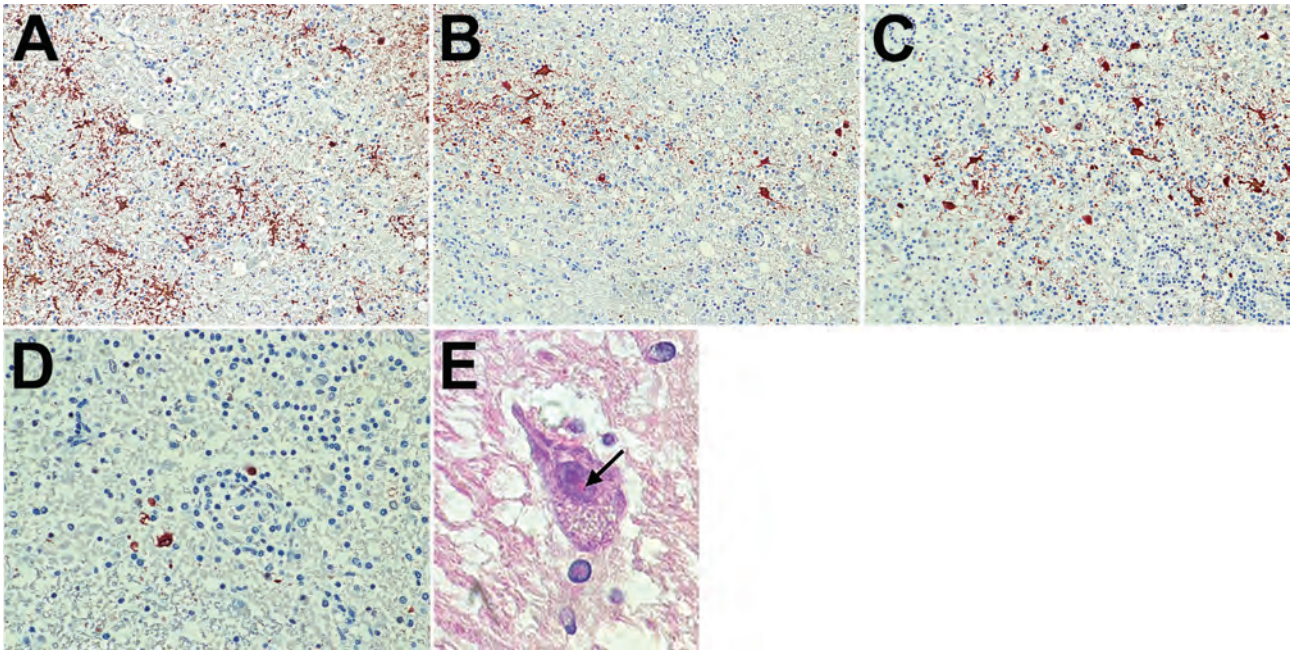


**Figure 3.** Immunohistochemical and histologic slides of brain of patient who died of limbic encephalitis caused by variegated squirrel bornavirus 1 (VSBV-1), Germany, 2013. Immunohistochemistry of viral antigen in subcortical areas of the brain was performed by using a polyclonal antiserum against VSBV-1 N protein. Viral antigen was present in neurons and glial cells in nuclei and cytoplasm. A) Substantia nigra. Immunoperoxidase stain with hematoxylin counterstain; original magnification ×200. B) Striatum. Immunoperoxidase stain with hematoxylin counterstain; original magnification ×200. C) Subcortical area next to the hypothalamus. Immunoperoxidase stain with hematoxylin counterstain; original magnification ×200. D) Subcortical area next to the hippocampus. Immunoperoxidase stain with hematoxylin counterstain; original magnification ×400. E) Intranuclear eosinophilic inclusion body resembling a bornavirus-like Joest-Degen body (arrow). Hematoxylin and eosin stain; original magnification ×600.

**Table 2.** Serologic test results for zoo animal caretaker who died of encephalitis, other zoo animal caretakers, and healthy blood donors, Germany, 2013*

| Patient or group, sample type | IgG immunoblot† | | | | BoDV IgG IFAT | VSBV-1 IgG ELISA‡ | | Contact with Prevost's squirrels |
|---|---|---|---|---|---|---|---|---|
| | BoDV-P | BoDV-N | VSBV-P | VSBV-N | | VSBV-P | VSBV-N | |
| Encephalitis patient, age, y/sex, CSF | | | | | | | | |
| 45 y/F | **+** | – | **+** | **+** | **1:2,560** | Pos | Pos | Regularly |
| 14 zoo animal caretakers, age, y/sex, serum | | | | | | | | |
| 44 y/F | – | – | – | **(+)** | Neg | Neg | Neg | Regularly |
| 32 y/M | – | – | – | – | Neg | Neg | Neg | Regularly |
| 25 y/F | – | **+** | – | **(+)** | Neg | Neg | Neg | Rarely |
| 33 y/F | – | – | – | – | Neg | Neg | Neg | Occasionally |
| 26 y/F | – | – | – | **(+)** | **1:160§** | Neg | Neg | Occasionally |
| 27 y/M | – | **+** | – | – | Neg | Neg | Neg | Occasionally |
| 29 y/F | – | – | – | – | Neg | Neg | Neg | Rarely |
| 48 y/F | – | **(+)** | – | **+** | **1:40** | Neg | Neg | Rarely |
| 35 y/M | – | – | – | – | Neg | Neg | Neg | Occasionally |
| 24 y/F | – | – | – | – | Neg | Neg | Neg | Regularly |
| 18 y/F | – | **+** | – | – | Neg | Neg | Neg | Regularly |
| 21 y/F | – | – | – | **(+)** | Neg | Neg | Neg | Occasionally |
| 37 y/M | – | – | – | **(+)** | Neg | Neg | Neg | Regularly |
| 20 y/F | – | – | – | – | Neg | Neg | Neg | Regularly |
| 150 healthy blood donors, %, serum | | | | | | | | |
| Positive | 1.5 | 0 | 1.5 | 1.5 | NA | Neg | Neg | No |
| Weakly positive | 4.5 | 4.5 | 0 | 12 | NA | Neg | Neg | No |

*Boldface indicates positive response. BoDV, borna disease virus; CSF, cerebrospinal fluid; ELISA; enzyme-linked immunosorbent assay; IFAT, immunofluorescence antibody test; NA, not available; neg, negative; OD, optical density; pos, positive; VSBV-1, variegated squirrel bornavirus 1;. +, strongly positive; (+), weakly positive; –, no reaction by immunoblot.
†Results were obtained densitometrically using EUROLineScan software (EUROIMMUN AG, Lübeck, Germany) and transformed into semiquantitative categories (negative, –, 0–13; weakly positive, (+), 14–20; positive, +, 21–255).
‡Final OD values for serum dilutions of 1:400 were regarded as positive if the mean OD exceeded the mean OD + 3 SD obtained with negative control samples.
§The IgG endpoint titer is defined as the reciprocal of the highest analyte dilution that gives a positive signal in IFAT.

### Serologic Screening Results for Zoo Animal Caretakers

Serum samples from all 14 animal caretakers were analyzed by ELISA, IFAT, and immunoblot. Of these 14 caretakers, 6 had regular contact with the squirrels (feeding, cleaning), 5 had occasional contact, and 3 had only rare contact. By immunoblot, of the 14 samples, 6 showed a positive or weakly positive reaction with VSBV-1 N and 4 with BoDV N but not with the respective P antigens. IFAT showed low titers for 2 of the 14 samples, whereas ELISA results were negative (Table 2). Serologic reactivity did not correlate with the reported intensity of squirrel contact. By immunoblot, samples from 13.5% of blood donors showed a positive or weakly positive reaction with VSBV-1 N, 4.5% with BoDV N, 1.5% with VSBV-1 P, and 6% with BoDV P antigens (Table 2). None of the blood donor samples reacted against >1 bornavirus antigen.

## Discussion

The association of a fatal case of encephalitis with the recently discovered zoonotic VSBV-1 was achieved by molecular assays, specific immunohistochemistry, and epitope-resolved serology. We found Joest-Degen inclusion bodies typical for a bornavirus infection in the brain tissue of the human patient. Genetic characterization and phylogenetic tree reconstruction of the VSBV-1 sequence from the patient demonstrated spillover infection originating from the infected Prevost's squirrel from the zoologic garden. This spillover hypothesis is supported by our finding of a unique amino acid substitution ($N_{390}T$) in the G gene and 3 unique synonymous nucleotide substitutions of VSBV-1 in both strains. The patient was therefore most likely infected during her work with the squirrels in the zoo. VSBV-1–infected *Sciuridae* squirrels show only mild inflammation of the brain and no clinical symptoms (*10*,*22*) and are therefore probably a natural reservoir for VSBV-1. In addition to virus presence in the brain of exotic squirrels with confirmed VSBV-1 infection, high viral genome loads were detected in skin, salivary glands, kidneys, and urinary bladder (*22*). The virus is probably transmitted by scratches and bites (*10*); however, infective viral particles have yet to be demonstrated in animal secretions or skin scalings.

The origin of this virus is unknown. Squirrel species from 2 subfamilies in different holdings in Europe were infected: Central America squirrel species (subfamily *Sciurinae*: *S. variegatoides* and *S. granatensis*) and Southeast Asia squirrel species (subfamily *Callosciurinae*: *C. prevostii*, *C. finlaysonii*, and *T. swinhoei*) (*9*). Phylogenetic analysis of the complete genome and complete p40 gene from VSBV-1 from the animal caretaker and comparison with sequences from previously identified infected exotic squirrel species and squirrel breeders further strengthen our previous reports (*9*,*10*,*22*) that the VSBV-1 strains form a distinct and highly divergent monophyletic lineage within the bornavirus phylogeny.

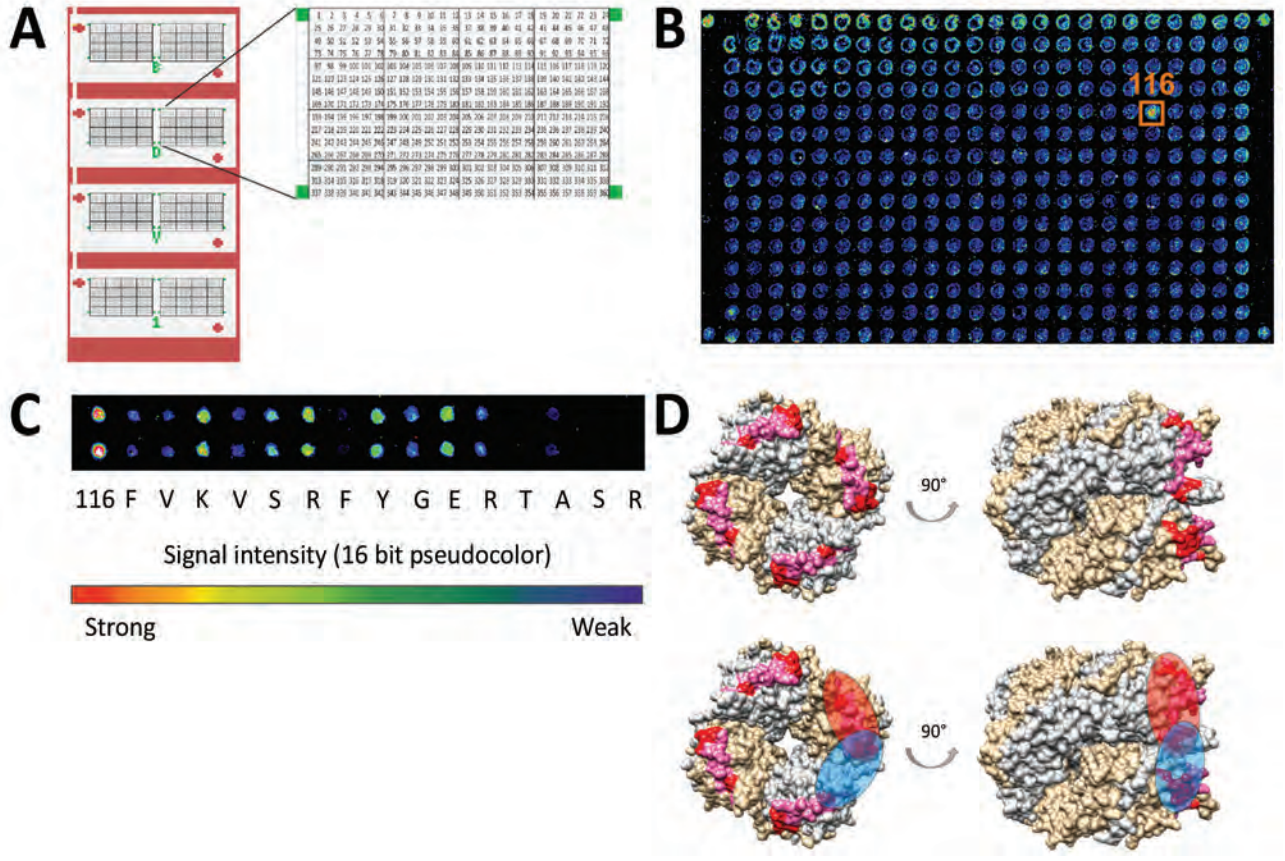


**Figure 4.** N protein (p40) peptide microarray–based epitope mapping of variegated squirrel bornavirus 1 from patient who died of limbic encephalitis, Germany, 2013. A) The N protein–based peptide microarray chip consists of 8 identical arrays composed of 360 15-mer peptides with an offset of 1 aa. Each subarray was bordered by biotin spots (green). B) Representative single-channel readouts from 1 subarray in 16-bit pseudocolor is given for the protein A–purified patient cerebrospinal fluid sample. Signals were classified positive if they appeared in each of the 8 subarrays. The detected signal is surrounded by an orange box and labeled by the corresponding spot number. C) The alanine scan of spot 116 showed highest antibody binding for the original sequence and less bound antibodies after substitution of the indicated amino acids by alanine. D) Mapping of antibody target sequences onto the tetrameric P40 nucleoprotein 3D-structure (PDB:1N93). Monomers are depicted in gray and gold, amino acids necessary for antibody binding (red) and inconsequential (pink) related to spot 116. Potential binding sites of antibodies for spot 116 are shown in the lower panels as red (1 monomer) and blue (spanning 2 adjacent monomers and forming a binding pocket) overlay.

The case we describe contrasts in several aspects with the previously reported cluster of fatal encephalitis cases in 3 elderly male private squirrel breeders in eastern Germany (*10*), which were associated with Central America variegated squirrel species. The infection we report emerged as an occupation-associated disease in an animal caretaker in a zoo in northern Germany and was linked to a different species of exotic squirrel, a Southeast Asia Prevost's squirrel. Moreover, the patient we report was female, <60 years of age, and had no medical preconditions. The myoclonus and thrombosis reported for the squirrel breeders (*10*) was absent in the zoo worker. However, the fever, ataxia, coma, duration of illness (subacute-onset encephalitis), and late appearance of MRI changes in the zoo worker are similar to findings in the previous cluster. In the case we describe, VSBV-1 in the central nervous system of the zoo worker was in a limbic distribution. In a rat model, the related BoDV has a preferential limbic tropism (*23*). Limbic encephalitis in humans is regarded as a regional autoimmune encephalitis that predominantly affects the limbic system. The disease is associated with paraneoplastic (epiphenomenal) nonpathogenic autoantibodies directed against intracellular epitopes (Hu, Ri, Ma2, and GAD), and pathogenic neuronal cell surface autoantibodies (against AMPA, GABA, and voltage-gated potassium-receptor complexes) (*2–4*), none of which were detected in the zoo worker. Recently, the triggering of an autoantibody-positive, nonparaneoplastic limbic encephalitis by human herpesvirus 6B was speculated (*24*), similar to the induction of NMDA (N-methyl-D-aspartate) receptor encephalitis as clinical relapse after herpes simplex virus encephalitis (*25*). Of note, several cases of limbic encephalitis without autoantibodies and with

unknown etiology have been reported (*5–8*). Hypothesizing that the pathogenesis of such seronegative limbic encephalitis cases without neoplasia is infectious or postinfectious remains tempting. In concordance with limbic encephalitis, for the patient reported here, electroencephalogram changes demonstrated epileptic activity, histologic examination showed lymphocyte involvement, and CSF showed a lymphocytic pleocytosis with raised protein levels (*2*,*5*). Treatment for seronegative limbic encephalitis thus far comprises immunomodulatory drugs, including intravenous immunoglobulins (*5–7*).

No curative treatment has been established for human bornavirus infections. Ribavirin, which has shown in vitro effectiveness against various bornaviruses (*26–28*), was not administered to the patient we describe. In the VSBV-1 encephalitis cluster among squirrel breeders (*10*), ribavirin had been given to 1 person but had no clinical effect. In animals, BoDV pathology is caused by the host's immune response (*29–31*). Whether an immunosuppressive treatment, as administered here and previously to 2 squirrel breeders (*10*), is clinically beneficial for patients with bornavirus infections remains unknown.

We developed novel serologic assays that showed a good correlation of ELISA, IFAT, and immunoblot results for the encephalitis patient. IFAT and immunoblot showed serologic cross-reactivity against BoDV antigens. In the patient's CSF sample, a single spot reaction to the viral N protein could be demonstrated by peptide microarray. We were able to project the epitope onto the surface of the 1N93 3D-structure. In the small number of healthy animal caretakers tested (14), the constellation of antibody reactivity was unclear and inconsistent. Whether the weak immunoblot and IFA antibody responses against bornavirus antigens in 2 healthy animal caretakers reflect a past contact with VSBV-1 remains speculative. A low percentage of positive reactions to single bornavirus antigens was detected by the immunoblot in healthy blood donors. This finding is probably nonspecific because none of the healthy persons screened in our study exhibited the antibody constellation that was seen in the confirmed VSBV-1–infected patient. Future studies, including T-cell assays, will address the immunologic response in humans in more detail. More seroprevalence studies of predominantly zoo animal caretakers and squirrel breeders are under way to evaluate the serologic tests and shed more light on the human exposure to VSBV-1.

Our investigation highlights the risk for VSBV-1 transmission from zoo animals to humans, especially in view of the previously described relatively high rate of infection among squirrels of these 2 families (*Callosciurinae,* 8.5%; *Sciurinae,*1.5% [*9*]). These findings further emphasize the need to test all exotic squirrels for VSBV-1 to prevent further spillover infections. Reasonable precautions should be taken, such as having zoo employees, zoo visitors, and private breeders avoid direct contact with exotic squirrels. Moreover, for patients with signs of limbic encephalitis without underlying autoimmunopathology (seronegative limbic encephalitis), differential diagnostics should be adapted, possible infection with VSBV-1 should be investigated, and patients should be asked whether they have had contact with exotic squirrels. According to results of in vitro studies with other bornaviruses (*26–28*), early and extended treatment with ribavirin might be considered for humans with VSBV-1 infection.

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# Genomic Sequencing of *Bordetella pertussis* for Epidemiology and Global Surveillance of Whooping Cough

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*Bordetella pertussis* causes whooping cough, a highly contagious respiratory disease that is reemerging in many world regions. The spread of antigen-deficient strains may threaten acellular vaccine efficacy. Dynamics of strain transmission are poorly defined because of shortcomings in current strain genotyping methods. Our objective was to develop a whole-genome genotyping strategy with sufficient resolution for local epidemiologic questions and sufficient reproducibility to enable international comparisons of clinical isolates. We defined a core genome multilocus sequence typing scheme comprising 2,038 loci and demonstrated its congruence with whole-genome single-nucleotide polymorphism variation. Most cases of intrafamilial groups of isolates or of multiple isolates recovered from the same patient were distinguished from temporally and geographically cocirculating isolates. However, epidemiologically unrelated isolates were sometimes nearly indistinguishable. We set up a publicly accessible core genome multilocus sequence typing database to enable global comparisons of *B. pertussis* isolates, opening the way for internationally coordinated surveillance.

Whooping cough (or pertussis) is a vaccine-preventable disease caused mainly by the bacterium *Bordetella pertussis*, and to a lesser extent by *B. parapertussis*. The infection is most severe in infants who are too young to be vaccinated or are not yet fully vaccinated. The burden of disease is still high: 24 million pertussis cases and 160,700 deaths from pertussis in children <5 years of age in 2014 (*1*). The introduction of vaccination using whole-cell vaccines in the 1950s, and the switch to acellular vaccines targeting only some antigens in the 1980–1990s, have played a central role in the control of whooping cough. However, increasing incidence of the disease and large outbreaks have been reported recently in many countries (*2*–*5*). The observed resurgence of whooping cough underlines the need for reinforced surveillance of strain evolution, local spread, and global transmission. For example, the relative contributions of intercountry spread compared with local, independent evolution of strains that do not express pertactin (*6*–*8*), one of the components of acellular vaccines, are unknown. This gap limits our ability to interpret the local prevalence of pertactin-negative isolates and to define the effects of country-specific vaccine strategies on the emergence of antigen-deficient isolates.

Until now, strain genotyping for surveillance and epidemiology has been based mostly on pulsed-field gel electrophoresis (PFGE), antigen and virulence factor genotyping, 7-gene multilocus sequence typing (MLST), or multilocus variable-number tandem-repeat analysis (MLVA) (*9*–*11*). PFGE achieves some level of resolution given the high structural dynamics of *B. pertussis* genomes, driven by insertion sequence elements dynamics (*12*), and is more discriminatory than MLVA or MLST. However, *B. pertussis* clinical isolates exhibit strong genetic homogeneity (*11*,*13*). Therefore, these traditional typing methods have largely failed to define local chains of transmission.

Whole-genome sequencing (WGS) provides the highest possible resolution of genetic differences among individual isolates. Working with WGS of an international collection of *B. pertussis* isolates collected through 2010, Bart et al. (*13*) provided a global phylogenetic structure of *B. pertussis* and analyzed genome evolutionary dynamics across the prevaccine and vaccine eras. This pioneering study found genotype mixing across countries at shallow phylogenetic depth, revealing frequent long-distance spread of *B. pertussis* isolates and underlining the importance of defining standard genotyping methods that would allow tracing international transmission. Core genome MLST (cgMLST), using the set of genes conserved among isolates of a given bacterial group, represents an approach that combines the high resolution of genome-level variation and the high reproducibility and portability of MLST (*14*). cgMLST genotyping strategies were recently implemented for international coordinated surveillance of several pathogenic bacterial species (*15*–*20*).

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We report on the development and evaluation of a cgMLST scheme for genotyping of *B. pertussis* clinical isolates. We demonstrate the resolution power of this approach to recognize groups of intrafamilial isolates or multiple isolates recovered from the same patient. We also show that, in some cases, temporally or geographically unrelated isolates can be nearly undistinguishable, illustrating the rapid diffusion of isolates through hidden chains of transmission. We made the cgMLST strategy for *B. pertussis* isolate characterization publicly available through a Web-accessible genotyping platform (http://bigsdb.pasteur.fr/bordetella), providing a novel tool for tracking the international spread of *B. pertussis* variants.

## Materials and Methods

### Isolates and DNA Preparation

We sequenced a set of 55 isolates, (online Technical Appendix 1 Table 1, https://wwwnc.cdc.gov/EID/article/24/6/17-1464-Techapp1.pdf). Of these, 24 isolates corresponded to 11 related groups of isolates: 8 isolates originated from 4 different pairs of intrafamilial transmission cases and 16 isolates corresponded to multiple isolates collected from 7 patients (6 pairs and 1 quadruplet); 30 corresponded to a random selection of temporally cocirculating isolates. We used as reference the Tohama isolate (GenBank accession no. NC_002929).

We grew isolates at 36°C for 72 hours on Bordet-Gengou agar (Becton Dickinson, Le Pont de Claix, France) supplemented with 15% defibrinated horse blood (BioMérieux, Marcy l'Étoile, France) and subcultured them in the same medium for 24 hours. We suspended the bacteria in physiologic salt to reach an optical density at 650 nm of 1, and pelleted 400 µL. We suspended the pellets in 100 µL of 1× phosphate-buffered saline, 100 µL of lysis buffer (Roche), and 40 µL of proteinase K; heated them at 65°C for 10 minutes and then at 95°C for 10 minutes; and used them for DNA extraction.

### PFGE

We obtained PFGE profiles using the *XbaI* enzyme, as described previously (*9*,*21*). We conduated analyses by using BioNumerics version 6.6 (Applied-Maths, Sint-Martens-Latem, Belgium).

### MLVA Analysis

We identified variable-number tandem-repeat (VNTR) sequences (*22*) on each whole-genome sequence using blastn (https://blast.ncbi.nlm.nih.gov/Blast) with Tohama alleles as query. To define Tohama alleles, we located the loci using the primer sequences defined for each locus (VNTR-1, -3, -4, -5, and -6) in the Protocols and Tables section of the Netherlands' National Institute for Public Health and the Environment's MLVA website (http://www.mlva.net/bpertussis/default.asp). We defined alleles by counting the number of repeats in the retrieved sequences. We then determined MLVA types using the Single Profile Query section at the same website.

### WGS, Definition of the Core Genome, and Data Analysis

We describe WGS, our definition of the core genome, and data analysis in online Technical Appendix 1. The study accession number in the European Nucleotide Archive is PRJEB21744, including samples ERS1869830–ERS1869884 and their corresponding sequence data.

## Results

### Constitution of the cgMLST Scheme

We identified protein-coding genes of *B. pertussis* that were found in ≥95% of a set of 300 genomes of *B. pertussis* gathered from publicly available data and from our sequencing of isolates from France. We subjected these genes to several filters designed to ensure robustness of genotyping data (online Technical Appendix 1). We then chased artifactual variation of allele calls using assemblies available for 3 reference strains obtained from different sequencing methods and assemblies of 17 isolates from France sequenced with different Illumina (San Diego, CA, USA) sequencing systems (HiSeq and NextSeq; online Technical Appendix 1 Table 2). We also assessed the dependency of allele calls to assembly coverage depth, by using randomly selected read pairs from raw sequencing data of 1 isolate (FR6072), and eliminated the loci that showed variation above 20× coverage depth. These steps led to a final set of 2,038 gene loci, together constituting a *B. pertussis* cgMLST scheme that should minimize artifactual variation caused by the use of different sequencing platforms or sequencing depths. The set of 2,038 core genes had a total length of 1,751,253 bp, covering 42.9% of the Tohama reference genome. The median gene length was ≈1,000 bp (online Technical Appendix 1 Figure 1). Eleven loci were >3,000 bp long and corresponded to genes encoding large proteins such as BrkA, DnaE, RpoB or CyaA (online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/24/6/17-1464-Techapp2.xlsx). Most core genes had <10 alleles within our selection of 300 *B. pertussis* genomes used to define the cgMLST scheme, consistent with previous estimates of sequence variation within this homogeneous pathogenic species (*11*,*13*). Core genes belonged to diverse gene classification categories (online Technical Appendix 1 Figure 2).

### Phylogenetic Analysis of cgMLST

Phylogenetic analysis of the 55 isolates of the study based on concatenated alignments of the 2,038 gene sequences

showed 2 early diverging branches comprising the reference strain Tohama, which belongs to the early-diverging *ptxP1* clade (*13*), and *ptxP1* strain FR6022 (Figure 1). Of the clinical isolates, 49 belonged to clade *ptxP3* and 4 belonged to the previously described clade *ptxP21*, which is derived from *ptxP3* (*23*) (online Technical Appendix 1 Table 1). These 53 non-*ptxP1* isolates were separated according to their *fim3* allele, either *fim3-1* or *fim3-2*. These results are congruent with previous phylogenetic analyses (*13*). Cluster analysis of the cgMLST allelic profiles led to a very similar grouping of isolates (online Technical Appendix 1 Figure 3), indicating that this method can be used for rapid classification purposes.

Groups of intrafamilial or of multiple isolates from the same patient were largely distributed across the phylogenetic tree (Figure 1). Although most groups formed a distinct branch (Figure 1), 3 sets of isolates (FR3916 and FR3917, FR5859 and FR5860, and FR5791 and FR5793), were intermixed in the tree with isolates with no documented epidemiologic link.

### Numbers of Allelic Differences among Epidemiologically Related or Nonrelated Isolates

When considering the 55 isolates of the study, we found only 1 or 2 uncalled allele(s) among the 2,038 loci of the cgMLST scheme: 53.6% of the isolates had 2,038 core

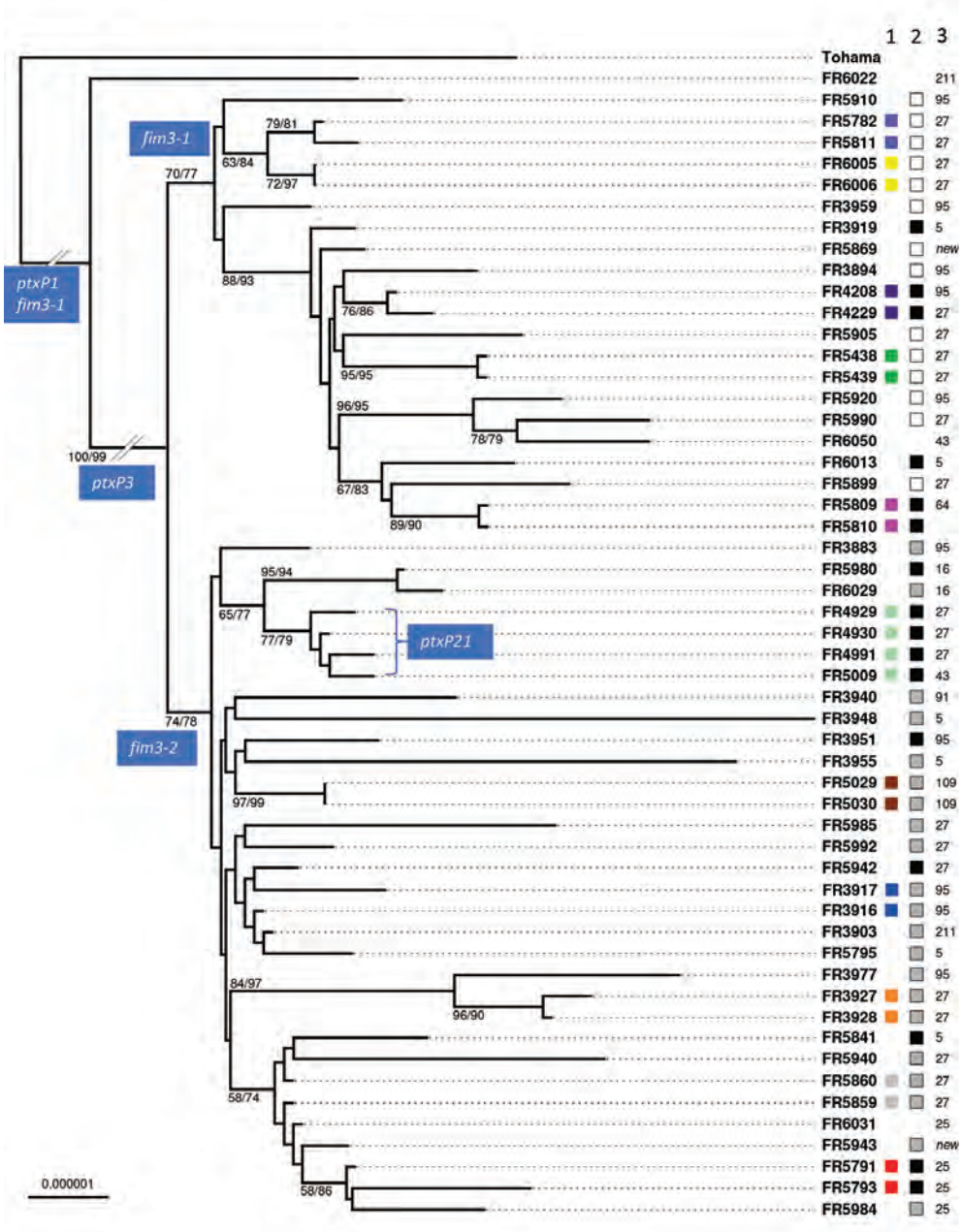


**Figure 1**. Maximum-likelihood phylogenetic tree for *Bordetella pertussis* isolates based on the concatenated multiple sequence alignments of 2,038 core genome multilocus sequence typing loci. The tree was rooted on the Tohama reference isolate (GenBank accession no. NC_002929). Only branch support values >50 are labeled (bootstrap/aLRT-SH). Column 1 to the right of the isolates' names shows colors indicating the intrafamilial groups or groups of multiple isolates from single patients (corresponding to colors shown in inner circle of Figure 2). Column 2 shows pulsed-field gel electrophoresis types (French nomenclature; white, IVα; gray, IVβ; black, IVγ). Column 3 shows multilocus variable-number tandem-repeat analysis types. Scale bar indicates nucleotide substitutions per site.

genes tagged, 42.8% had 1 missing allele, and 3 (5.4%) isolates had 2 missing alleles. We evaluated the pairwise comparisons of allelic profiles and recorded the number of mismatches, defined as allelic differences at loci where both isolates had an allele called. We found the highest numbers of allelic mismatches (close to 66 allelic differences) for the comparisons of non-*PtxP1* isolates with the Tohama reference strain, consistent with this strain belonging to a distant lineage. In turn, comparisons of the *ptxP1* isolate FR6022 with the non-*ptxP1* clinical isolates showed 15–25 allelic differences. Among non-*ptxP1* clinical isolates, the number of allelic mismatches varied from 0 to 15 (median 9). All pairwise comparisons between pairs of intrafamilial isolates or pairs of multiple isolates collected from the same patient showed a median value of 1 allelic mismatch (maximum 2). However, 2.8% (39 of 1,415) pairs of nonrelated isolates also showed either 1 or 2 mismatches only.

### Comparison of cgMLST with PFGE and MLVA

Based on PFGE, all clinical isolates belonged to PFGE group IV. This group is highly predominant in France among contemporaneous (post-2000) isolates, and is subdivided into 3 different subgroups, IVα, IVβ, and IVγ (*24*,*25*), which were all represented in our selection. Analysis of the distribution of PFGE profiles along the cgMLST-based phylogenetic tree (Figure 1) revealed that subgroups IVα and IVβ were separated into 2 clades, corresponding with *fim3-1* (associated with IVα) and *fim3-2* (associated with IVβ). In contrast, PFGE subgroup IVγ was found interspersed in these 2 clades, indicating that it does not represent a natural (monophyletic) grouping of *B. pertussis* isolates. As expected, the same PFGE subgroups were shared by nonrelated and related isolates (Figure 1).

We extracted MLVA profiles from whole-genome sequence assemblies. The main MLVA types were MLVA-27 (38.9%), MLVA-95 (18.5%), MLVA-5 (11.1%), and MLVA-25 (7.4%). The first 3 genotypes were distributed widely across the phylogenetic tree (Figure 1), indicating that, similar to PFGE subgroups, they do not represent proper phylogenetic clades.

### Comparison of cgMLST with a Whole-Genome Single-Nucleotide Polymorphism–Based Approach

We used a mapping approach against the Tohama strain genome as reference (GenBank accession no. NC_002929), and compared the derived single-nucleotide polymorphism (SNP)–based phylogenetic tree to the one inferred from the concatenated multiple sequence alignments obtained from the cgMLST loci (online Technical Appendix 1 Figure 4). Both approaches were highly congruent, grouping the isolates in nearly identical clades. The SNP-based approach led to the identification of 721 variable positions. The highest numbers of SNPs were found for the comparisons of recent isolates (*ptxP3* and *ptxP21*) with the Tohama reference strain (266 ± 10 SNPs) and with the *ptxP1* isolate FR6022 (98 ± 5 SNPs). Among unrelated *ptxP3* clinical isolates, the number of SNPs was 34 ± 9. In the cgMLST gene sequences, there were 206 variable positions, 83.5% of which were included in those identified in the SNP-based analysis. The genome-wide SNP approach might thus be useful as a complementary approach when very high resolution is needed. Comparisons among the 11 related cases showed a very low number of SNPs (no SNP in 9 out of 11 comparisons, 1 SNP in 1 comparison, and 2 SNPs in the remaining comparison), consistent with the cgMLST results. When considering the 3 pairs of isolates not fully resolved using cgMLST, we noticed that FR3916 and FR3917 displayed no SNP between each other, and no SNP with cocirculating isolate FR3903; FR5859 and FR5860 displayed no SNP between each other but ≥18 SNPs compared with FR5940 and 12 SNPs compared with FR5841; and FR5791 and FR5793 displayed no SNP between each other but 3 or 4 SNPs with cocirculating isolate FR5984. These observations show that, except for the first case, whole-genome SNPs discriminate the related pairs from epidemiologically nonrelated isolates better than cgMLST does. Altogether, these results emphasize that, for the highly monomorphic *B. pertussis*, genotyping data will need to be complemented with epidemiologic data to unravel transmission chains.

### Application of cgMLST to Study of Outbreaks from Different Countries

We analyzed publicly available whole-genome sequences corresponding to 3 outbreaks that occurred in California and Vermont (*26*,*27*), USA, and in the United Kingdom (*4*) (online Technical Appendix 1 Table 3). Figure 2 illustrates the phylogenetic relationships of these isolates compared with those from France, based on cgMLST gene sequences. We observed that all pairs of intrafamilial isolates from France and all pairs of multiple isolates recovered from the same patient remained grouped. Isolates from each of the 3 US and UK outbreaks were found in different branches of the phylogenetic tree, consistent with previous results showing that they did not result from the spread of a unique strain (*4*). This finding confirms that the outbreaks of pertussis disease we analyzed corresponded to the simultaneous emergence of multiple strains, consistent with the hypothesis of the silent maintenance of a genetically heterogeneous pool of *B. pertussis* strains in the human population (*4*,*27*).

## Discussion

We developed a cgMLST scheme for *B. pertussis*, one of the most monomorphic bacterial pathogens (*11*,*13*,*28*). Resolving groups of related isolates (such as intrafamilial cases or multiple isolates recovered from the same patient) from nonrelated cocirculating isolates is challenging,

given that the *B. pertussis* population is very homogeneous. Consistent with expectations, few of the 2,038 gene loci of the genotyping scheme were variable among non-*ptxP1* isolates. Despite this low level of genetic diversity, the cgMLST scheme grouped most sets of isolates from direct transmission chains and distinguished them most of the time from cocirculating unrelated isolates. Therefore, this genotyping approach will help define chains of transmission of *B. pertussis*. Nevertheless, close genetic relatedness does not demonstrate direct epidemiologic relatedness. Conversely, isolates from different outbreaks (as defined by the notable increase of clinical cases in defined geographic areas) were genetically heterogeneous, demonstrating a diversity of isolates circulating during pertussis epidemics.

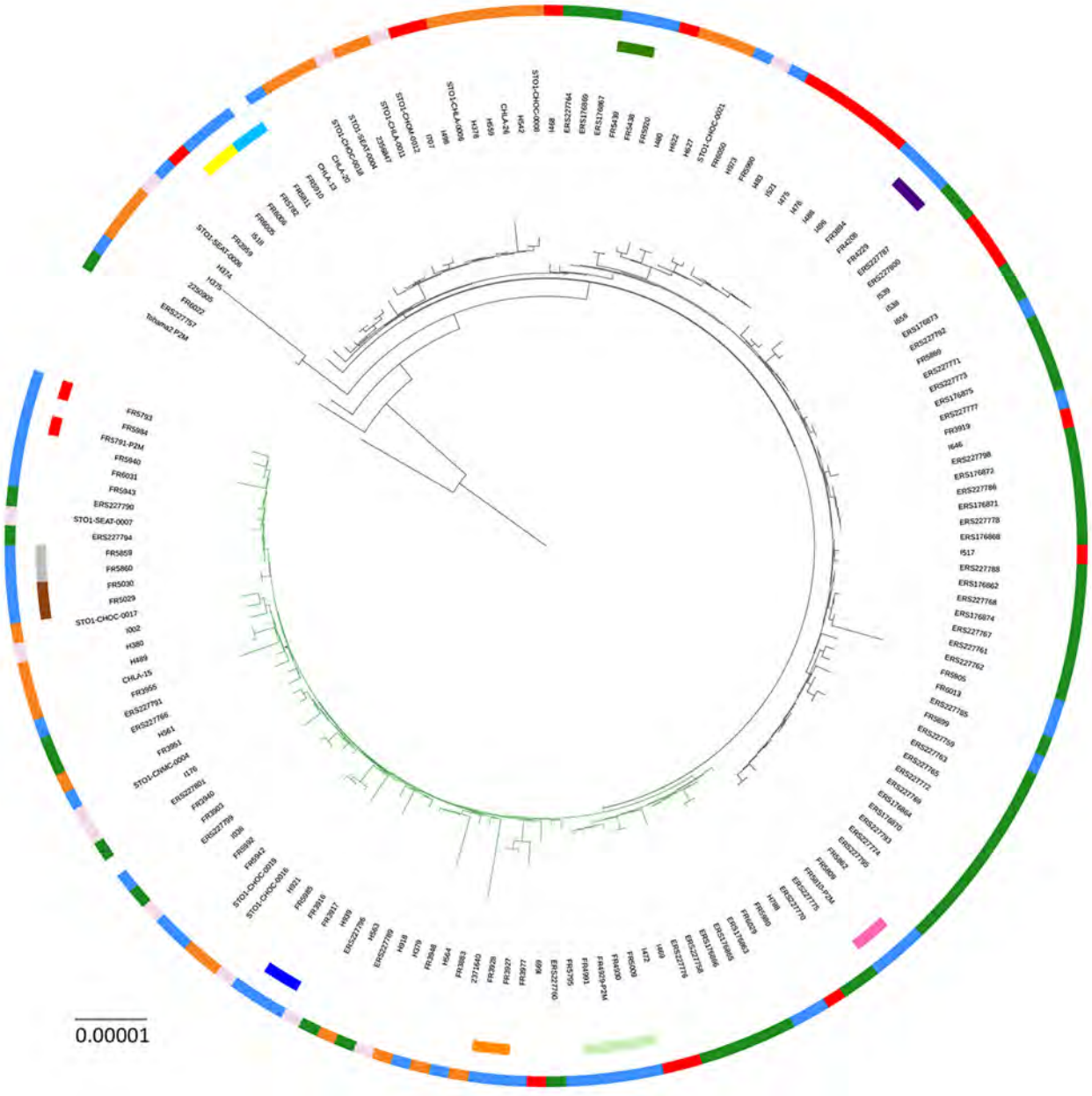


**Figure 2**. Maximum-likelihood phylogenetic tree for *Bordetella pertussis* based on the concatenated alignments of the 2,038 cgMLST loci sequences of isolates from France (this study) and isolates from outbreaks in the United States and the United Kingdom. The tree was rooted on the Tohama reference isolate (GenBank accession no. NC_002929). Black tree branches indicate *fim3-1* clade and green tree branches indicate *fim3-2* clade. Intrafamilial groups of isolates and multiple groups of isolates recovered from the same patient are represented by sectors of the internal circle surrounding the tree (corresponding to colors in column 1 of Figure 1). The external circle indicates the geographic origin of isolates (blue, France; red, Vermont, USA; orange, California, USA; light pink, other United States; green, United Kingdom). Scale bar indicates nucleotide substitutions per site.

Given our ambition to develop a universally applicable cgMLST *B. pertussis* genotyping scheme, we selected the core genes using 300 *B. pertussis* genomes of international origins. Although we cannot exclude that some sublineages of *B. pertussis*, not represented among the 300 isolates, may have lost several of the core loci selected here, we regard this possibility as unlikely and believe that most cgMLST gene loci selected here will be present in most *B. pertussis* isolates.

Remarkably, cgMLST was much more discriminant than PFGE or MLVA, 2 reference epidemiologic typing methods, which appeared unable to distinguish related intrafamilial isolates or isolates collected from the same patient from cocirculating unrelated ones. PFGE and MLVA are widely used and will likely continue to be used until WGS is largely implemented (*29*–*32*), especially in settings in which WGS cannot be achieved because of cost considerations. Our work provides a correspondence between whole-genome–based phylogenetic data and both PFGE and MLVA genotypes and thus provides essential information for the accurate interpretation of typing data from these legacy typing methods.

Even though standardization efforts have been made, it remains difficult to compare PFGE data from different countries. Using isolates from France and the French nomenclature for PFGE profiles (*9*), we demonstrated that some PFGE subtypes, such as type IV-γ, conflate phylogenetically distinct *B. pertussis* sublineages. MLVA typing is more comparable across laboratories but even less discriminatory than PFGE. In contrast to these 2 legacy typing methods, the phylogenetic tree based on cgMLST genes was highly congruent with that obtained from whole-genome SNPs. Therefore, the cgMLST loci, used in combination, represent powerful phylogenetic markers and will enable identification of meaningful groupings of *B. pertussis* isolates. The cgMLST scheme developed here may therefore be a powerful approach for identifying emerging *B. pertussis* sublineages. Because the cgMLST scheme covers only 43% of the genome, a complementary full-genome analysis will be required to define the particular biologic features, such as the loss of vaccine antigen expression, of emerging sublineages.

The cgMLST scheme developed here forms the basis of a unified allele nomenclature database, which was made openly accessible online at http://bigsdb.pasteur.fr/bordetella. This novel gene-by-gene genotyping strategy (*14*) opens the prospects of an internationally unified surveillance, whereby emerging genotypes and sublineages can be recognized in real time by surveillance laboratories. The ability of different national reference centers, microbiology laboratories, and public health agencies to compare *B. pertussis* genotypes will facilitate understanding of transmission dynamics. Further, the harmonization of epidemiologic typing practice by the use of the same genotyping approach will facilitate sharing of experiences among national surveillance systems and has the potential to promote collaboration. Finally, phylogenetic comparisons of isolates from different countries and world regions will facilitate the much-needed studies of the impact of whole-cell or acellular vaccines and the various vaccination strategies in use (*33*,*34*) on the transmission success of particular *B. pertussis* lineages, such as those that evolve toward a lack of expression of vaccine antigens.

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# Use of Bead-Based Serologic Assay to Evaluate Chikungunya Virus Epidemic, Haiti

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The index case of chikungunya virus (CHIKV) in Haiti was reported during early 2014; the vector, the pervasive *Aedes aegypti* mosquito, promoted rapid spread throughout the country. During December 2014–February 2015, we collected blood samples from 4,438 persons at 154 sites (62 urban, 92 rural) throughout Haiti and measured CHIKV IgG by using a multiplex bead assay. Overall CHIKV seroprevalence was 57.9%; differences between rural (mean 44.9%) and urban (mean 78.4%) areas were pronounced. Logistic modeling identified the urban environment as a strong predictor of CHIKV exposure (adjusted odds ratio 3.34, 95% CI 2.38–4.69), and geographic elevation provided a strong negative correlation. We observed no correlation between age and antibody positivity or titer. Our findings demonstrated through serologic testing the recent and rapid dissemination of the arbovirus throughout the country. These results show the utility of serologic data to conduct epidemiologic studies of quickly spreading mosquitoborne arboviruses.

Chikungunya virus (CHIKV) is an arbovirus, transmitted by *Aedes aegypti* and *Ae. albopictus* mosquitoes, that can cause transient but debilitating disease in humans. The World Health Organization reported the first cases of CHIKV on the island nation of Haiti in April 2014; by June 2014, a total of 6,318 cases had been reported there and in 16 other countries or territories in the Caribbean and South America; 103,018 suspected cases were reported (*1*). Additional evidence that CHIKV was introduced into Haiti in 2014 came from evaluation of a longitudinal cohort of children in the coastal town of Leogane during 2011–2014. Before 2014, these children tested negative for CHIKV antibodies, but samples collected in 2014 showed CHIKV IgG; 78.9% of all children seroconverted within the span of 1 year (*2*).

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Confirmation of active CHIKV infection is accomplished through reverse transcription PCR or detection of CHIKV IgM (*3*,*4*). Although confirming infection aids in determining the causative agent of symptoms, only supportive care is currently available for chikungunya, because CHIKV-specific antiviral drugs have not been identified (*5*). Furthermore, using these assays would require persons to have been sampled during active or recent viremia, whereas CHIKV IgG could persist for longer periods of time (*4*,*6*). We present data from a nationwide survey in Haiti in which we used a bead-based serologic assay to determine the overall presence of CHIKV IgG, which provides evidence of past and current exposure.

## Materials and Methods

### Study Population

Our group, which includes Population Services International (PSI), conducted a survey to evaluate malaria prevalence in Haiti during December 2014–February 2015 as part of the malaria control activities supported by the Global Fund (https://www.theglobalfund.org/en/malaria/). In addition to planned multiplex serologic testing for malaria, we chose antigens for nonmalarial febrile diseases before starting data collection; these tests were approved by the Haitian Ministry of Health. The Institut Haïtien de Statistiques et d'Informatique (http://www.ihsi.ht/) had previously subdivided the nation into 12,000 enumeration areas (sections d'énumération, SDEs) on the basis of population density; we chose 154 of these SDEs for this survey through proportional sampling of predicted malaria risk strata within the country, as had been determined by predictive modeling (*7*). The SDEs were classified as urban if they were within the administrative boundary of any of the 140 municipal cities in Haiti and were otherwise considered rural. Field teams randomly selected 20 households within each SDE; all members of selected households were offered participation. Following verbal consent by the participant (or parents or guardian if <15 years of age), blood was collected by finger prick on Whatman 903 Protein Saver cards (GE Healthcare, Marlborough, MA, USA), dried overnight, and

individually stored in plastic bags with desiccant. We assigned samples unique identification numbers that were not traceable to the individual persons. The study protocol was approved by the Haitian Ministry of Health and approved as a nonresearch activity by the US Centers for Disease Control and Prevention (CDC; Center for Global Health determination #2015-04).

A total of 4,438 participants, 1–99 years of age, were included in the survey; the median number of persons sampled per site was 30. During sample collection, we logged global positioning system coordinates for each SDE; we later obtained elevation above sea level in meters by using a digital elevation map of Haiti that was accurate within 1 m.

### Antigen Coupling to Microbeads and Direct Comparison with Anti-CHIKV IgG ELISA

The CHIKV IgG bead assay was designed by CDC laboratories (*2*), and the assay was conducted at CDC laboratories (Atlanta, GA, USA). Carboxyl groups on the surface of specifically classified spectral polystyrene microspheres (BioPlex microbeads; Bio-Rad, Hercules, CA, USA) were converted to reactive esters by using the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide method (Calbiochem, Woburn, MA, USA). The recombinant CHIKV wild-type and mutant (A226V) envelope 1 (E1) antigens (CTK Biotech, San Diego, CA, USA), 7.5 μg each, were covalently linked to 1 mL ($12.5 \times 10^6$ microbeads) of activated microspheres by amide bonds by using phosphate-buffered saline (pH 7.2). To confirm the coupling reaction, the serum we tested was previously found to be highly reactive to the antigens; these showed high median fluorescence intensity (MFI) minus background (MFI-bg) values, indicating appropriate antigen coupling to the microbeads. A seropositivity MFI-bg cutoff value for the antigen-coupled microbeads was determined by using 86 serum specimens from adults from the United States who had not traveled internationally. Of the 86 specimens, 2 outliers had MFI-bg readings >2 SDs above the mean and were eliminated from the analysis as influential outliers. We defined the lower limit for seropositivity to IgG as 594 MFI-bg, which was the fluorescence intensity 3 SD above the mean MFI for the remaining 84 samples.

We evaluated the sensitivity and specificity of the bead assay in comparison to the anti-CHIKV IgG ELISA protocol used by CDC's Division of Vector-Borne Diseases, National Center for Emerging and Zoonotic Infectious Diseases (*2*–*4*). For the ELISA, the viral antigen came from the brain of a suckling mouse and was captured with a monoclonal antibody. Any IgG from a test serum that reacted with this antigen was probed by using goat anti-human IgG linked to alkaline phosphatase. We developed color by using disodium p-nitrophenyl phosphate and read at 405 nm. We subtracted optical density (OD) of a blank well containing sample diluent from the test serum to report a final value of OD minus background (OD-bg). We tested serum samples (n = 50) from CHIKV endemic and nonendemic regions by using ELISA and the bead assay; test comparisons for continuous data are shown in online Technical Appendix Figure 1, panel A (https://wwwnc.cdc.gov/EID/article/24/6/17-1447-Techapp1.pdf) and binary IgG positive/IgG negative data in online Technical Appendix Figure 1, panel B. When we used ELISA as the standard assay, the sensitivity of the bead assay was estimated to be 90% and specificity was 85%. The bead assay signal for anti-CHIKV IgG in Haitians was basically nonexistent before CHIKV introduction (online Technical Appendix Figure 2).

### Dried Blood Spot Elution and Data Acquisition

Elution of whole blood from dried blood spots (DBSs), detection of the IgG bound to the CHIKV antigen-coupled microbeads, and data acquisition by the multiplex bead assay have been described (*8*). In brief, we took a 6-mm circular punch corresponding to 14 μL whole blood from the center of each DBS for elution. Samples were shaken overnight at room temperature in 140 μL protein elution buffer containing PBS (pH 7.2), 0.05% Tween-20, and 0.05% sodium azide. Samples were then stored at 4°C until analysis. Elution from blood spots provided an initial 1:10 dilution of whole blood, and samples were further diluted 1:40 in sample diluent for a final whole blood dilution of 1:400, corresponding to a serum dilution of ≈1:800 on the basis of the assumption of 50% hematocrit in whole blood. We diluted samples in a blocking buffer (sample diluent) containing 0.5% polyvinyl alcohol (Sigma, St. Louis, MO, USA), 0.8% polyvinylpyrrolidine (Sigma), 0.1% casein (ThermoFisher Scientific, Waltham, MA, USA), 0.5% bovine serum albumin (Millipore, Burlington, MA, USA), 0.3% Tween-20, 0.1% sodium azide, and 0.01% *E. coli* extract to prevent nonspecific binding. Assay reagent diluent (Buffer C) consisted of PBS-Tween (ThermoFisher Scientific, Waltham, MA, USA) plus 0.5% bovine serum albumin and 0.02% sodium azide. We prewetted filter bottom plates (Multiscreen 1.2 μmol/L, Millipore) with PBS-Tween, added 1,500 microbeads/classification each well, and incubated with sample in duplicate for 1.5 h under gentle shaking. We then added secondary antibodies tagged with biotin (1:500 anti-human $IgG_{1-3}$; Southern Biotech, Birmingham, AL; 1:2,500 anti-human $IgG_4$; Sigma) and incubated for 45 min. Next, we added streptavidin-phycoerythrin (1:200; Invitrogen, Carlsbad, CA, USA) and incubated for 30 min. Plates had a final wash incubation with Buffer C for 30 min and were read on a Bio-Plex 200 instrument by generating the median fluorescence signal for 50 microbeads/analyte. We calculated the mean from duplicate wells, each with an MFI (1 – 32,766 channels) by using Bio-Plex Manager 6.1 software (Bio-Rad). We subtracted background from a DBS blank from all sample MFI values to give a final MFI-bg value that we used for analysis.

### Statistical Methods

We used the Mann-Whitney rank sum test to determine differences between groups for continuous variables and the z-test to determine the significance of differences between 2 groups for proportions. We considered $p<0.05$ statistically significant. We modeled the relationship between IgG against chikungunya E1 antigen and urban environment, elevation, and age by using logistic regression (GENMOD procedure in SAS version 9.4; SAS Institute, Cary, NC, USA) and reported 95% Wald CIs with a null hypothesis of $\beta_x = 0$. The final logistic regression model was written as logit P(chikpos = 1) = $\beta_0$ + $\beta_1$(urban setting) + $\beta_2$(elevation[m]) + $\beta_3$(age[years]). We used generalized estimating equations to account for clustering at the SDE level when obtaining p values and 95% CIs.

## Results

### Prevalence of IgG Responses to CHIKV

The overall CHIKV antibody seroprevalence was 57.9% (2,570/4,438 persons). However, rates of seropositivity were highly variable: 78.4% (1,350/1,722) prevalence in urban areas (range 20%–100%) and 44.9% (1,220/2,716) in rural areas (range 8%–100%) (Figure 1). Many of the rural areas sampled were located inland, away from the coast and at higher elevations. In contrast, many of the urban sampling sites were located nearer the coast at lower elevations. Median IgG responses in urban areas (MFI-bg 1,856) were significantly higher ($p<0.001$) than median IgG responses in the rural areas (MFI-bg 298), and median elevation above sea level in the urban areas (38 m) was significantly lower than the median elevation in the rural areas (228 m; $p<0.001$).

### Relationship between Seroprevalence and IgG Titer with Elevation

The prevalence of positivity for CHIKV IgG and MFI-bg assay signal (indicating the magnitude of a person's IgG response), by elevation categories, for urban and rural areas is shown in Figure 2. Modeling to account for the effects of the urban setting, elevation, and age showed a substantial increase in likelihood of a patient's sample testing CHIKV IgG–positive in an urban setting (adjusted odds ratio of 3.34; Wald $\chi^2$ 48.7; 95% CI 2.38–4.69), considering the rural setting as the referent and accounting for the within-subject factor of sampling multiple persons within an SDE. For every 100 m increase in elevation, the log odds of CHIKV IgG positivity decreased 14.8%; the interaction between elevation and the urban setting was not significant (Wald $\chi^2$ 0.56; $p = 0.45$). Seroprevalence estimates remained higher for populations sampled in urban settings <600 m elevation and steadily decreased among increasing elevation of rural communities (Figure 2, panel A). In directly comparing urban versus rural CHIKV IgG seroprevalence for each elevation category, only the difference within the 100–200 m category was found to be significant ($p = 0.024$), although urban/rural differences for other categories at elevations <600 m approached statistical significance ($p = 0.06$–$0.09$). We found no difference in the rate of IgG positivity when comparing urban and rural settings at >600 m ($p = 0.29$), but this segment only accounted for 9.5% of the study population.

We saw a more striking difference between urban and rural areas when maintaining a continuous scale for antibody titer. At all elevation categories <600 m, we found a significantly higher IgG titer for persons living in urban versus rural areas ($p<0.001$ for all elevations), but we found no difference in MFI-bg for persons living at elevations >600 m ($p = 0.26$) (Figure 2, panel B). Altogether, we found a significantly higher median IgG response ($p<0.001$) for persons living at elevations <600 m (1,217 MFI-bg) than for persons living at elevations >600 m (66 MFI-bg). When the analysis was restricted to persons living ≤600 m elevation, modeling for the relationship between IgG titer and elevation showed a sharp decrease of 216 MFI-bg units (F value 47.0; $p<0.001$) in rural settings for every 100 m increase in elevation. In the urban setting, we found a predicted increase in IgG titer for every 100 m increase in elevation, but this increase was not significant ($p = 0.12$).

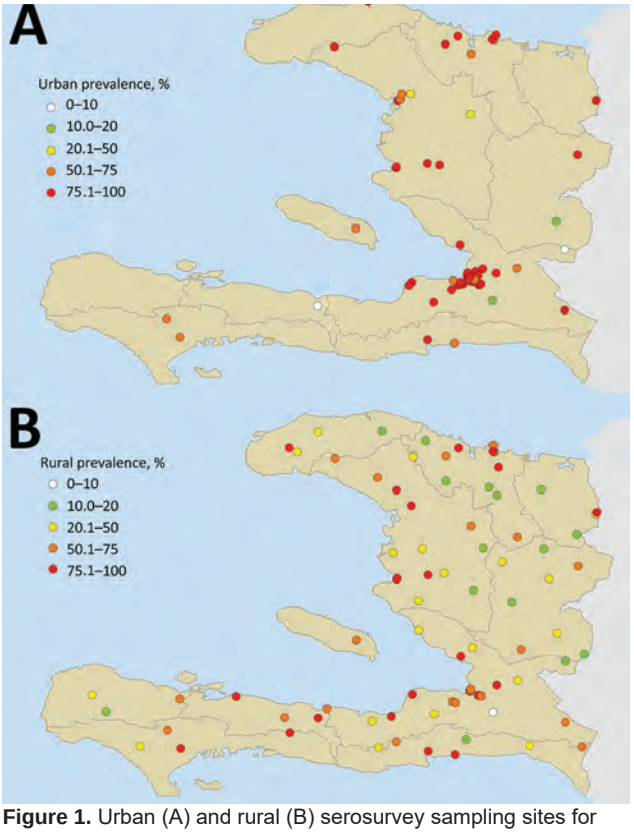


**Figure 1.** Urban (A) and rural (B) serosurvey sampling sites for chikungunya prevalence, Haiti, December 2014–February 2015. Geolocated point seroprevalence is shown as the percentage of the sampled population positive for chikungunya IgG.

### Relationship between Seroprevalence and IgG Titer and Age by Urban and Rural Setting

Seroprevalence of CHIKV IgG and a plot of median MFI-bg (magnitude of IgG response) by age categories for persons living in urban versus rural areas are shown in Figure 3. These graphs show similar patterns; seroprevalence and MFI-bg responses were consistently higher from persons living in urban versus rural areas, regardless of age. Median MFI-bg was higher in urban areas when compared with rural areas for all age groups ($p<0.01$ for all categories except age >80 y, $p = 0.03$) (Figure 3, panel B). By using regression models, we did not find age to be a significant predictor when considering either seroprevalence of CHIKV IgG ($p = 0.34$) or magnitude of the IgG response ($p = 0.19$), indicating equivalent probability of lifetime exposure for all Haiti residents, regardless of age. Children 0–10 years of age in rural areas showed exceptionally low median MFI-bg compared with urban children of the same age group.

## Discussion

From this serosurvey, we found 57.9% of the 4,438 residents of Haiti that were tested had IgG responses to CHIKV by early 2015. Although our sample was not nationally representative, we may have underestimated true exposure rates, because 38.8% of persons sampled resided in urban areas, and population estimates for Haiti calculated the percentage of the population in cities at 48.8% (*9*). We found a seroprevalence of 78.4% in urban areas, similar to the 78.9% seroprevalence previously found in the urban area of Leogane in August 2014 (*10*). Considering that the first confirmed cases of CHIKV in Haiti were reported in April 2014 (the same month confirmed cases were seen in the Dominican Republic [*1*]), it is notable that >50% of the population would have evidence of exposure to this arbovirus within 9 months of introduction. The fast spread of CHIKV has been observed before: in the French island of La Reunion in the 2005–2006 outbreak, more than one third of the population was believed to have been exposed, and 63% of the persons living on the island of the Union of the Comoros were exposed to CHIKV during the initial outbreak there (*11*,*12*).

One of our most striking findings was that, even after controlling for age and elevation, persons in urban areas had a >3-fold increase in odds (adjusted odds ratio 3.34) of CHIKV exposure. This result, in combination with the increased likelihood of CHIKV exposure at lower elevation, is directly in line with the consistent observation that the *Aedes* vector house index (HI) is typically higher in these settings. Multiple studies have observed an increase in *Ae. aegypti* vector HI and breeding sites in urban areas when compared with rural locations (*13*–*17*). Previous findings showing increased prevalence of *Ae. aegypti* mosquito colonization in areas of high-density housing and higher water temperature allude to the importance of the urban environment for this vector (*18*). These similar behavioral patterns have recently been observed in Haiti, where *Ae. aegypti* mosquitoes are readily observed in urban and periurban settings and have a propensity for manmade habitats (*19*,*20*). Although *Aedes* sp. mosquito populations have not been mapped by elevation in recent surveys in Haiti, past studies showed substantial decreases in prevalence of hemagglutination inhibition antibodies to dengue virus (as well as to the vector) in moving from low-lying coastal to mountainous inland areas (*21*). We observed a gradual and consistent decrease in seroprevalence to CHIKV IgG for rural areas of increasing elevation ≥600 m, but this same

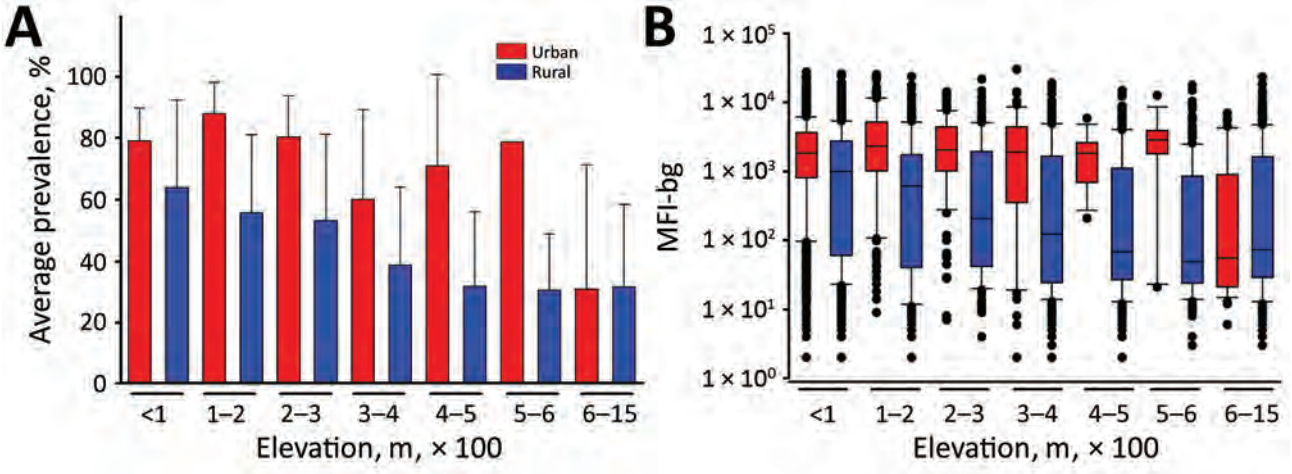


**Figure 2.** Seroprevalence and chikungunya IgG levels among persons living in urban and rural areas at different elevations, Haiti, December 2014–February 2015. A) Seroprevalence mean of persons sampled in urban or rural areas at different elevations; error bars indicate SEM. B) Chikungunya IgG median fluorescence intensity minus background signal by urban and rural sampling sites at different elevations. Boxes indicate interquartile ranges; horizontal lines within boxes indicate medians; black dots indicate values <10th or >90th percentiles; error bars indicate 10th and 90th percentiles of data. M, meters; MFI-bg, median fluorescence intensity minus background.

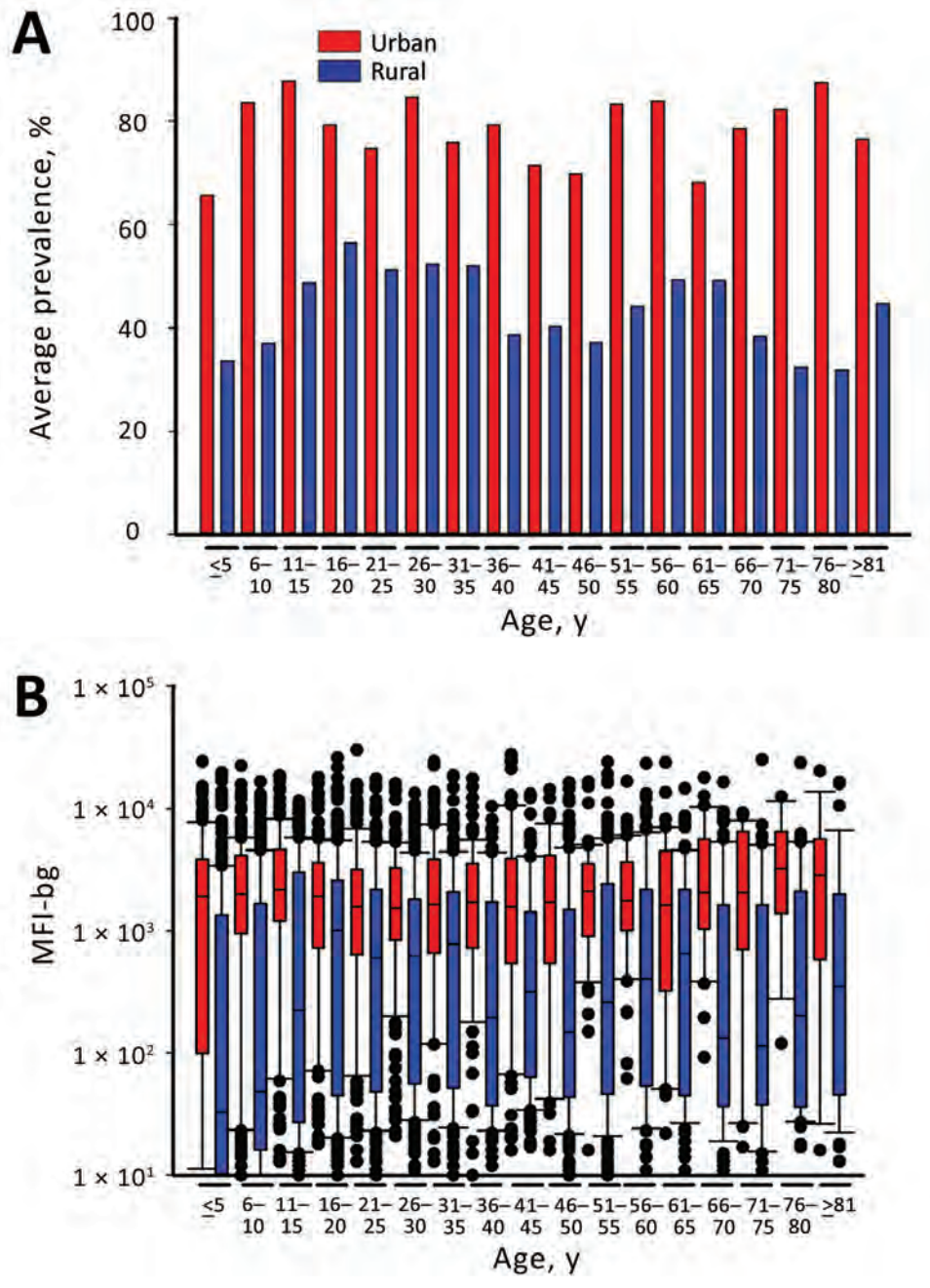


**Figure 3**. Seroprevalence and chikungunya IgG levels among persons living in urban and rural areas, by age group, Haiti, December 2014–February 2015. A) Mean seroprevalence by urban or rural setting and age category. B) Range of median fluorescence intensity minus background (IgG responses) to chikungunya antigens for the same age categories. Bars indicate interquartile ranges; horizontal lines within bars indicate medians; black dots indicate values >10th or <90th percentiles; error bars indicate 10th and 90th percentiles of data. y, years; MFI-bg, median fluorescence intensity minus background.

pattern was less striking for persons sampled from urban areas. This finding may indicate the preservation of *Ae. aegypti* mosquito habitat in urban settings, even at elevations in the hundreds of meters in Haiti; previous entomological work has found limits of distribution of this vector to be largely related to temperature (*22*).

Persons residing >600 m elevation showed no difference in seroprevalence or CHIKV IgG titers regardless of whether they lived in an urban or rural setting. This finding shows that urban environments in Haiti become less suitable for vector habitat when located at areas of increasing elevation and decreasing temperatures (*23*). Unfortunately, because <10% of our study population resided at elevations >600 m, we were unable to further segregate higher elevation categories.

A notable limitation to our study is the assumption of persons acquiring CHIKV infection at the geographic location of residence, as this was the location in which persons were sampled. Travel history was not gathered for participants in this study.

In our study, all age groups living in urban areas showed substantially higher MFI-bg signal intensity (IgG responses) to the CHIKV antigens compared with the same age groups living in rural areas. Differences were not seen between IgG prevalence or titer between younger and older age categories, which is atypical for serologic studies of infectious diseases (*2*,*9*,*24*,*25*), because an older age indicates more life-years of potential exposure to an endemic infectious disease and, thus, acquisition of IgG. These serologic findings provide strong evidence for the rapid dissemination of CHIKV by the *Aedes* vector. It appears all age groups in the population were uniformly susceptible to a high rate of transmission, with a preference for urban populations. Of note was the finding that children <10 years of age living in rural areas had lower CHIKV seroprevalence and titers; possible explanations for this include reduced mosquito exposure or transmission efficiency in this setting, or differential behavior patterns among these children as compared to their urban counterparts.

The multiplex bead assay is an excellent serosurvey platform that can simultaneously analyze IgG responses to multiple antigens from multiple pathogens. This study shows the utility of IgG detection by using this assay for monitoring exposure history of a population to an infectious disease and predicting populations most susceptible to exposure after arboviral introduction to a naive population. Because the initial aim of laboratory data collection for this serosurvey was to measure serologic responses to malaria in Haiti, the addition of microbeads coupled to the CHIKV-E1 antigen to the laboratory assay cost <$700 USD, and no additional time was required for data collection. Future serosurveys representative of a population may offer opportunities to collect data on arboviruses and other infectious diseases to provide health officials with quality data to direct disease programs.

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# Widespread *Treponema pallidum* Infection in Nonhuman Primates, Tanzania

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We investigated *Treponema pallidum* infection in 8 nonhuman primate species (289 animals) in Tanzania during 2015–2017. We used a serologic treponemal test to detect antibodies against the bacterium. Infection was further confirmed from tissue samples of skin-ulcerated animals by 3 independent PCRs (*polA*, *tp47*, and *TP_0619*). Our findings indicate that *T. pallidum* infection is geographically widespread in Tanzania and occurs in several species (olive baboons, yellow baboons, vervet monkeys, and blue monkeys). We found the bacterium at 11 of 14 investigated geographic locations. Anogenital ulceration was the most common clinical manifestation; orofacial lesions also were observed. Molecular data show that nonhuman primates in Tanzania are most likely infected with *T. pallidum* subsp. *pertenue*–like strains, which could have implications for human yaws eradication.

The geographic distribution of infection with the bacterium *Treponema pallidum* in nonhuman primates (NHPs) in Africa has been reported to closely match the one seen in human yaws in Africa before the first yaws eradication campaign (*1*). Some Africa countries, such as Tanzania, have a history of human yaws but lack recent epidemiologic data that support elimination (*2*). At the same time, many of these countries report NHP infection with *T. pallidum* strains that are highly similar to the human yaws–causing *T. pallidum* subsp. *pertenue* (TPE) (*3*,*4*; S. Knauf et al., unpub. data, https://www.biorxiv.org/content/early/2017/05/10/135491) and thus make NHP infection an important issue for a One Health approach.

The first published report of *T. pallidum* infection in Tanzanian NHPs came from anogenital ulcerated olive baboons (*Papio anubis*) at Gombe National Park (GNP) in the late 1980s (*5*), followed by cases reported from olive baboons at Lake Manyara National Park (LMNP) (*3*,*6*,*7*) and Serengeti National Park (SNP) (*3*). Clinical manifestations of *T. pallidum* infection in NHPs ranged from asymptomatic to severe skin ulceration mainly affecting the face or genitalia (*8*). Although early serologic investigations conducted by Fribourg-Blanc in West Africa confirmed widespread infection in several NHP species (e.g., baboons [*Papio* sp.], guenons [*Cercopithecus* sp.], red colobus [*Piliocolobus badius*], and chimpanzees [*Pan trogoldytes*]) (*9*), the infection in Tanzania was exclusively reported from olive baboons in northern parts of the country. Despite the close genetic relationship to human yaws–causing TPE strains (*3*,*4*; S. Knauf et al., unpub. data, https://www.biorxiv.org/content/early/2017/05/10/135491), and in the absence of recent reports of human yaws in Tanzania (*10*), it is currently unclear whether NHP strains naturally infect humans.

As a starting point and basis for advanced epidemiologic investigations, our main objective was to investigate the geographic distribution and host species composition of *T. pallidum* infection in free-ranging NHPs in Tanzania. We hypothesized that, in Tanzania, A) NHPs other than olive baboons are infected with the *T. pallidum* bacterium and B) that infection is not restricted to northern parts of the country.

## Materials and Methods

### Study Design, Sampling Sites, and Animals

We applied a cross-sectional study design using semirandom selection of free-ranging NHPs in selected areas in

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[1]Deceased.

Tanzania. Selection of NHPs was biased toward animals with visible skin ulcers. Sampling took place at Arusha National Park (ANP), GNP, Katavi NP (KNP), LMNP, Mahale NP (MNP), Mikumi NP (MKNP), Ngorongoro Conservation Area (NCA), Ruaha NP (RNP), Selous Game Reserve (SGR), SNP, Tarangire NP (TNP), Udzungwa NP (UNP), and Issa Valley (Issa), as well as Jozani-Chwaka Bay NP–Masingini Forest (JCBNP) on Unguja Island, Zanzibar (Figure 1). We investigated the following species: olive baboon, yellow baboon (*Papio cynocephalus*), blue monkey (*Cercopithecus mitis*), red-tailed monkey (*Cercopithecus ascanius*), vervet monkey (*Chlorocebus pygerythrus*), Udzungwa red colobus (*Piliocolobus gordonorum*), Zanzibar red colobus (*Piliocolobus kirkii*), and Ugandan red colobus (*Piliocolobus tephrosceles*). Using FreeCalc (http://epitools.ausvet.com.au/content.php?page=FreeCalc2), and based on our previous study at LMNP (*6*) that showed a disease prevalence of 85%, we calculated a sample size of ≥4 (expected disease prevalence 85%) to 21 (expected disease prevalence 25%) per sample site as statistically sufficient to demonstrate freedom from *T. pallidum* infection using imperfect tests and allowing for small populations (online Technical Appendix 1, https://wwwnc.cdc.gov/EID/article/24/6/18-0037-Techapp1.pdf).

### Anesthesia and Sampling

We studied the animals in accordance with applicable regulations and guidelines (online Technical Appendix 1). The sampling of blood and skin tissue followed a standardized protocol that we previously applied for baboons (*6*,*11*). In brief, the NHPs were chemically immobilized by remote distance injection of 10.0 mg ketamine/kg body mass (Kyron Laboratories, Johannesburg, South Africa) in combination with 0.2 mg/kg medetomidine (Domitor; Pfizer, Berlin, Germany). Anesthetics were intramuscularly injected using a cold-gas immobilization rifle (MOD JM; Dan-Inject ApS, Børkop,

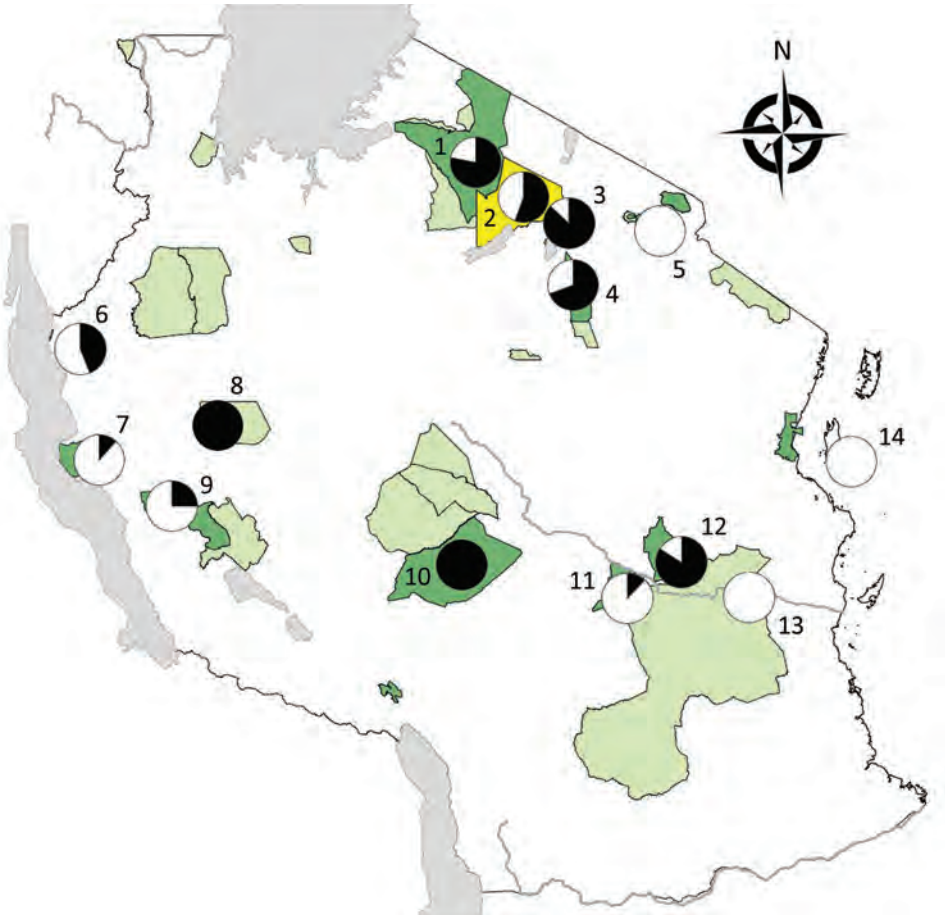


**Figure 1**. Protected areas and sites where free-ranging nonhuman primates (NHPs) were sampled in a study of *Treponema pallidum* infection, Tanzania. 1, Serengeti National Park (41 NHPs); 2, Ngorongoro Conservation Area (18 NHPs); 3, Lake Manyara National Park (38 NHPs); 4, Tarangire National Park (26 NHPs); 5, Arusha National Park (14 NHPs); 6, Gombe National Park (32 NHPs); 7, Mahale National Park (17 NHPs); 8, Issa Valley (2 NHPs); 9, Katavi National Park (12 NHPs); 10, Ruaha National Park (18 NHPs); 11, Udzungwa National Park (25 NHPs); 12, Mikumi National Park (25 NHPs); 13, Selous Game Reserve (8 NHPs); 14, Jozani-Chwaka Bay National Park–Masingini Forest on Unguja Island, Zanzibar (13 NHPs). Dark green indicates national parks; light green indicates game reserves; yellow indicates conservation area. Circle graphs: black, NHPs *T. pallidum*–positive (serology and/or PCR); white, NHPs T. pallidum–negative (serology and PCR). The map was produced with ArcMap version 10.0 (ESRI, Redlands, CA, USA) by using shape files available from ESRI (national boundary of Tanzania, water bodies of Africa, main rivers of Africa). The shape files of the conservation areas of Tanzania were provided by the Tanzania National Park Authority and are available free from http://www.arcgis.com/home/item.html?id = 9b06fe723ad14991b30b1b85953224c1. Prevalence circles were generated using Excel version 15.38 (Microsoft, Redmond, WA, USA).

Denmark) and appropriate projectiles. Immobilized NHPs were continuously observed for vital parameters such as respirations, pulse frequency, and internal body temperature. We monitored pulse frequency and blood oxygen saturation using a Nellcor OxiMax N65 Pulse Oximeter (Tyco Healthcare Deutschland GmbH, Neustadt, Germany). Anesthetized animals underwent a standardized health check with special focus on skin lesions. We collected whole blood from the femoral vein using an S-Monovette closed blood collection system (Sarstedt, Nümbrecht, Germany) mounted with a 20G needle. We collected two 9-mL serum tubes under aseptic conditions. We then centrifuged serum tubes at 55,000 relative centrifugation force for 15 min, transferred serum into cryovials, and stored the vials in liquid nitrogen. In animals with skin lesions, we took a 6-mm biopsy from the skin ulcer using a sterile dermal biopsy punch. From each animal (and ulcer), we preserved tissue samples in lysis buffer (10 mmol/L Tris [pH 8.0], 0.1 EDTA, and 0.5% sodium dodecyl sulfate).

We treated animal wounds with Silverspray (Silver Aluminum Aerosol; Henry Schein, Langen, Germany) and allowed animals to recover under close supervision. Samples were temporarily stored at –80°C at the Tanzania Wildlife Research Institute headquarters (Arusha, Tanzania). Aliquots were exported to the German Primate Center (Göttingen, Germany) for further analysis and additional confirmation.

### Serologic Testing

We used a commercially available treponemal test (ESPLINE TP; Fujirebio Diagnostics, Hannover, Germany) to check all serum samples for *T. pallidum* antibodies. The assay has been validated for use in baboons (*12*), where it performed with 97.7% (95% CI 87.7%–99.9%) sensitivity and 96.0% (95% CI 79.7%–99.9%) specificity. We tested serum samples on the day of sampling and operated and interpreted test cassettes according to the manufacturer's guidance.

### DNA Extraction from Skin Tissue

We extracted DNA following the standard protocol of the QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany), with some minor modifications. In brief, we cut ≈25 mg tissue into small pieces and incubated in 180 μL lysis buffer, in which the sample had been stored since collection. After adding 20 μL proteinase K, samples were digested overnight at 56°C and 900 rpm (Thermomixer Comfort; Eppendorf, Hamburg, Germany). We added an additional washing step using 300 μL AW1 buffer and eluted the DNA twice with 100 μL AE buffer. We further purified extracted DNA using glycogen precipitation according to the protocol published in Knauf et al. (*13*).

### *TP_0105* (*polA*) Amplification and Sequencing

We performed PCR targeting the polymerase 1 gene (*TP_0105*, *polA*) of *T. pallidum* by using primers designed by Liu et al. (*14*). This assay has a reported sensitivity of 95.8% and a specificity of 95.7% and has been demonstrated to segregate pathogenic *T. pallidum* subspecies from nonpathogenic treponemes, other spirochetes, and 59 species of bacteria and viruses including those causing genital ulcers in humans (*14*). The 50-μL reaction volume comprised 25 μL 2 × Universe High-Fidelity Hot Start DNA Polymerase Master Mix (Biotool, Munich, Germany), 17 μL RNAase free water, 2 μL of each 10 μmol/L primer, 1 μL DNA polymerase (1 U/μL), 1 μL of 10 mmol/L each dNTP, and 2 μL template DNA, independent of DNA concentration. We conducted amplification in a SensoQuest Labcycler using the following thermocycling conditions: predenaturation at 95°C for 3 min, followed by 50 cycles each with 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s. The profile was completed with a postextension step at 72°C for 5 min and indefinite cooling of the PCR product at 8°C. All *polA* PCR products were run on a 1% agarose gel to check for PCR performance and correct amplicon size. We gel extracted a representative subset of the PCR products (n = 19), purified with the QIAGEN Gel Extraction Kit (QIAGEN), and Sanger sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the amplification primers. Sequencing was performed by Seqlab Sequence Laboratories (Microsynth, Göttingen, Germany).

### *TP_0574* (*tp47*) Quantitative PCR

We performed TaqMan real-time PCR targeting a 132-bp fragment of the *TP_0574* gene. Primers and probe used were published elsewhere (*15*). The reaction encompassed 10 μL TaqMan Universal MasterMix II (no Uracil-N glycosylase; Applied Biosystems) and 1.8 μL of each 10 μmol/L primer and the probe. Total genomic DNA concentration added to each reaction was normalized to 100 ng. Molecular-grade water was used to adjust the reaction volume to 20 μL. Cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 50 cycles each at 95°C for 15 s and 60°C for 60 s. Reactions were run on a StepOnePlus Real-Time PCR System (Applied Biosystems). We measured all samples as triplicates and analyzed data using StepOne version 2.3 software (Applied Biosystems).

### *TP_0619* Amplification and Sequencing

We performed PCR targeting the *TP_0619* gene of *T. pallidum* to distinguish infection with TPE or *T. pallidum* subsp. *endemicum* (TEN) strains from infection with *T. pallidum* subsp. *pallidum* (TPA) strains. At this locus, TPA differs from TPE and TEN in ≥73 positions (online Technical

Appendix 1 Figure). We used primers 5′-TTACCCAGACATTTTTCTCCACATA-3′ and 5′-TACAAGCTCCCACAATGCCA-3′ to amplify a 608-bp fragment. The PCR conditions and working steps were identical to the PCR targeting the *polA* gene, except that the annealing temperature was adjusted to 55°C.

### Data Analysis

We performed statistical analyses using GraphPad Prism version 7.0c (GraphPad Software, La Jolla, CA, USA), and R version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria). We compared variables such as the presence of *T. pallidum* antibodies and clinical manifestations per species by using $2 \times 2 \times n$ contingency tables and a 2-tailed Fisher exact test. We used a $\chi^2$ test to compare the outcome of >2 sampling sites using $n \times 2$ contingency tables. Proportions were tested at a critical probability of 0.05 and 95% CI. We considered $p \leq 0.05$ as statistically significant.

We analyzed and edited retrieved sequence data using 4Peaks 1.8 (http://www.nucleobytes.com) and SeaView 4.5.4 software (*16*). We compared sequences with respective orthologs available in GenBank using a standard nucleotide BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

## Results

### NHP Species

We sampled 289 NHPs (Table) and confirmed previously reported *T. pallidum* infection in olive baboons at GNP (*5*,*17*,*18*), SNP (*3*), NCA (*3*), and LMNP (*3*,*6*,*7*). In addition, we report *T. pallidum* infection in yellow baboons, vervet monkeys, and blue monkeys in different regions of Tanzania (Table; Figure 1; online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/24/6/18-0037-Techapp2.xlsx).

The overall mean seropositivity of *T. pallidum* infection in the NHP samples was 53.3% (154/289). More female (82/135 [60.7%]) than male (72/154 [46.8%]) NHPs had *T. pallidum* antibodies. Overall, 35/45 (77.8%) vervet monkeys, 85/137 (62.0%) olive baboons, 33/75 (44.0%) yellow baboons and 1/15 (6.7%) blue monkeys had antibodies against the bacterium. Most (94 [61.0%]) of the 154 seropositive NHPs appeared healthy without any clinical skin lesions. The association between *T. pallidum* antibodies and skin ulceration was tested using 2-tailed Fisher exact test and was significant in olive baboons (n = 137; odds ratio [OR] 15.95 [95% CI 4.7–51.1]; p<0.0001) and yellow baboons (n = 75; OR 11.04 [95% CI 1.7–126.8]; p = 0.0185), but not in vervet (n = 45; OR ∞ [95% CI 0.0–1.0]; p = 0.0888) and blue monkeys (n = 15; OR 0.00 [95% CI 0.0–126.0]; p>0.9999 [dataset is provided in online Technical Appendix 2]). No *T. pallidum* antibodies were detected in the 10 Zanzibar red colobus, 3 Udzungwa red colobus, 2 Ugandan red colobus, and 2 red-tailed monkeys sampled. Moreover, none of these 4 species showed any kind of skin ulceration (Table).

### Clinical Manifestations

Among the 156 *T. pallidum*–seropositive and/or PCR-positive NHPs (including 2 serologically negative but PCR-positive animals) and across the different sampling sites, we found anogenital ulcers associated with the infection (Figure 2, panel A) in 59.8% ± 23.9% of the yellow baboons (mean ± SEM, 6 investigated sites; data were analyzed as fraction of *T. pallidum*–infected animals with anogenital lesions per sampling site); 45.6% ± 16.2% of the olive baboons (mean ± SEM, 6 investigated sites); and 31.6% ± 9.4% of the infected vervet monkeys (mean ± SEM, 9 investigated sites). One of the 2 *T. pallidum*–infected blue monkeys showed anogenital skin ulceration; the second animal was clinically healthy. Orofacial lesions (Figure 2, panel B) were exclusively observed in

**Table.** Test results of *Treponema pallidum* infection in samples of free-ranging nonhuman primate species, Tanzania*

| | | Total/seropositive/skin lesion/PCR positive† | | | | | |
|---|---|---|---|---|---|---|---|
| | | Positive | | | Negative | | |
| Species | No. (%) | Total | Male | Female | Total | Male | Female |
| Olive baboon (*Papio anubis*) | 137 (47.4) | 86 | 34/34/12/12 | 52/51/31/30‡ | 51 | 29/0/1‡/NA | 22/0/1‡/NA |
| Yellow baboon (*Papio cynocephalus*) | 75 (26.0) | 33 | 17/17/2/2 | 16/16/5/5 | 42 | 27/0/1§/NA | 15/0/0/NA |
| Vervet monkey (*Chlorocebus pygerythrus*) | 45 (15.6) | 35 | 21/21/10/8‡ | 14/14/1/1 | 10 | 7/0/0/NA | 3/0/0/NA |
| Blue monkey (*Cercopithecus mitis*) | 15 (5.2) | 2 | 1/0/1/1 | 1/1/0/0 | 13 | 8/0/0/NA | 5/0/0/NA |
| Red-tailed monkey (*Cercopithecus ascanius*) | 2 (0.7) | 0 | NA | NA | 2 | 2/0/0/NA | NA |
| Zanzibar red colobus (*Piliocolobus kirkii*) | 10 (3.5) | 0 | NA | NA | 10 | 4/0/0/NA | 6/0/0/NA |
| Udzungwa red colobus (*Piliocolobus gordonorum*) | 3 (1.0) | 0 | NA | NA | 3 | 2/0/0/NA | 1/0/0/NA |
| Ugandan red colobus (*Piliocolobus tephrosceles*) | 2 (0.7) | 0 | 0/0/0/0 | 0/0/0/0 | 2 | 2/0/0/NA | NA |
| Total | 289 (100.0) | 156 | 73 | 83 | 133 | 81 | 52 |

*Results are based on the consensus of detected *T. pallidum* antibodies (ESPLINE TP) and PCR results of 3 independent gene targets (*polA*, *tp47*, and *TP_0619*). NA, not applicable.
†PCR was conducted only on animals with skin lesions.
‡No skin sample was available for some positive animals.
§Skin lesion at the genitalia most likely from fight; no tissue sample available.

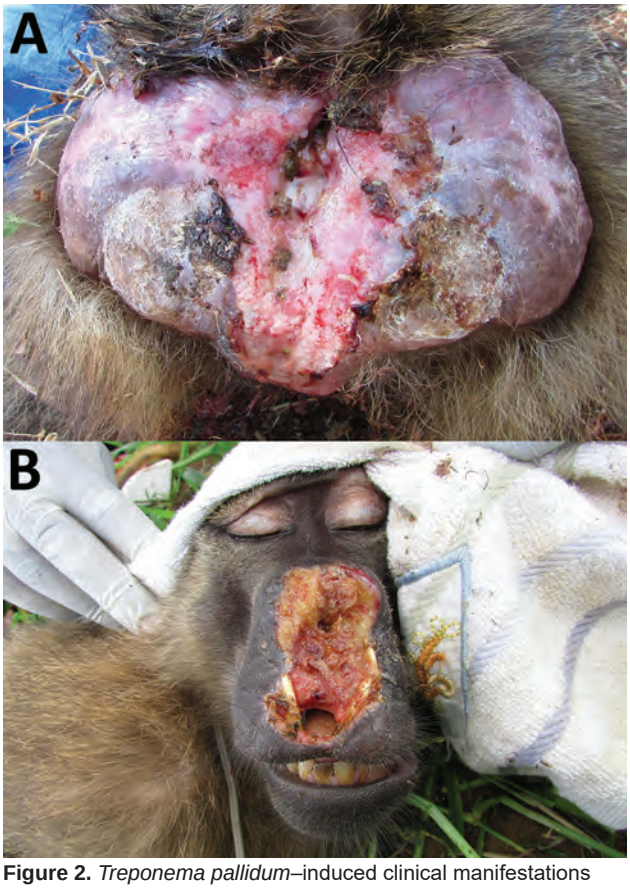


**Figure 2.** *Treponema pallidum*–induced clinical manifestations affecting olive baboons (*Papio anubis*), Tanzania. A). Lesions on the anogenital area of animal at Lake Manyara National Park. B) Facial lesions of animal at Tarangire National Park. Orofacial lesions were found only in olive baboonsA color version of this figure is available online (http://wwwnc.cdc.gov/EID/article/24/6/18-0037-F2.htm).

olive baboons at SNP, TNP, and LMNP, of which 2 olive baboons at TNP and 1 at SNP were included in our study. These animals represent 3.5% of the 86 *T. pallidum*–seropositive and/or PCR-positive sampled olive baboons. One animal from TNP had concurrent orofacial and anogenital skin ulcerations. We also observed these ulcerations in olive baboons at LMNP, although capture and sampling of these animals was not possible.

### Geographic Distribution

Our results provide evidence for *T. pallidum* infection in NHPs at 11 of the 14 sites investigated (Figure 1; online Technical Appendix 2). The only sites where infection was not detected were ANP (14 NHPs), SGR (9 NHPs), and JCBNP (13 NHPs). We found *T. pallidum*–positive vervet monkeys in all areas where the species was examined (GNP, KNP, LMNP, MKNP, MNP, RNP, SNP, TNP, UNP) except for the 1 animal from Zanzibar (JCBNP). One PCR-positive and anogenital ulcerated blue monkey from LMNP had reproducibly negative serologic results. Because sampling was biased toward animals with skin lesions, we more objectively compared field sites by analyzing data from animals that appeared to be clinically unaffected. Healthy-looking olive baboons were significantly more often *T. pallidum*–positive at LMNP (n = 6/6) than at any other sampling area in Tanzania where the species is present (ANP [n = 0/12], GNP [n = 8/23], NCA [1/9], SNP [n = 16/25], TNP [n = 12/17]; 6 × 2 contingency table: $\chi^2 = 30.15$, df = 5; p<0.0001). Likewise, clinically unaffected yellow baboons were significantly more often *T. pallidum*–infected at MKNP (n = 16/19) than at any of the other sampling areas in Tanzania where the species is present (KNP [n = 0/6], MNP [n = 0/10], RNP [n = 8/16], SGR [n = 0/7], UNP [n = 2/17]; 6 × 2 contingency table: $\chi^2 = 38.39$, df = 5; p<0.0001). In the vervet monkeys, we found no differences among sampling sites (GNP [n = 3/3], KNP [n = 2/5], LMNP [n = 1/2], MKNP [n = 2/3], MNP [n = 1/2], RNP [n = 4/4], SNP [n = 8/8], TNP [n = 3/6], JCBNP [n = 0/1]; 9 × 2 contingency table: $\chi^2 = 12.97$, df = 8; p = 0.1130), but sample size per site was low (online Technical Appendix 2).

### Molecular Characterization of *T. pallidum* Samples

In the 65 animals with skin ulcers, we confirmed *T. pallidum* by amplification of a part of the *polA* gene (classic PCR) and/or the *tp47* locus (quantitative PCR; 59/60 animals tested positive; online Technical Appendix 2). For 5 animals, we did not perform PCR because of limited quantities of samples. All obtained sequences were identical. We deposited a representative sequence of the *polA* gene from a yellow baboon (16RUF8140716) in GenBank (accession no. MF627733). Of 58 tested animals, 56 were positive in the PCR targeting the *TP_0619* locus. For 7 NHPs, no PCR was performed because of sample limitations. Again, all 41 sequences obtained were identical. We deposited a representative sequence from a vervet monkey (4KNF2121016) in GenBank (accession no. MF754122). The haplotype was identical to those derived from TPE and TEN strains but different from TPA strains in ≥73 positions (online Technical Appendix 1 Figure).

## Discussion

We confirmed *T. pallidum* infection in 4 free-ranging NHP species at 11 of 14 investigated sites in Tanzania. Our data for GNP must be interpreted with caution. GNP has a history of treating infected baboons with antimicrobial drugs (*17*), which might have affected prevalence rates and clinical manifestations. The finding that clinically unaffected olive baboons at LMNP, but also many animals at SNP and TNP, were infected with the bacterium (as indicated by serology; Table) shows that clinical manifestations are not representative of the actual prevalence of the disease. This finding is consistent with reports from an earlier

investigation of olive baboons at LMNP in 2007 (*6*) and in Guinea baboons (*Papio papio*) in the Niokolo Koba National Park, Senegal (*11*). In the context of human *T. pallidum* infection, where a latent stage is a key feature of infection (*19*) and which equally features positive serology in the absence of active skin lesions (*20*), this finding could argue for a similarity of disease progression in the NHP host. However, in the absence of long-term monitoring data for infected NHPs, relapsing cases, which would indicate the latent stage, cannot be identified, and standardized laboratory infection might be needed to obtain those data.

Although reduced susceptibility for *T. pallidum* infection is possible in some of the investigated species (colobines), it is likely that infection is not yet present because of behavioral and ecologic constraints between the infected and noninfected species. At least in a recent publication, a Ugandan red colobus was described with suspected active yaws-like lesions in Uganda (*21*). Consequently, we note that our sample size for colobines and red-tailed monkeys was insufficient. As a result, a conclusive evaluation on possible *T. pallidum* infection in these species was not possible. The same applied for sites where the number of infected NHPs was critically low, for example, UNP and MNP or the negative tested areas at ANP (14 animals), SGR (9 animals), and JCBNP (13 animals), as well as the NCA crater region where all 8 olive baboons were tested negative. We found *T. pallidum*–infected vervet monkeys with and without skin ulcers in 9 of the 10 sites where the species has been investigated. This finding and the larger number and geographic extent of *T. pallidum* infection in *Chlorocebus* sp. (*4*,*11*,*22–24*) deserve further attention in prospective studies, especially in areas where the species is present but has not yet been tested.

All *T. pallidum*–positive NHPs in this study revealed a *TP_0619* sequence that points toward infection with either TPE or TEN strains (online Technical Appendix 1 Figure). In the context of the geographic distribution of TEN strains (dry areas in Sahelian Africa and western Asia) (*25*) as well as the information obtained from the whole-genome sequences of the Tanzanian simian strains LMNP1 and 2 (S. Knauf et al., unpub. data, https://www.biorxiv.org/content/early/2017/05/10/135491), which are considered TPE strains, we assume that TPE is the dominant, if not exclusive, *T. pallidum* subspecies infecting Tanzanian NHPs. Further clarification will be achieved when multilocus strain typing data and whole-genome sequence data of the NHP samples become available.

In humans, TPE is mainly transmitted by direct skin-to-skin contact (*26*). A possible important alternate route of infection has been discussed through the involvement of flies as a vector (*27*,*28*). Although both options are at least theoretically possible for NHPs (*13*), direct contact should be considered the most likely way of intraspecies and interspecies transmission. Such transmission is further supported by reports of the close association and interaction (play, fight, or hunt) among different NHP species (*29–31*). Again, multilocus strain typing and whole-genome sequence data of the strains infecting NHPs in Tanzania are likely to contribute to a better understanding of host–pathogen coevolution and will provide details of the relatedness of the *T. pallidum* subspecies that infect the different NHP taxa.

Human yaws is known to be endemic to 13 countries, but Tanzania is among the 76 countries with a known history of the disease that lack recent epidemiologic data (*2*). More precisely, the disease was reported to be endemic in humans in the western areas along Lake Tanganyika and in southern Tanzania (*32*). Extensive elimination efforts decreased the reported incidence of human yaws in Tanzania from 120,000 cases in 1927 to 52,000 in 1950 (*33*) and 71 in 1978 (https://web.gideononline.com). At the same time, the wide distribution of *T. pallidum* infection in NHP on Tanzania's mainland (*7*) and the chronic infection with locally high prevalence rates (e.g., LMNP [*6*]) suggest the pathogen has been present in the respective NHP populations for at least several decades. However, current data are insufficient to develop a conclusive biogeographic scenario about the origin and spread of the infection. The first published report on *T. pallidum* infection in NHPs in 1989 (*5*) involved olive baboons at GNP. Although this is no evidence for the origin of *T. pallidum* infection in NHPs in Tanzania, it is interesting in the context of a possible anthropozoonotic introduction of the disease. GNP is in the region that has been historically classified as an area to which human yaws in Tanzania is endemic (*33*). Furthermore, GNP is close to the Democratic Republic of the Congo, a country that still reports cases of human yaws (*34*). However, all of this is speculative, and whole-genome data are needed from NHPs and human strains from the same area to provide a deeper understanding on the origin and transmission of *T. pallidum* in NHPs in Tanzania.

In a larger context, neighboring countries currently do not report NHPs with *T. pallidum*–confirmed skin lesions, although animals from East Africa (not further classified) (*22*) and Kenya (*3*) have tested serologically positive. Because *T. pallidum* infection in NHPs in Africa is widespread (*1*), further investigations should specifically include more East Africa countries, particularly those that share their borders with Tanzania.

We showed that *T. pallidum* infection in NHPs in Tanzania is geographically widespread and present in several Old World monkey species, namely olive and yellow baboons, vervet monkeys, and blue monkeys (hypothesis A). We identified the pathogen in almost all investigated sites

covering large parts of Tanzania's mainland (hypothesis B) and showed that NHPs in Tanzania are most likely infected by TPE strains. Nevertheless, our overall sample size does not permit a conclusive statement on *T. pallidum* prevalence in NHPs at any of the sampled sites. Further studies on the spatial distribution of NHP infection with *T. pallidum* and advanced genetic characterization of simian strains are crucial for identifying NHPs as a possible reservoir for human infection (*35*). In light of the data and for a sustainable eradication of human yaws, a One Health approach in which animal and human health is investigated (*36*) is needed.

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## About the Author

Mr. Chuma is a veterinarian and PhD student at the Sokoine University of Agriculture in Tanzania. His research focuses on the epidemiology and characterization of nonhuman primate infection with *T. pallidum* under the aspect of One Health.

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# Genomic Epidemiology of Global Carbapenemase-Producing *Enterobacter* spp., 2008–2014

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We performed whole-genome sequencing on 170 clinical carbapenemase-producing *Enterobacter* spp. isolates collected globally during 2008–2014. The most common carbapenemase was VIM, followed by New Delhi metallo-β-lactamase (NDM), *Klebsiella pneumoniae* carbapenemase, oxacillin 48, and IMP. The isolates were of predominantly 2 species (*E. xiangfangensis* and *E. hormaechei* subsp. *steigerwaltii*) and 4 global clones (sequence type [ST] 114, ST93, ST90, and ST78) with different clades within ST114 and ST90. Particular genetic structures surrounding carbapenemase genes were circulating locally in various institutions within the same or between different STs in Greece, Guatemala, Italy, Spain, Serbia, and Vietnam. We found a common NDM genetic structure (NDM-GE-U.S.), previously described on pNDM-U.S. from *Klebsiella pneumoniae* ATCC BAA-214, in 14 different clones obtained from 6 countries spanning 4 continents. Our study highlights the importance of surveillance programs using whole-genome sequencing in providing insight into the molecular epidemiology of carbapenemase-producing *Enterobacter* spp.

The emergence of carbapenem resistance is a major public health concern because these agents are regarded as one of the last effective therapies available for treating serious infections caused by *Enterobacteriaceae* (*1*). Carbapenemases are important causes of carbapenem resistance because they can be transferred between members of the *Enterobacteriaceae*. The most common carbapenemases among clinical *Enterobacteriaceae* are the *Klebsiella pneumoniae* carbapenemases (KPCs) (Amber class A), IMPs, VIMs, New Delhi metallo-β-lactamase (NDMs) (class B or metallo-β-lactamases), and oxacillin (OXA) 48–like (class D) enzymes (*2*).

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Recent surveillance studies have shown that *Enterobacter* spp. are often the second or third most common *Enterobacteriaceae* species associated with carbapenemases (*3*,*4*). Typically, KPCs are common among *Enterobacter* spp. from the United States and South America (*5*). VIMs are often limited to Europe, NDMs to the Indian subcontinent, and OXA-48 to North Africa and the Middle East (*5*).

Comprehensive global data regarding the different *Enterobacter* species and molecular epidemiology are currently limited. We designed a study that used short-read whole-genome sequencing to describe the molecular characteristics and international distribution of *Enterobacter* spp. with different carbapenemases (n = 170) obtained from 2 global surveillance systems during 2008–2014.

## Materials and Methods

### Bacterial Isolates

We included 170 clinical, nonrepeat *Enterobacter* spp. collected from 2 global surveillance programs, namely the Merck Study for Monitoring Antimicrobial Resistance Trends (SMART) (2008–2014) and the AstraZeneca global surveillance program (2012–2014), presently known as the INFORM Global Surveillance Study of Antimicrobial Resistance (online Technical Appendix 1 Table 1, https://wwwnc.cdc.gov/EID/article/24/6/17-1648-Techapp1.xlsx; online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/24/6/17-1648-Techapp2.pdf). The isolates initially underwent phenotypic identification and microdilution panel susceptibility testing, and all carbapenem-nonsusceptible isolates underwent molecular screening for $bla_{KPC}$, $bla_{VIM}$, $bla_{NDM}$, $bla_{OXA-48}$–like, $bla_{IMP}$, and $bla_{GES}$ as described previously (*6*). We obtained a total of 142,226 *Enterobacteriaceae* from the period 2008–2014, and 6,457 (4.5%) were identified as *Enterobacter* spp.; 682 were nonsusceptible to 1 of the carbapenems, and 170/6,457 (2.6%) were positive for $bla_{KPC}$, $bla_{OXA-48}$–like, $bla_{NDM}$, $bla_{VIM}$, and $bla_{IMP}$ and thus included in our study.

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### Whole-Genome Sequencing

We used the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA) to prepare libraries for sequencing. We multiplexed and sequenced samples on an Illumina NextSeq500 for 300 cycles (151 bp paired-end).

### Genomic Analysis

We obtained draft genomes by using SPAdes version 3.10.1 (*7*). We identified species based on the *hsp60* gene sequences (*8*). We created whole-genome phylogenetic trees, including reference strains for identification of *E. cloacae* complex (*9*; online Technical Appendix 1 Table 2).

To define the presence of genes and their alleles, we accessed BLAST in combination with the following databases or typing schemes: BLAST (http://blast.ncbi.nlm.nih.gov/Blast), National Center for Biotechnology Information (NCBI) Beta-Lactamase Data Resources (http://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources), ResFinder (*10*), PlasmidFinder (*11*), and *Enterobacter cloacae* Multilocus Sequence Typing (MLST) Databases (http://pubmlst.org/ecloacae). We classified integrons according to INTEGRALL (http://integrall.bio.ua.pt).

### Phylogenetic Analysis

We created a recombination-free, core single-nucleotide polymorphism (SNP)–based phylogenetic tree and identified SNPs by mapping the reads or aligning the genomes against *E. xiangfangensis* type strain LMG27195 (*9*) using the RedDog pipeline (https://github.com/katholt/RedDog). We removed recombination sites according to Gubbins (*12*) and removed prophages identified by PHAST (*13*). We included core SNPs and sites that were present in all genomes to create a maximum-likelihood tree using RAxML with the general time-reversible plus gamma substitution model (*14*). We visualized the tree by using iTOL version 3 (*15*).

To identify clades within certain sequence types (STs), we used a phylogeny-free population genetics approach of core SNPs, conducting hierarchal clustering analysis with the Bayesian Analysis of Population Structure program (*16*). We included all 1,048 available *Enterobacter* spp. genomes in the NCBI Reference Sequence Database (http://www.ncbi.nlm.nih.gov/refseq) as of June 20, 2017. An in silico MLST analysis identified 282 STs from 950 typeable genomes. We included a total of 201 genomes of STs 78, 90, 93, 105, 108, 114, and 171 for the clustering analysis (online Technical Appendix 1 Table 3). For each *E. hormaechei* subspecies or *E. xiangfangensis*, the hierarchal Bayesian Analysis of Population Structure clustering analysis (*16*) was conducted with 3 nested levels with a priori upper bound of the number of clusters between one fourth to one half of the total number of isolates. We defined clades by using the second level of clustering.

### Sequence Data Accession Numbers

We deposited the sequencing data in the DNA Data Bank of Japan and NCBI (NCBI BioProjects PRJNA259658 and PRJNA398291) databases (accession nos. DRA004879, SRP046977, and SRR2960053–SRR2960159 [SMART isolates] and SRR5939895–SRR5939952 [AstraZeneca isolates]). The sequences of new integrons or genetic environments described in this study were GenBank accession nos. LC224310–2, MF288916–351991, and MF327263–71.

## Results

### Global Distribution of Carbapenemases among *Enterobacter* spp.

We included a total of 170 carbapenemase-producing *Enterobacter* strains in the study. The VIMs (VIM-1, 4, 5, 23, and 31; n = 51 [46 were only positive for VIM, and 5 co-produced OXA-48]) were the most common carbapenemase among this collection, followed by NDMs (NDM-1, 6, and 7; n = 43 [41 were positive only for NDM, 1 also co-produced OXA-48. and 1 co-produced KPC-2]); KPCs (KPC-2, 3, 4, and 5; n = 38 [37 were only positive for KPC, and 1 co-produced NDM]); OXA-48 (n = 31 [25 were only positive for OXA-48, 5 co-produced VIM, and 1 co-produced NDM]); and IMPs (IMP-1, 4, 8, 13, and 14; n = 14). *Enterobacter* spp. with $bla_{VIM}$ were mostly limited to Europe; isolates with $bla_{NDM}$ were present predominantly in the Balkans, India, and Vietnam; isolates with $bla_{KPC}$ were mainly found in the United States and South America; isolates with $bla_{OXA-48}$ were largely present in North Africa and the Middle East; and isolates with $bla_{IMP}$ occurred mostly in the Philippines, Taiwan, and Australia. The global distribution of isolates from this study was similar to what had previously been reported for other members of *Enterobacteriaceae*, especially *Klebsiella* spp. with carbapenemases (*5*,*17*).

### *E. aerogenes* Distant from *E. cloacae* Complex

We identified 10 isolates as *E. aerogenes*. These results are described in online Technical Appendix 2.

### *E. xiangfangensis* Identified as the Most Common Species

The *E. cloacae* complex (n = 160) from our study was obtained from intraabdominal (n = 69), urine (n = 56), skin and soft tissue (n = 19), blood (n = 2), and respiratory specimens (n = 14). We identified 8 species among *E. cloacae* complex (*E. xiangfangensis* [n = 65], *E. hormaechei* subsp. *steigerwaltii* [n = 47], *E. cloacae* cluster III [n = 14], *E. cloacae* subsp. *cloacae* [n = 13], *E. cloacae* cluster IV [n = 9], *E. hormaechei* subsp. *oharae* [n = 6], *Enterobacter asburiae* [n = 5], and *Enterobacter kobei* [n = 1]). These species

were associated with different types of carbapenemases and showed global distribution (Figure 1; online Technical Appendix 2 Table 1). *E. xiangfangensis* was frequent in the Balkans (e.g., Croatia, Romania, and Serbia), whereas *E. hormaechei* subsp. *steigerwaltii* was mostly prevalent in Greece and Vietnam (online Technical Appendix 2 Table 1). This overrepresentation was attributable to the presence of particular STs among these species (online Technical Appendix 2 Table 2).

### Dominant Sequence Types Identified among 4 Species in *E. cloacae* Complex

*E. xiangfangensis* from our study comprised 18 different STs, including 1 dominant ST, ST114 (19/65; 29%).

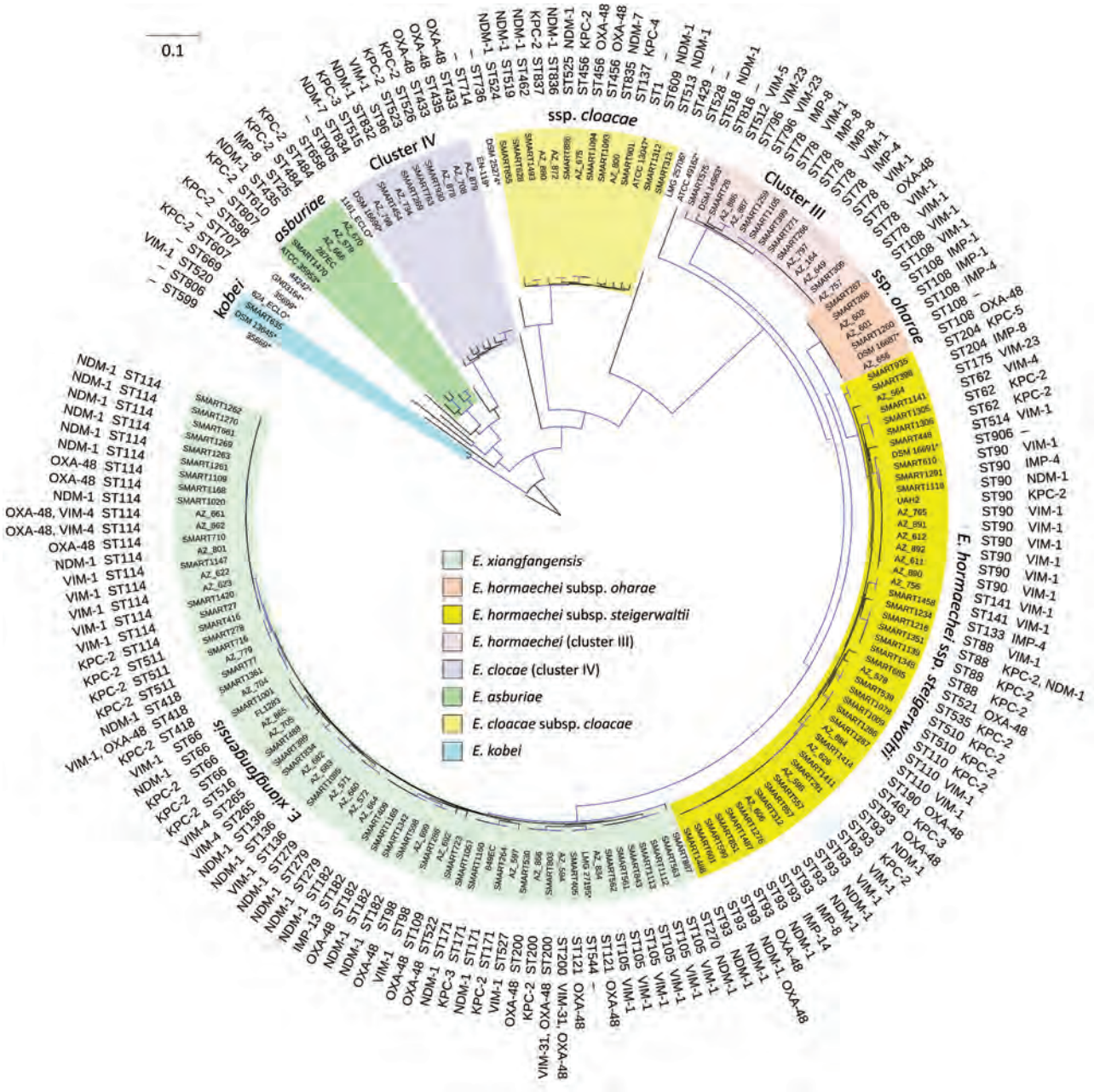


**Figure 1.** Phylogenetic tree of the different species and sequence types among 160 *Enterobacter cloacae* complex isolates identified from *Enterobacter* spp. isolates collected in the Merck Study for Monitoring Antimicrobial Resistance Trends, 2008–2014, and the AstraZeneca global surveillance program, 2012–2014. The tree is rooted with *E. cloacae* complex Hoffmann cluster IX (Chavda group R) strain 35,699. A total of 369,123 core single-nucleotide polymorphisms were found; 4,010 were used to draw the tree (after phages and recombination sites were excluded). KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamase; OXA, oxacillin; ST, sequence type; –, information missing; *, isolate identified in another study. Scale bar indicates nucleotide substitutions per site.

*E. hormaechei* subsp. *steigerwaltii* comprised 15 different STs, including 2 dominant STs, ST90 (10/47; 21%) and ST93 (14/47; 30%). *E. cloacae* cluster III comprised 4 different STs, including 1 dominant ST, ST78 (10/14 [71%). All 6 of the *E. hormaechei* subsp. *oharae* isolates belonged to ST108 (Figure 1). The remaining species did not contain a dominant ST, and we found new STs among *E. cloacae* cluster IV (ST832 and ST834) and *E. cloacae* subsp. *cloacae* (ST835, ST836, and ST837).

### Major and Minor Sequence Types among *Enterobacter cloacae* Complex

Among the *E. cloacae* complex, we identified 4 major STs (≥10 isolates/ST), ST114, ST93, ST90, and ST78. We also identified 2 minor STs (5–9 isolates/ST), ST105 and ST108.

ST114 (n = 19) from *E. xiangfangensis* was the most common ST and divided into 4 clades. Isolates representing 3 of the clades (ST114A, ST114B, and ST114C) were from this study, and isolates representing clade ST114D were from a different study (*9*; Figure 2; online Technical Appendix 2 Table 2). ST114 had a global distribution (Greece, Italy, Kuwait, Morocco, Romania, Serbia, Tunisia, and the United States) and was associated with different carbapenemases (VIM-1, VIM-4+OXA-48, NDM-1, KPC-2, and OXA-48) (online Technical Appendix 2 Table 2). The largest clade (ST114A [n = 13]) was present in Serbia, Romania (with $bla_{\text{NDM-1}}$), Tunisia, Morocco, and Kuwait (with $bla_{\text{OXA-48}}$) (online Technical Appendix Table 2). Clade ST114B (n = 4) with $bla_{\text{VIM-1}}$ was obtained from Greece and Italy, and clade ST114C (n = 2; 1 with $bla_{\text{VIM-1}}$ and 1 with $bla_{\text{KPC-2}}$) was found in the United States. ST114 is a common global human *Enterobacter* clone (*18*) and is also present in companion animals (*19*). This international clone is associated with various antimicrobial resistance

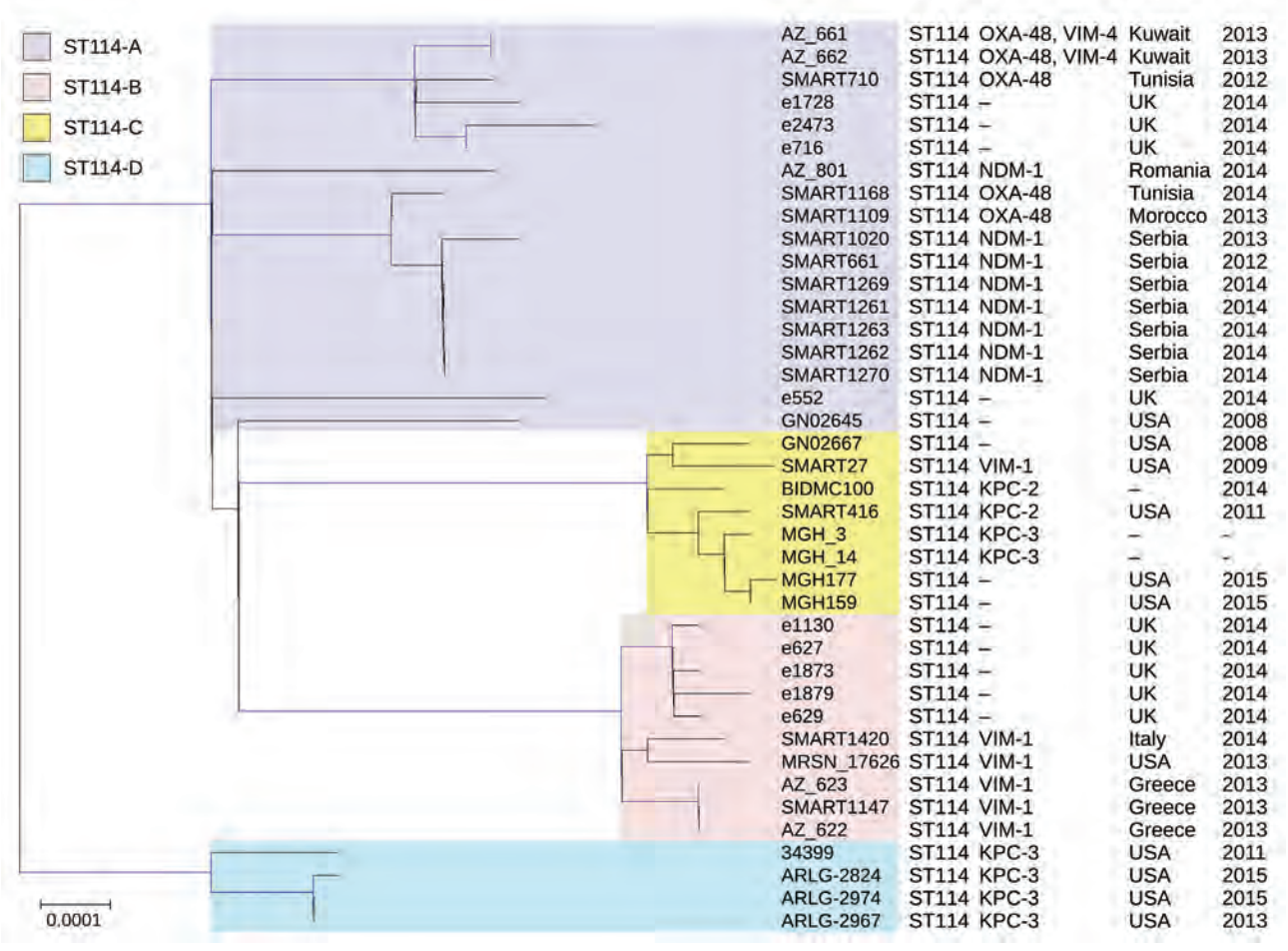


**Figure 2.** Phylogenetic tree of the different clades among 40 *Enterobacter xiangfangensis* ST14 isolates identified from *Enterobacter* spp. isolates collected in the Merck Study for Monitoring Antimicrobial Resistance Trends, 2008–2014, and the AstraZeneca global surveillance program, 2012–2014. The tree is rooted with *E. hormaechei* subsp. *hormaechei* isolate ATCC49162. A total of 317,867 core single-nucleotide polymorphisms were found; 27,705 were used to draw the tree (after phages and recombination sites were excluded). The isolates from other studies were negative for carbapenemases. KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamase; OXA, oxacillin; ST, sequence type; –, information missing. Scale bar indicates nucleotide substitutions per site.

determinants (*20*) and was responsible for a prolonged nosocomial outbreak involving KPC-3 in the United States (*21*).

ST93 (n = 14), from *E. hormaechei* subsp. *steigerwaltii*, was the second most common ST in this collection and consisted of 1 clade (Figure 3; online Technical Appendix 2 Table 2). ST93 had a global distribution (Australia, Belgium, China, Romania, Spain, Thailand, United States, and Vietnam) and was associated with different carbapenemases (IMP-8, IMP-14, VIM-1, NDM-1, KPC-2, and OXA-48). ST93 was mostly present in Vietnam (n = 7), where it contained $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ (online Technical Appendix 2 Table 2).

ST90 (n = 10), from *E. hormaechei* subsp. *steigerwaltii,* and ST78 (n = 10), from *E. cloacae* cluster III, were the next most common STs in our collection. ST90 was divided into 3 clades; isolates from 2 of the clades (ST90B and ST90C) were from this collection, whereas isolates representing clade ST90A were from a different study (*22*; Figure 3; online Technical Appendix 2 Table 2). ST90C with $bla_{\text{VIM-1}}$ (n = 7) was from Greece, whereas clade ST90B showed an international distribution (ST90C with IMP-4 from Australia, KPC-2 from Canada, and NDM-1 from Romania).

ST78 from *E. cloacae* cluster III consisted of 1 clade. This ST was associated with VIM-1 (Greece, Italy, and Spain), IMP-4 (Philippines), IMP-8 (Taiwan), and OXA-48 (Turkey) (online Technical Appendix 2 Table 2).

The minor STs, including ST105 and ST108 (both with 6 isolates), were distinguished on the basis of their molecular epidemiology. ST105 from *E. xiangfangensis* belonged to a single clade and was only present in Croatia, where it contained $bla_{\text{VIM-1}}$. All the *E. hormaechei* subsp. *oharae* isolates belonged to ST108, which was divided into 5 clades; isolates from 2 of the clades (ST108C and ST108D) were from this collection, whereas isolates representing the other clades were from different studies (*23*; Figure 4). Clade 108C (n = 4) was present in Spain with $bla_{\text{VIM-1}}$ (n = 2) and China with $bla_{\text{IMP-1}}$ (n = 2), and ST108D (n = 2) was found in Australia (with $bla_{\text{IMP-4}}$) and Israel (with $bla_{\text{OXA-48}}$).

### β-lactamases, Antimicrobial Resistance Determinants, and Plasmid Analysis

For each of the 170 isolates, we tabulated the study number, GenBank accession number, species, date, country of isolation, ST, and clade. The β-lactamases, antimicrobial resistance determinants, plasmid replicon types, and plasmid STs are shown in online Technical Appendix 1 Table 1 and online Technical Appendix 2.

### Genetic Environments Surrounding the Carbapenemase Genes

We were able to successfully characterize the immediate genetic environments surrounding the carbapenemase genes in 8/14 *E. cloacae* complex with IMP, 28/33 with KPC (including 4 novel structures named KPC-GE01, KPC-GE02, KPC-GE03, and KPC-GE04), 42/42 with NDM (including 4 novel structures named NDM-GE01, NDM-GE02, NDM-GE03, and NDM-GE04), 17/27 with OXA-48 (including 4 novel structures named OXA-GE01, OXA-GE02, OXA-GE03, and OXA-GE04), and 46/51 with VIM (including the novel integrons In*1372*, In*1373*, and In*1374*) (online Technical Appendix 2 Table 3). We have also described the novel structures found in *E. aerogenes* (online Technical Appendix 2).

The $bla_{\text{KPC}}$ were mainly associated with the Tn*4401b* isoform (including the 4 novel structures), whereas $bla_{\text{OXA-48}}$ was always associated with Tn*1999* (including the 4 novel structures). Isolates with NDM contained IS*Aba125* upstream and $ble_{\text{MBL}}$ downstream of the $bla_{\text{NDM}}$, and the $bla_{\text{VIM}}$ and $bla_{\text{IMP}}$ were situated within diverse class I integrons from various countries (online Technical Appendix 2 Table 2).

### Integrons Harboring $bla_{\text{VIM-1}}$ Circulating Locally within the Same or between Different STs in Spain, Greece, and Italy

In237 was present in ST78 (obtained in 2013) and ST90C (obtained in 2014) from the same institution in Greece. In916 was identified in ST78 (obtained in 2010) and ST114B (obtained in 2014) from the same institution in Italy. In624 was harbored in ST78, ST96, and ST108 from the same institution in Spain (all obtained in 2010). In*87* was detected in ST98, ST110, and ST141 from 2 different institutions in Greece (obtained in 2010 and 2014). In4873 was identified in ST114B from 2 different institutions in Greece (obtained in 2013) (online Technical Appendix 2 Tables 1, 2). In110 with $bla_{\text{VIM-1}}$ was present in ST105 from Croatia (obtained in 2013) and ST520 from Spain (obtained in 2012).

### Global Distribution of a Common NDM-1 Genetic Structure

The most common genetic structure immediately surrounding the $bla_{\text{NDMs}}$ (named NDM-GE-U.S.) in our collection was identical to that previously described on a 140.8 kb IncA/C plasmid (pNDM-U.S.; GenBank accession no. CP006661.1) found in *K. pneumoniae* ATCC BAA-2146 with $bla_{\text{NDM-1}}$ (*24*). This bacterium was isolated in 2010 from the urine of a US hospital patient who had previously received medical care in India (*25*). NDM-GE-U.S., a 3,063-bp fragment consisting of ΔIS*Aba125*-$bla_{\text{NDM-1}}$-$ble_{\text{MBL}}$-*trpF*-*dsbC*, was present in 16/42 of NDM *E. cloacae* complex isolates among 14 different STs (88, 90B, 93, 114A, 279, 136, 182, 270, 435, 513, 524, 525, 609, and 832) obtained from Colombia, Romania, Philippines, Vietnam, South Africa, and Kenya (online Technical Appendix 2 Table 3).

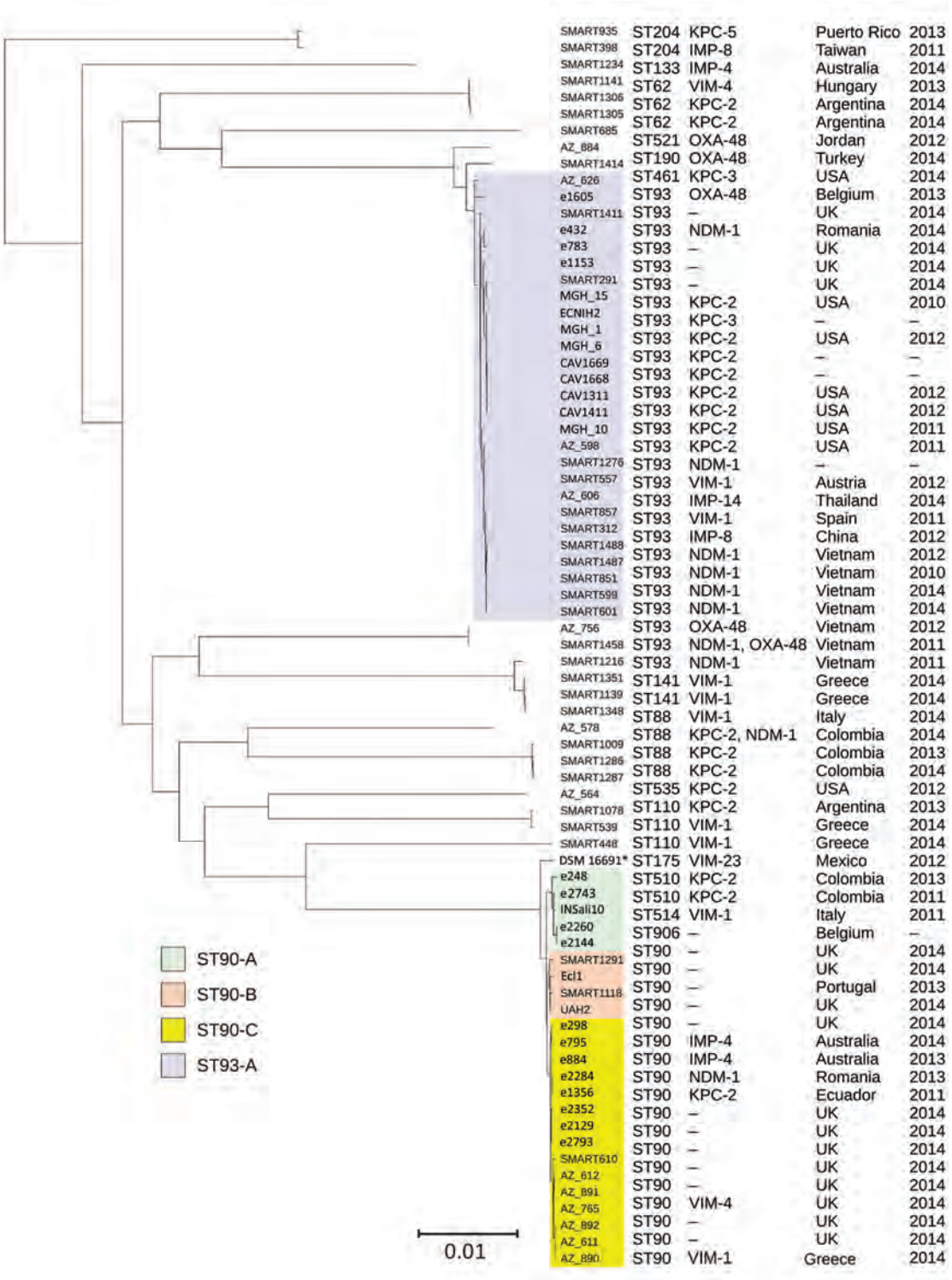


**Figure 3.** Phylogenetic tree of the different clades among 51 *Enterobacter hormaechei* subsp. *steigerwaltii* ST90 and ST93 isolates identified from *Enterobacter* spp. isolates collected in the Merck Study for Monitoring Antimicrobial Resistance Trends, 2008–2014, and the AstraZeneca global surveillance program, 2012–2014. The tree is rooted with *E. hormaechei* subsp. *hormaechei* isolate ATCC49162. A total of 317,867 core single-nucleotide polymorphisms were found; 27,705 were used to draw the tree (after phages and recombination sites were excluded). The isolates from other studies were negative for carbapenemases. Clades are grouped by color. KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamase; OXA, oxacillin; ST, sequence type; –, information missing. Scale bar indicates nucleotide substitutions per site.

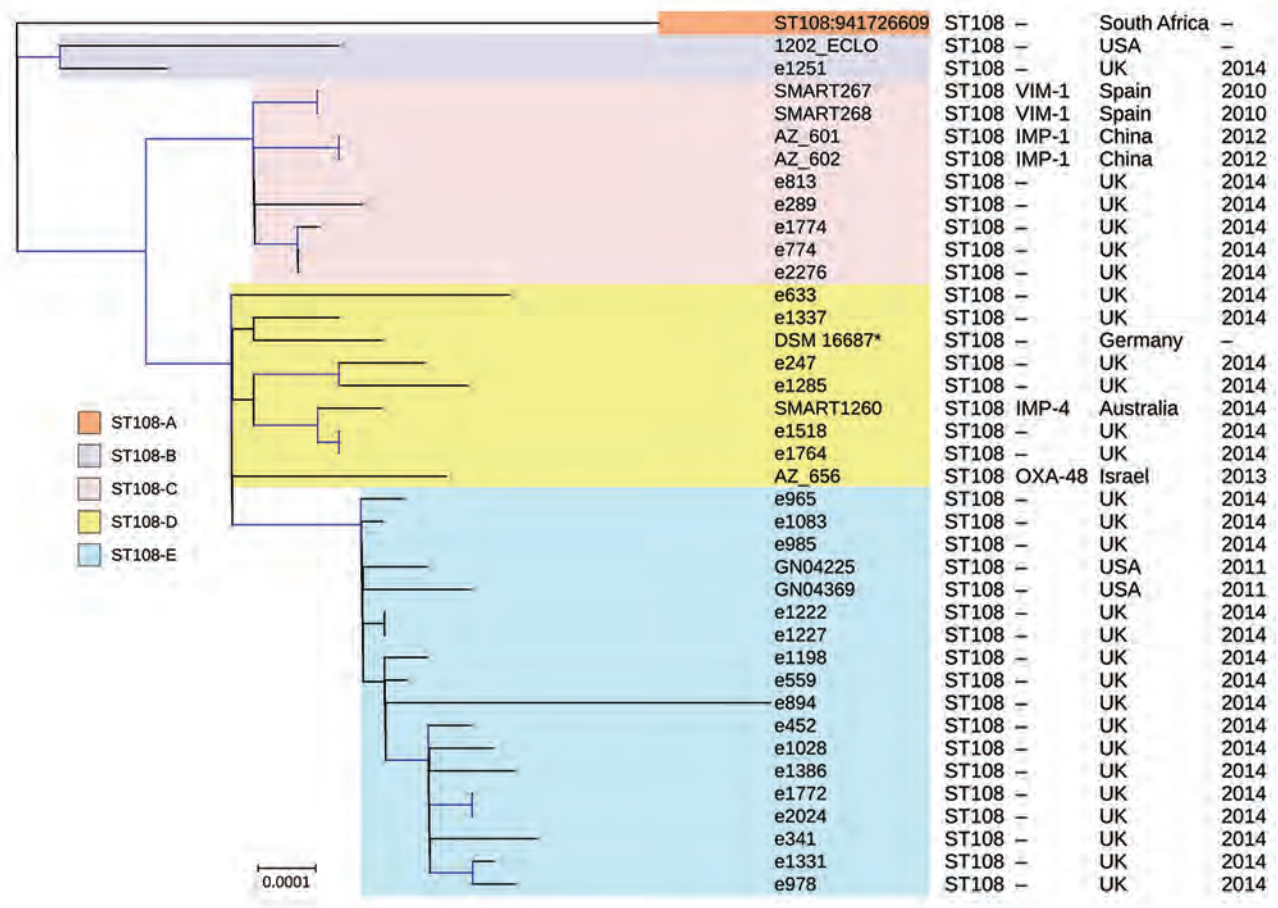


**Figure 4.** Phylogenetic tree of the different clades among 39 *Enterobacter hormaechei* subsp. *oharae* ST108 isolates identified from *Enterobacter* spp. isolates collected in the Merck Study for Monitoring Antimicrobial Resistance Trends, 2008–2014, and the AstraZeneca global surveillance program, 2012–2014. The tree was rooted with *E. hormaechei* subsp. *hormaechei* isolate ATCC49162. A total of 317,867 core single-nucleotide polymorphisms were found; 27,705 were used to draw the tree (after phages and recombination sites were excluded). The isolates from other studies were negative for carbapenemases. Clades are grouped by color. KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Delhi metallo-β-lactamase; OXA, oxacillin; ST, sequence type; –, information missing. Scale bar indicates nucleotide substitutions per site.

We determined the sequence similarity of the isolates with NDM-GE-U.S. to previously sequenced plasmids in the GenBank database. The similarity to pNDM-U.S. ranged from 7% to 81%, suggesting that different plasmids contained NDM-GE-U.S. Twelve of the isolates showed high similarity (range 93%–100%) to pK518_NDM1, a 106.8-kb IncFII plasmid with $bla_{\text{NDM-1}}$ from China (GenBank accession no. CP023187). The remaining 4 isolates showed high similarity (range 98%–100%) with the 54-kb IncX3 plasmid pNDM-HN380 (n = 3) from China (*26*) and the 178.2-kb IncA/C plasmid p6234–178.193kb (n = 1) from the United States (GenBank accession no. CP010391).

## Discussion

The most common carbapenemase among *Enterobacter* spp. from our study was VIM, followed by NDM, KPC, OXA-48, and IMP. Carbapenemase-producing *Enterobacter* spp. was dominated by 2 global species, namely *E. xiangfangensis* with 1 major clone (ST114) and *E. hormaechei* subsp. *steigerwaltii* with 2 major clones (ST90 and ST93). ST114 and ST90 were divided into different clades; some of the clades (e.g., 90C and 114B) were located in certain geographic regions affiliated with specific carbapenemases, whereas other clades (114A and 90B) were distributed globally in association with different types of carbapenemases.

The taxonomy of *E. cloacae* complex is confusing, and uncertainty still remains about what species make up this complex. In the early 2000s, Hoffmann and Roggenkamp (*8*) sequenced *hsp60* and established 12 genetic clusters (I to XII) in *E. cloacae* complex. In 2005, the same authors further defined the taxonomy of *E. cloacae* complex and named cluster VII as *E. hormaechei* subsp. *hormaechei*, cluster VI as *E. hormaechei* subsp. *oharae*, and cluster VIII as *E. hormaechei* subsp. *steigerwaltii* (*27*). In 2014,

Gu et al. (*28*) described a novel *Enterobacter* species obtained from sourdough in China named *E. xiangfangensis*, which clustered closest to *E. hormaechei*.

The first study that described the global distribution of *E. cloacae* clones was undertaken by Izdebski et al (*18*), who performed MLST on 173 cephalosporin-resistant *E. cloacae* isolates obtained from Israel and several countries in Europe. MLST identified 88 STs among this collection, with ST78, ST114, ST108, and ST66 being the most common and widespread clones. A ST78 isolate was positive for KPC-2, and a ST114 isolate was positive for VIM-1 (*18*). With the exception of this study from Izedebski et al (*18*), limited information is available regarding the global distribution of ST93, ST90, ST78, ST105, and ST108 and consists mainly of sporadic reports (*29*–*32*).

Chavda et al. (*9*) characterized 74 carbapenem-resistant *Enterobacter* spp. (more than half of the isolates were obtained from New Jersey, USA), and most possessed different $bla_{KPC}$s, whereas only 2 isolates had $bla_{NDM-1}$. *E. xiangfangensis* also dominated, and ST171 was the most common clone. ST171 was rare in our collection (n = 4) but did show genetic and geographic diversity. ST171 was divided into 3 clades: 171A, 171B, and 171C (online Technical Appendix 2 Figure). Clades 171B and 171C are associated with $bla_{KPC}$ from the United States and United Kingdom (online Technical Appendix 2 Figure). Clade 171B (n = 2) contained $bla_{KPC-2}$ from Colombia and $bla_{NDM-1}$ from Guatemala. Clade 171A (n = 1) with $bla_{NDM-1}$ was obtained from South Africa, and clade 171C with $bla_{KPC-3}$ was obtained from the United States.

We noted interesting associations and geographic distribution between genetic structures surrounding carbapenemase genes and clades, clones, and species. First, identical genetic structures were situated in various STs within the same or different institutions of the same country (e.g., NDM-GE01 with $bla_{NDM-1}$ in Vietnam; In87 and In237 with $bla_{VIM-1}$ in Greece; In916 with $bla_{VIM-1}$ in Italy; In624 with $bla_{VIM-1}$ in Spain; and NDM-GE03 with $bla_{NDM-1}$ in Guatemala). Second, identical genetic structure was present in different STs (ST105 and ST520), from different countries (e.g., In110 with $bla_{VIM-1}$ in Croatia and Spain). Third, different genetic structures were present in the same STs and clades obtained from different countries (e.g., ST78 with In237 from Greece, ST78 with In916 from Italy, ST78 with In624 from Spain, ST114A with NDM-GE02 from Serbia, and ST114A with pNDM-U.S. from Romania). Last, an identical genetic structure (NDM-GE-U.S.) was found in different global species, STs, and clades.

These associations demonstrate that certain mobile genetic elements with carbapenemase genes have the ability to move between clones and clades of *Enterobacter* spp. on a global scale. This ability is highlighted by ST78 with $bla_{VIM-1}$ within different integrons (In237, In916, and In624) that circulate between various countries (Greece, Italy, and Spain). As some STs are introduced into different countries, they apparently acquire the local genetic elements prevalent in that country. Of special concern is the description of a common NDM genetic structure, named NDM-GE-U.S., previously found on pNDM-U.S. and first described in a *K. pneumoniae* from the United States (*24*). NDM-GE-U.S. was present in different species, clones, and clades obtained from 6 countries spanning 4 continents. Sequence similarity analysis suggested that it was present on different types of plasmids (pK518_NDM1 and pNDM-HN380) among *Enterobacter* spp. with $bla_{NDM}$.

Our results support the current understanding that the carbapenem resistance pandemic is the consequence of circulating clones and the spread of mobile genetic elements. We found that certain clones and clades (ST78, ST90C, ST96, ST114A, ST114C, and ST141) containing particular genetic structures (In87, In624, In916, In237, NDM-GE01, NDM-GE02, and NDM-GE03) and carbapenemases were circulating locally within the same or between different institutions in certain countries (Greece, Guatemala, Italy, Spain, Serbia, and Vietnam). Other global clones and clades (ST90B, ST93, and ST108) contained various genetic structures and carbapenemases.

A limitation of this study was that plasmids harboring carbapenemases were not reconstructed because of the limitations of short-read sequencing (*33*). The characterization of plasmids is vital to fully comprehend the molecular epidemiology of *Enterobacter* spp. with carbapenemases, and a follow-up study using long-read sequencing is currently under way. In the meantime, our study highlights the importance of surveillance programs using whole-genome sequencing to provide insight into the characteristics and global distribution of clones and clades as well as their association with mobile genetic elements surrounding the different carbapenemase genes.

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# Influenza D Virus Infection in Feral Swine Populations, United States

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Influenza D virus (IDV) has been identified in domestic cattle, swine, camelid, and small ruminant populations across North America, Europe, Asia, South America, and Africa. Our study investigated seroprevalence and transmissibility of IDV in feral swine. During 2012–2013, we evaluated feral swine populations in 4 US states; of 256 swine tested, 57 (19.1%) were IDV seropositive. Among 96 archived influenza A virus–seropositive feral swine samples collected from 16 US states during 2010–2013, 41 (42.7%) were IDV seropositive. Infection studies demonstrated that IDV-inoculated feral swine shed virus 3–5 days postinoculation and seroconverted at 21 days postinoculation; 50% of in-contact naive feral swine shed virus, seroconverted, or both. Immunohistochemical staining showed viral antigen within epithelial cells of the respiratory tract, including trachea, soft palate, and lungs. Our findings suggest that feral swine might serve an important role in the ecology of IDV.

Influenza D virus (IDV), first isolated in 2011 from a domestic pig with influenza-like symptoms, has genomic similarity to influenza C virus (ICV) (*1*). IDV has 7 genomic RNA segments like ICV but exhibits a broader cellular and host tropism than ICV (*1*), which might be attributable to IDV's open receptor-binding cavity (*2*). Evidence suggests that IDV circulates in domestic animals, including swine, cattle, camelids, and small ruminants, throughout North America, Asia, Africa, and South America (*1*,*3*–*15*). Among these species, cattle are proposed to be the natural reservoir of IDV (*13*,*15*). Susceptibility and seroprevalence of IDV in domestic and wild animal species is largely unknown.

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Swine were introduced into what is now the United States in the 15th century. Since that time, populations of free-ranging swine have spread to ≈40 states. These swine are escaped domestic animals, imported wild boar, or hybrids of the two, and they now number ≈5 million (Figure 1, panel A) (*16*–*19*). Feral swine transmit diseases that are swine-specific (feral and domestic) as well as diseases that can be transmitted to domestic species (cattle, sheep, goats, horses, and dogs) and wild mammals; some of the more important diseases include porcine circovirus-2, pseudorabies virus, *Brucella suis*, and influenza A viruses (IAVs) as well as vesicular diseases (*16*,*17*,*20*–*25*). Feral swine have been shown to have contact with domestic swine in transitional and commercial settings (*17*,*20*). Moreover, feral swine also frequently interact with free-ranging cattle near shared water sources (*16*). Of particular concern, feral swine populations are increasing and pose potential threats to domestic swine and human public health (*26*).

Little is known regarding seroprevalence of IDV in feral swine. In this study, we conducted serologic surveillance to estimate seroprevalence of IDV in the feral swine population in the United States. We also conducted infection experiments to determine the pathogenesis and transmission of IDV in feral swine.

## Materials and Methods

### Viruses

We used influenza viruses D/bovine/C00046N/Mississippi/2014 virus (D/46N) and D/bovine/C00013N/Mississippi/2014 virus (D/13N). Before use, the viruses were isolated and passaged twice in HRT-18G cells (American Type Culture Collection, Manassas, VA, USA) with Opti-MEM supplemented with 1× Pen Strep and 12.5× 7.5% bovine serum albumin (GIBCO Life Technologies, Carlsbad, CA, USA) and 1:2000 *N*-tosyl-L-phenylalanine chloromethyl ketone-Trypsin (Sigma-Aldrich, St. Louis, MO, USA).

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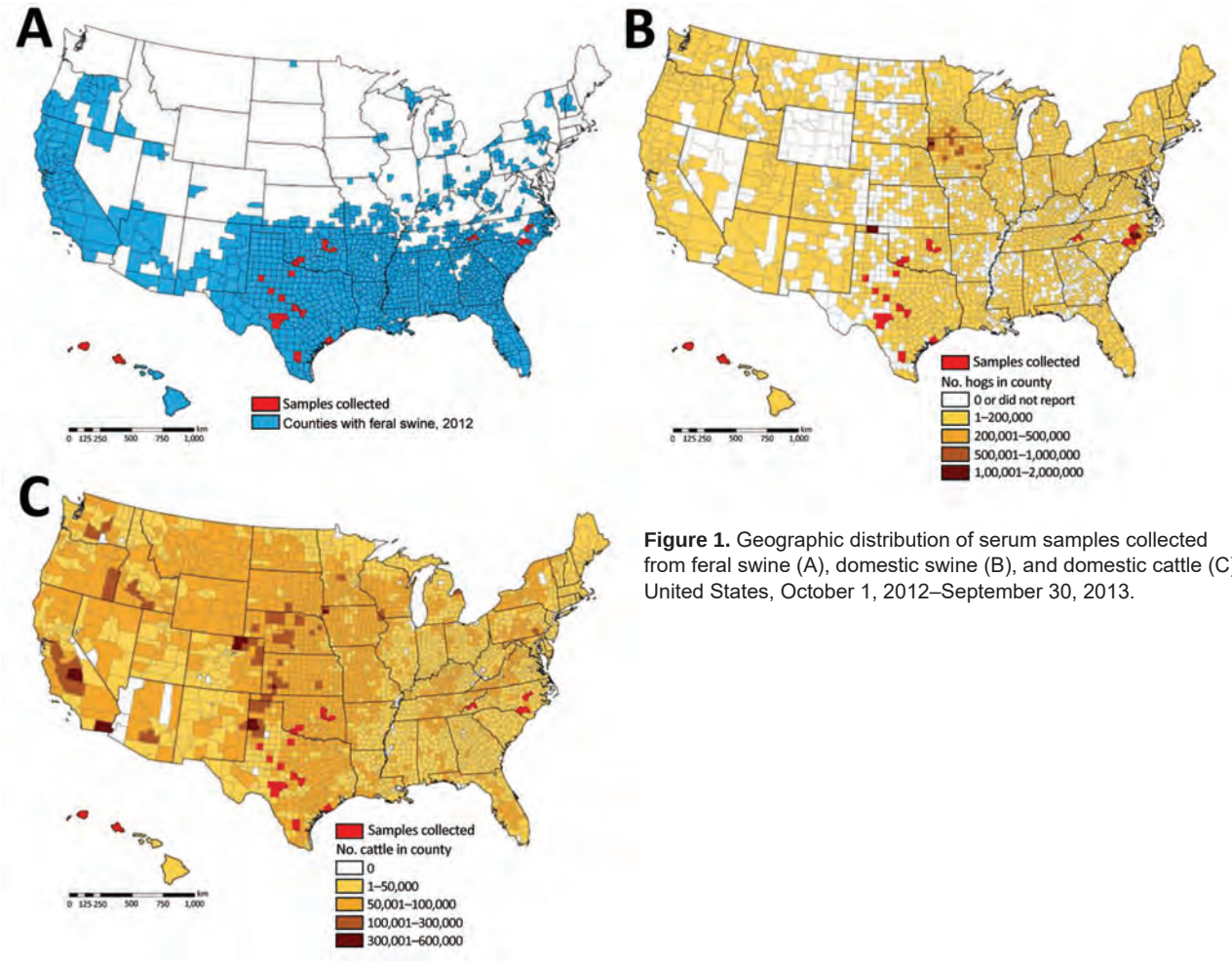


**Figure 1.** Geographic distribution of serum samples collected from feral swine (A), domestic swine (B), and domestic cattle (C), United States, October 1, 2012–September 30, 2013.

### Serum Samples

A total of 256 convenient serum samples were available from a collection of feral swine serum archived by the US Department of Agriculture's National Wildlife Research Center. The 256 samples constituted feral swine serum samples collected during October 1, 2012–September 30, 2013, in 4 US states: Hawaii (n = 73 samples), North Carolina (n = 64), Oklahoma (n = 49), and Texas (n = 70) (Table 1). North Carolina, Oklahoma, and Texas have large domestic swine populations; Texas and Oklahoma have large cattle populations; and Hawaii, Oklahoma, and Texas have large feral swine populations. Of the 256 serum samples, 118 were from male feral swine, 135 were from female feral swine, and 3 were from feral swine of unknown sex; in addition, 32 of the samples were from juveniles (<2 mo of age), 43 from subadults (2–12 mo of age), and 181 from adults (>1 y of age) (Table 2).

Previous studies have suggested that feral swine have been exposed to IAV (*24*,*25*). To determine whether feral swine could have been exposed to both IDV and IAV, we identified 96 IAV-seropositive samples. Of the total 256 samples we described, 13 were IAV seropositive and were included in the IAV-seropositive sample set. We selected 83 additional convenient serum samples collected during October 1, 2012–September 30, 2013, from archived feral swine serum samples that had been previously determined to be IAV seropositive using the IDEXX AI MultiS-Screen Ab Test (IDEXX, Westbrook, ME, USA) (*9*,*10*). We sampled these 96 IAV-seropositive samples from 16 US states: Alabama (n = 7 samples), Arizona (n = 1), California (n = 5), Florida (n = 6), Georgia (n = 8), Hawaii (n = 4), Illinois (n = 2), Kansas (n = 5), Louisiana (n = 2), Missouri (n = 1), North Carolina (n = 8), New Mexico (n = 1), Oklahoma (n = 9), South Carolina (n = 1), Tennessee (n = 2), and Texas (n = 34).

### Hemagglutination Assays

We performed hemagglutination (HA) and HA inhibition (HAI) assays as previously described (*11*). In brief, we treated serum samples with receptor-destroying enzyme (Denka Seiken Co., Tokyo, Japan) at 37°C for ≥18 hr, followed by heat inactivation at 55°C for 30 min. We diluted inactivated serum to a final concentration of 1:10

**Table 1**. Seroprevalence of influenza D virus among 256 feral swine, by state, United States, October 1, 2012–September 30, 2013*

| State, no. samples | D/13N Seropositive swine, no. (%) | D/13N GMT (range) | D/46N Seropositive swine, no. (%) | D/46N GMT (range) | Total seropositive swine, no. (%) |
|---|---|---|---|---|---|
| Hawaii, n = 73 | 11 (16.4) | 53.4 (1:40–1:80) | 4 (5.5) | 67.3 (1:40–1:80) | 15 (20.5) |
| North Carolina, n = 64 | 4 (6.3) | 67.3 (1:40–1:160) | 3 (4.7) | 40 (1:40–1:40) | 5 (7.8) |
| Oklahoma, n = 49 | 13 (26.5) | 49.5 (1:40–1:80) | 3 (6.1) | 50.4 (1:40–1:80) | 14 (28.6) |
| Texas, n = 70 | 10 (14.3) | 85.7 (1:40–1:160) | 8 (11.4) | 63.5 (1:40–1:160) | 15 (21.4) |

*D/13N and D/46N were used in HAI assays with 0.5% turkey red blood cells. Seropositivity defined as HAI titer ≥1:40. D/13N, influenza D/bovine/C00013N/Mississippi/2014 virus; D/46N, influenza D/bovine/C00046N/Mississippi/2014 virus; GMT, geometric mean titer; HAI, hemagglutination inhibition.

with 1× phosphate-buffered saline. We added turkey red blood cells to the serum (concentration 1:20) at 4°C for 30 min and then centrifuged the serum at 13,000 rpm for 1 min to pellet the red blood cells. We conducted the HA and HAI assays with 0.5% turkey red blood cells at 4°C against a testing IDV; we considered samples with an HAI titer ≥1:40 as IDV seropositive. We tested all serum samples against influenza D/46N and D/13N.

### Infection Experiments

We trapped a total of 26 feral swine over the course of 100 trap nights in Mississippi and transported them to the US Department of Agriculture's Mississippi Field Station under state permits (nos. 894, 896, and 908). All 26 feral swine tested seronegative for pseudorabies, brucellosis, and IDVs (D/13N and D/46N).

We randomly separated the 26 feral swine into 3 groups: virus-inoculated animals (n = 12), contact animals (n = 8), and control animals (n = 6). We used 12 pens to house the 26 feral swine; 4 pens contained 1 virus-inoculated animal plus 1 contact animal, 4 pens contained 2 virus-inoculated animals plus 1 contact animal, and 3 pens contained 2 control animals. Pens housing control feral swine were in an animal room separate from pens housing contact and inoculated feral swine. We inoculated each animal for the virus-inoculated group intranasally with 1 mL of D/46N ($10^6$ 50% tissue culture infective dose [$TCID_{50}$]) and each control animal with 1 mL of phosphate-buffered saline. At 2 days postinoculation (dpi), we moved 1 contact animal into the pen housing 1 or 2 virus-inoculated feral swine. At 0, 3, 5, 7, 9, 11, and 21 dpi, we collected nasal washes, rectal swab samples, and blood from all animals. We stored nasal washes and rectal swab samples at –80°C and serum samples at –20°C.

We euthanized feral swine at 3 dpi (1 virus-inoculated, 1 contact, and 1 control animal), 5 dpi (3 virus-inoculated, 1 contact, and 2 control animals), 7 dpi (3 virus-inoculated, 1 contact, and 1 control animal), 9 dpi (2 virus-inoculated, 1 contact, and 1 control animal), 11 dpi (2 virus-inoculated and 1 contact animal), and 21 dpi (1 virus-inoculated, 3 contact, and 1 control animal). We collected nasal turbinate, soft palate, trachea, bronchi, and lung at necropsy and stored tissues at –80°C before virologic characterization or fixed tissues in 10% neutral buffered formalin for histologic and immunohistochemical analysis.

### Calculation of $TCID_{50}$

We serially diluted homogenate supernatants from tissue samples and nasal washes from $10^{-1}$ to $10^{-6}$ and titrated them in HRT-18G cells. We tested each sample in triplicate and calculated the $TCID_{50}$ by using the Reed-Muench method (*27*).

### Immunohistochemical Staining

We performed IDV immunohistochemical staining as previously described (*28*). In brief, we heated slides at 65°C overnight. We deparaffinized and retrieved slides in an antigen retrieval solution at pH 6.1 (Dako, Carpinteria, CA, USA) by using a decloaking chamber. We used Tris-buffered saline (TBS) with 0.5% Tween to wash slides and used 3% $H_2O_2$ to quench peroxidase activity; we then blocked slides with 10% normal goat serum (Invitrogen, Carlsbad,

**Table 2**. Seroprevalence of influenza D virus among 256 feral swine, by age group and sex, Hawaii, North Carolina, Oklahoma, and Texas, United States, October 1, 2012–September 30, 2013*

| Characteristic, no. samples | D/13N Seropositive swine, no. (%) | D/13N GMT (range) | D/46N Seropositive swine, no. (%) | D/46N GMT (range) | Total seropositive swine, no. (%) |
|---|---|---|---|---|---|
| Age | | | | | |
| Juvenile, n = 32 | 6 (18.8) | 63.5 (1:40–1:160) | 1 (3.1) | 80.0 (1:80–1:80) | 7 (21.9) |
| Subadult, n = 43 | 6 (14.0) | 40.0 (1:40–1:40) | 2 (4.7) | 40.0 (1:40–1:40) | 8 (18.6) |
| Adult, n = 181 | 27 (14.9) | 65.1 (1:40–1:160) | 15 (8.3) | 58.8 (1:40–1:160) | 34 (18.8) |
| Sex | | | | | |
| F, n = 135 | 23 (17.0) | 62.9 (1:40–1:160) | 10 (7.4) | 54.0 (1:40–1:160) | 28 (20.7) |
| M, n = 118 | 16 (13.6) | 56.6 (1:40–1:160) | 8 (6.8) | 61. 7 (1:40–1:160) | 21 (17.8) |

*D/13N and D/46N were used in HAI assays with 0.5% turkey red blood cells. Seropositivity defined as HAI titer ≥1:40. Sex unknown for 3 animals. D/13N, influenza D/bovine/C00013N/Mississippi/2014 virus; D/46N, influenza D/bovine/C00046N/Mississippi/2014 virus; GMT, geometric mean titer; HAI, hemagglutination inhibition.

CA, USA) for 1 h. We added bovine-generated antiserum to D/46N (diluted 1:200 in antibody diluent [Dako]) to the slides and incubated them at –4°C for 24 h. We then washed the slides with TBS with 0.5% Tween and incubated them for 30 min with biotinylated goat anti–bovine IgG polyclonal secondary antibody diluted 1:500 in TBS with 0.5% Tween. Slides were washed and then incubated with ABC reagent (Vectastain, Burlingame, CA) according to the manufacturer's instructions, exposed to 3,3′-diaminobenzidine and $H_2O_2$ for 5 min, counterstained with hematoxylin, and dehydrated; we then applied a coverslip.

### Biosafety and Animal Handling

We conducted laboratory and animal experiments under Biosafety Level 2 conditions in compliance with protocols approved (QA 2563) by the Institutional Animal Care and Use Committee of the US Department of Agriculture's National Wildlife Research Center. Before necropsy, we fully anesthetized the feral swine with 0.044 mL/kg TKX (Telazol 4.4 mg/kg, ketamine 2.2 mg/kg, and xylazine 2.2 mg/kg) and, once fully sedated, the swine were euthanized by administration of a barbiturate solution (1 mL/4.5 kg body weight).

## Results

To identify whether IDV is circulating among feral swine populations in the United States, we performed HAI against D/46N and D/13N on 256 serum samples from feral swine collected during October 1, 2012–September 30, 2013 (*11*). We selected D/46N and D/13N to represent 2 genetic clades of IDVs, which are antigenically different (*13*,*29*). Of the 256 samples, 39 (15%) were positive for D/13N (HAI geometric mean titer [GMT] 60.2, range 1:40–1:160), and 18 (7%) were positive for D/46N (HAI GMT 52.3, range 1:40–1:160); the overall seropositive rate was 19.1% for IDV. Of the 39 samples seropositive for D/13N, 8 were also seropositive for D/46N, but the other 31 samples were seronegative for D/46N; of the 18 samples seropositive for D/46N, 10 samples were also seropositive for D/13N, whereas the other 8 samples were seronegative for D/13N. These data suggest a greater prevalence of infection with viruses antigenically related to D/13N among the feral swine populations tested.

The overall seroprevalence rate for IDV (i.e., D/13N, D/46N, or both) was 21.9% in juveniles (n = 32), 18.6% in subadults (n = 43), and 18.8% in adults (n = 181) (Table 2). Overall, seroprevalence was 17.8% among female feral swine (n = 135) and 20.7% among male feral swine (n = 118) (Table 2). The sex of 3 feral swine was unknown, but the animals were seronegative for IDV. By state, IDV seropositive rates among feral swine were 20.5% in Hawaii, 7.8% in North Carolina, 28.6% in Oklahoma, and 21.4% in Texas (Table 1; online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/6/17-2102-Techapp1.xlsx).

Previous serologic surveillance showed that ≈4.9% of feral swine had been exposed to IAVs (*25*). We explored whether an opportunity exists for feral swine to be exposed to both IAV and IDV. Our results show that 13 (5.1%) of the 256 serum samples were IAV positive (Table 1, 2), and 5 (38.5%) of the 13 were IDV positive. To determine whether feral swine could have exposure to both IAV and IDV, we selected an additional 83 serum samples from the 294 IAV-positive samples collected during October 1, 2012–September 30, 2013. We tested the 96 IAV feral swine serum samples (including the 13 already discussed) against D/46N and D/13N; of the 96 samples, 41 (42.7%) were IDV seropositive for D/13N (n = 37; GMT 1:59.6, range 1:40–1:160), D/46N (n = 9; GMT 1:58.8, range 1:40–1:160), or both (n = 5; GMT1:59.3, range 1:40–1:160) (online Technical Appendix Table 2).

To evaluate the characteristics of IDV infection in feral swine, we inoculated D/46N virus intranasally into 12 feral swine. We chose D/46N because this virus was shown to cause infection and transmission as well as a substantial increase in neutrophil tracking in tracheal epithelia of the infected calves, and we intended to compare the pathogenesis in cattle with that in feral swine (*28*). The IDV-inoculated swine showed no clinical signs or changes in body temperature. Viral titration of nasal washes showed that, at 3 dpi, 7 of 12 D/46N-inoculated swine shed virus with a maximum titer of 2.199 $\log_{10}$ $TCID_{50}$/mL, and that, at 5 dpi, 6 of the 8 remaining virus-inoculated swine shed virus with a maximum titer of 2.366 $\log_{10}$ $TCID_{50}$/mL. None of the remaining 5 virus-inoculated swine shed virus at or after 7 dpi (Figure 2; online Technical Appendix Table 3). No virus was detected in any rectal swab samples from these experimentally infected feral swine.

HAI results indicated that 7 (63.6%) of 11 virus-inoculated animals seroconverted at 5 dpi and all 8 remaining virus-inoculated animals seroconverted at 7 dpi (online Technical Appendix Table 4). We did not detect virus in any fecal swab samples from virus-inoculated swine, nor did we detect virus in nasal washes or fecal swab samples from the control feral swine, which remained seronegative throughout the study.

The viral titrations of feral swine tissues demonstrated viral replication in the upper and lower respiratory tract as well as the soft palate. At 5 dpi, viral titers were highest in the trachea sections (2.699–2.366 $\log_{10}$ $TCID_{50}$/mL) and lowest in the left and right caudal lung and soft palate (0.699–2.199 $\log_{10}$ $TCID_{50}$/mL $_0$) (online Technical Appendix Table 3). At 7 dpi, we detected no virus in nasal swab samples; however, the highest (maximum) viral titer (3.866 $\log_{10}$ $TCID_{50}$/mL) was in the soft palate, and the lowest viral titers (0.699 $\log_{10}$ $TCID_{50}$/mL) in the lower trachea (online Technical Appendix Table 3).

Three of the 8 contact animals exposed to IDV-infected feral swine had detectable viral shedding: 1 animal shed virus at 5 days postexposure (titer 2.032 $\log_{10}$ $TCID_{50}$/mL), and 1 animal each shed virus at 7 and 9 days postexposure (both had a titer equal to the detection limit). At 19 days postexposure, 1 of the 3 remaining contact feral swine seroconverted, with an HAI titer of 1:40, indicating that IDV can be transmitted among feral swine (Table 3; online Technical Appendix Table 4). Nearly half of the contact animals exposed to IDV-infected feral swine were infected with IDV (Figure 2). We did not detect virus in any rectal swab samples from the contact feral swine. None of the contact animals showed any clinical signs or change in body temperature.

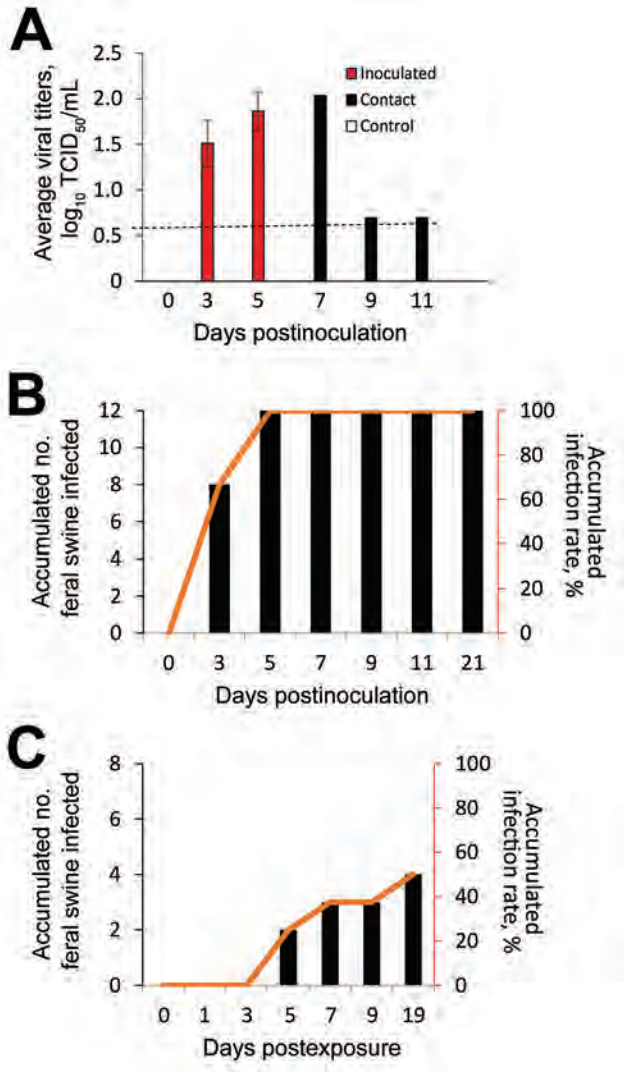


**Figure 2.** Infectivity and transmissibility of influenza D virus in feral swine populations, United States. A) Viral titers from nasal washes of feral swine. Feral swine were inoculated intranasally with $10^6$ $TCID_{50}$/mL of influenza D/bovine/C00046N/Mississippi/2014 virus. Naive feral swine were exposed to the virus by direct contact with D/bovine/C00046N/Mississippi/2014 virus–inoculated feral swine. On days 3, 5, 7, 9, and 11 after the inoculation group was inoculated, nasal washes were collected from all 3 groups of swine and in HRT-18G cells. Ending titers are expressed as $\log_{10}$ $TCID_{50}$/mL. The limit of virus detection was $10^{0.699}$ $TCID_{50}$/mL. Error bars represent standard error of viral titers. The dashed line indicates the lower limit of detection, which is $10^{0.699}$ $TCID_{50}$/mL. B) Accumulated number of feral swine infected and accumulated infection rate for the feral swine inoculated with influenza D virus. C) Accumulated number of feral swine infected and accumulated infection rate for the contact feral swine. A feral swine was considered infected if a viral titer was detected in nasal washes, serum samples, or both or if this feral swine seroconverted. $TCID_{50}$, 50% tissue culture infective dose.

Viral titration showed that virus was in the nasal turbinate, soft palate, trachea, lung tissues, or some combination of these tissues collected at 3–9 dpi. The tissue with the highest viral titer (5.366 $\log_{10}$ $TCID_{50}$/mL) was the lower trachea of an IDV-inoculated animal at 5 dpi. Immunohistochemical staining demonstrated the presence of IDV

**Table 3**. Summary of viral shedding and seroconversion in evaluation of characteristics of influenza D virus infection in feral swine*

| Timeline | HAI titer | Nasal titer |
|---|---|---|
| Inoculated swine, dpi, n = 12 | | |
| 3 | 0 (12) | 7 (12) |
| 5 | 6 (11) | 6 (11) |
| 7 | 8 (8) | 0 (8) |
| 9 | 5 (5) | 0 (5) |
| 11 | 3 (3) | 0 (3) |
| 21 | 1 (1) | 0 (1) |
| Control swine, dpi, n = 6‡ | | |
| 3 | 0 (6) | 0 (6) |
| 5 | 0 (5) | 0 (5) |
| 7 | 0 (3) | 0 (3) |
| 9 | 0 (2) | 0 (2) |
| 11 | 0 (1) | 0 (1) |
| 21 | 0 (1) | 0 (1) |
| Contact swine, dpe, n = 8§ | | |
| 1 | 0 (8) | 0 (8) |
| 3 | 0 (7) | 0 (7) |
| 5 | 0 (6) | 1 (6) |
| 7 | 0 (5) | 1 (5) |
| 9 | 0 (4) | 1 (4) |
| 19 | 1 (3) | 0 (3) |

*HAI titer data indicate number of swine that seroconverted (no. tested). Seropositivity defined as HAI titer ≥1:40. Nasal titer data indicate number of swine that shed virus (no. tested). Animals with a nasal wash viral titer ≥0.699 $\log_{10}$ $TCID_{50}$/mL were considered as shedding virus. dpe, days postexposure; dpi, days postinoculation; HAI, hemagglutination inhibition; $TCID_{50}$, 50% tissue culture infective dose.
†Feral swine inoculated with influenza D/bovine/C00046N/Mississippi/2014 virus.
‡Feral swine inoculated with sterile phosphate-buffered saline.
§Feral swine with direct-contact exposure to influenza D virus–inoculated swine.

antigen in epithelial cells of the soft palate, trachea, and lung. In the lung, we observed IDV immunostaining in type I pneumocytes, macrophages, and bronchiolar epithelial cells (Figure 3). The viral titrations on tissues from contact animals indicated that they were negative for IDV.

## Discussion

Since it was first identified in domestic swine in 2011, IDV has been detected in a wide range of animal hosts, mainly livestock such as bovids, goats, horses, and sheep, across multiple continents, indicating that IDV is a transboundary pathogen (*12*–*19*,*30*). In the United States, feral swine serve as an important vector between domestic and wild animals for multiple transboundary diseases, such as *Brucella suis* and IAV (*20*–*25*). Our study demonstrated that the seroprevalence rate of IDV in feral swine was 19.1%, which is similar to rates (13.5%–18.3%) reported for commercial beef cattle (*15*) but higher than the reported rate (<10%) in domestic swine (*1*). For example, a serologic study that used 220 pigs (3–20 weeks old) found that only 9.5% of tested domestic swine had an IDV titer ≥1:10, with a GMT of 1:20.7 (*1*). The difference in the seroprevalence between domestic and feral swine might be related to the fact that feral swine are mobile and have ample opportunities to come into contact with various domestic and wild animals; thus, compared with domestic swine, feral swine could have additional opportunities to be exposed to IDV. Among the 4 states that we sampled, the state with the highest IDV seroprevalence rate in feral swine also has the largest cattle population (Figure 1); however, whether IDV transmissions between bovids and feral swine is bidirectional is unknown. Two previous studies suggested that feral swine are likely to have indirect and direct contact with free-range bovine herds near water sources and that higher *B. suis* seroprevalence among free-range bovine herds was likely attributable to the bovine herd's close proximity to feral swine (*16*,*23*). Nevertheless, the seroprevalence rate reported in our study among feral swine was based on convenience samples; thus, an epidemiologic study will be needed to determine the enzootic status of IDV in the feral swine population.

Previous studies have suggested that domestic swine are major sources of IAV exposure for feral swine (*24*,*25*). IDV was shown not to cross-react with IAV (*1*), and our study showed that 42.3% of the IAV-seropositive feral swine had exposure to IDV, indicating that feral swine were exposed to both IDV and IAV. In addition, our results showed that the seroprevalence rate of IDV in IAV-seropositive feral swine was more than twice that observed among IAV-negative feral swine. However, a larger epidemiologic study covering larger geographic areas and longer

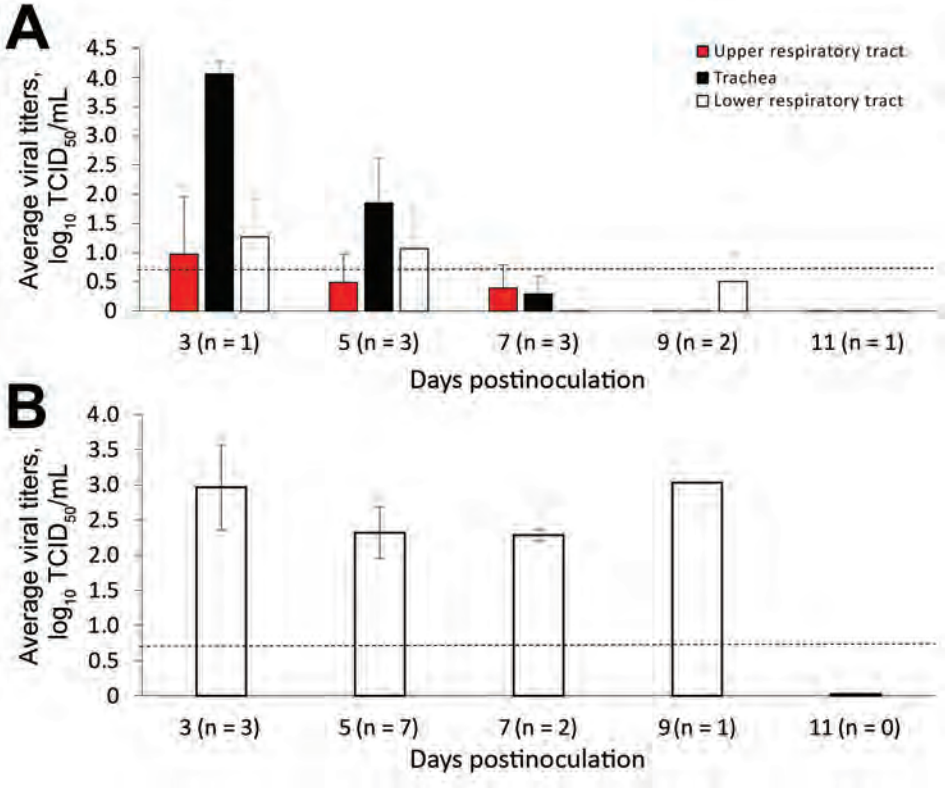


**Figure 3.** Influenza D viral titers in feral swine tissues. Feral swine were inoculated intranasally with $10^6$ $TCID_{50}$ of influenza D/bovine/C00046N/Mississippi/2014 virus or sterile phosphate-buffered saline (controls). At 3, 5, 7, 9, and 11 days postinoculation, they were humanely euthanized, and the following tissues were collected: turbinate; soft palate; upper, middle, and lower trachea; bronchus; left and right caudal lung; left and right medial lung; left and right cranial lung; and right accessory lung. A) The tissues were grouped as upper respiratory tract (turbinate and soft palate), trachea (upper, middle, and lower trachea, and bronchus), and lower respiratory tract (left and right caudal lung, left and right medial lung, and left and right cranial lung). B) All lung tissue sections at each time point that were influenza D virus–positive by $TCID_{50}$ titration in HRT-18G cells were averaged and plotted for each day postinoculation; day 9 has no error bars because only 1 positive tissue sample was found. Dashed lines indicate the lower limit of detection, which was $10^{0.699}$ $TCID_{50}$/mL. Error bars indicate SE. Numbers in parentheses indicate number of animals used in the analyses. $TCID_{50}$, 50% tissue culture infective dose.

periods is needed to test the hypothesis that IDV would be more prevalent in IAV-positive than IAV-negative feral swine. The effect of IDV infections on the pathogenesis of IAV, or vice versa, remains unknown, but findings from our study and previous studies suggest that future work ought to focus on whether feral swine act as a vector for transboundary disease between domestic swine and cattle.

The results of our animal challenge study show that IDV can be transmitted among feral swine; however, the infection resulted in limited clinical signs. Cattle infected with IDV shed virus up to 9 dpi, with a peak titer of 4.417 $\log_{10}$ $TCID_{50}$/mL (*28*), whereas the swine infected with IDV in our study shed virus only up to 5 dpi, with a peak titer of 2.366 $\log_{10}$ $TCID_{50}$/mL (Figure 2). Such a difference might be attributable to the distinct patterns of viral distributions in the respiratory tracts of the infected animals. In cattle, IDV was predominantly distributed in the tissues of the upper respiratory tract (i.e., turbinate and trachea) (*28*), but in swine the distribution was predominantly in the middle and lower respiratory tract (i.e., trachea and lung) (Figure 4, panel A). Previous studies have suggested that, under laboratory conditions, IDV replicates in the upper and lower respiratory tracts of guinea pigs infected with a bovine strain of IDV (*6*). In addition, bovine IDV infects ferrets and can be transmitted to IDV-naive ferrets through direct contact; however, IDV cannot infect naive ferrets through a fomite contaminated with nasal drainage from IDV-infected calves (*1*,*28*). Another swine infection study indicated that no viruses were detected in the lung of domestic swine that were infected with D/swine/Oklahoma/1334/2011 (*1*). The genetic variations between D/swine/Oklahoma/1334/2011and D/46N used in these studies might have led to the difference in viral tissue tropisms observed in these 2 studies.

Three IDV-inoculated animals and 1 contact animal in our study had transient viremia, a finding consistent with a previous study that found IDV in animal serum samples during IDV surveillance (*31*). We found IDV at moderate viral titers in the soft palate of feral swine at 3 dpi, and the viremia lasted ≥3 days for some animals (online Technical Appendix Table 3). The soft palate has been identified as a major site of influenza virus infection in ferrets (*32*). Previous studies have shown that several bacteria (e.g., *Streptococcus porcinus, Streptococcus dysgalactiae,*

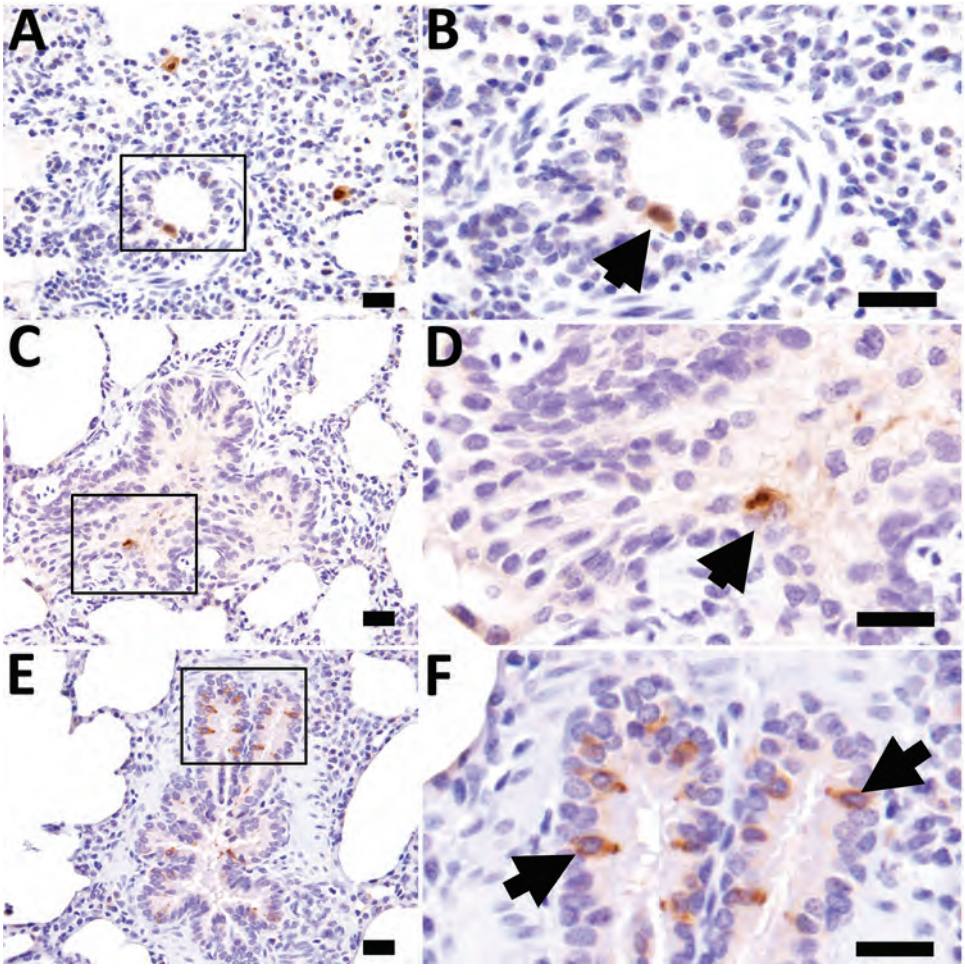


**Figure 4.** Influenza D virus immunohistochemistry in swine lung at 3 days (A and B), 5 days (C and D), and 7 days (E and F) postinoculation. Right column panels are higher magnification of boxed region in panels to the left. At all time points, scattered immunopositive bronchiolar epithelial cells were observed (arrows). Scale bars indicate 20 μm.

*Staphylococcus aureus, Staphylococcus hyicus, Streptococcus suis, Yersinia enterocolitica, Salmonella* spp., and *Listeria monocytogenes*) and viruses (e.g., porcine reproductive and respiratory syndrome virus and porcine circovirus-2) can be isolated from the soft palate (*33*). The rich distribution of lymphoid tissue in the soft palate might enable virus from the soft palate to enter the bloodstream and cause the transient viremia observed in feral swine that we observed in the experimentally infected feral swine.

The transmission ability of IDV through direct contact is similar to that in domestic swine; however, the transmission ability in feral and domestic swine seems to be less efficient than that in bovids, as suggested by viral load titers and durations of shedding (*1*,*28*). Given the limited transmissibility of IDV in feral swine, we would speculate that feral swine could have additional opportunities for exposure to IDV in addition to IDVs circulating in the feral swine populations.

In summary, our findings suggest that IDV has been circulating in the feral swine population across multiple states in the United States and that IDV can be transmitted among feral swine. Although the economic impact of IDV on commercial livestock remains unknown, our findings suggest that feral swine might be important in the ecology of IDV. Further studies are needed to understand whether other wild animals are infected by IDV and to what extent interspecies transmission contributes to IDV maintenance in domestic and wild populations.

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# Prion Disease in Dromedary Camels, Algeria

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Prions cause fatal and transmissible neurodegenerative diseases, including Creutzfeldt-Jakob disease in humans, scrapie in small ruminants, and bovine spongiform encephalopathy (BSE). After the BSE epidemic, and the associated human infections, began in 1996 in the United Kingdom, general concerns have been raised about animal prions. We detected a prion disease in dromedary camels (*Camelus dromedarius*) in Algeria. Symptoms suggesting prion disease occurred in 3.1% of dromedaries brought for slaughter to the Ouargla abattoir in 2015–2016. We confirmed diagnosis by detecting pathognomonic neurodegeneration and disease-specific prion protein ($PrP^{Sc}$) in brain tissues from 3 symptomatic animals. Prion detection in lymphoid tissues is suggestive of the infectious nature of the disease. $PrP^{Sc}$ biochemical characterization showed differences with BSE and scrapie. Our identification of this prion disease in a geographically widespread livestock species requires urgent enforcement of surveillance and assessment of the potential risks to human and animal health.

Prions are responsible for a group of fatal and transmissible neurodegenerative diseases named prion diseases. A misfolded and aggregated isoform of a cell-surface protein termed cellular prion protein ($PrP^{Sc}$) is the main, if not the sole, component of prions (*1*). Creutzfeldt-Jakob disease in humans and scrapie in small ruminants are the longest known diseases in this group, but prion diseases entered the public spotlight with the massive bovine spongiform encephalopathy (BSE) epidemic started in 1986 in the United Kingdom, revealing the zoonotic potential of animal prions.

Since the BSE epidemic begin, interest in these diseases has increased, and the prion universe has continued to expand (*2*). Several new prion diseases—including variant Creutzfeldt-Jakob disease, atypical/Nor98 scrapie of sheep, and atypical L- and H-type BSE—have been identified in the past 20 years, and chronic wasting disease (CWD) is spreading dramatically across cervid populations in North America and recently was discovered in Norway (*3*).

Public health concern increased markedly after variant Creutzfeldt-Jakob disease was demonstrated to be caused by the same prion strain responsible for the BSE epidemics (*4*). Unprecedented efforts were made to control the epidemics in cattle and to contain the exposure of humans to potentially infected cattle-derived materials.

In addition to having fatal consequences for infected animals, scrapie and BSE have a serious economic effect on the livestock industry. Scrapie brings economic damages through production loss, export loss, and increased cost for carcass disposal, which account for $10–$20 million annually in the United States (*5*). In the United Kingdom, where BSE was diagnosed in >180,000 cattle and up to 3 million were likely to have been affected, the cost to the public was >£5 billion (≈$7.1 billion US) (*6*).

Prion diseases can manifest as sporadic (putatively spontaneous), genetic, or infectious disorders (*1*). In animals, disorders resembling sporadic or genetic human prion diseases have been reported only recently, with the discovery of atypical/Nor98 scrapie in small ruminants (*7*) and L- and H-type BSE in cattle (*8,9*). Infectious prion diseases have been known for much longer and have been described in several animal species. Some diseases derived from accidental transmission, as is the case with BSE, which affected millions of cattle but also involved goats, domestic cats, nonhuman primates, and wild bovid and felid species, most likely fed with material contaminated by the BSE agent (*10*). Even the outbreaks of transmissible mink encephalopathy reported in the United States and various European countries in ranch-raised mink most likely originated from feedstuff accidentally contaminated by prions (*10*).

Despite the long list of susceptible animal species, prion diseases behave as infectious and naturally occurring conditions only in ruminants. Scrapie affects sheep and goats, and CWD affects different species of the *Cervidae* family: mule deer (*Odocoileus hemionus*), white-tailed

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deer (*O. virginianus*), elk (*Cervus canadensis*), and moose (*Alces alces*) (*11*). Furthermore, CWD has been recently diagnosed in reindeer (*Rangifer tarandus*) (*3*) and moose (*12*) from Norway.

We report prion disease in dromedary camels (*Camelus dromedarius*) from a Saharan population in Ouargla in southeastern Algeria, where the disease was observed in animals brought for slaughter at the Ouargla abattoir. Dromedaries are widespread throughout northern and eastern Africa, the Middle East, and part of Asia, where they are the means of subsistence for millions of families who live in the most hostile ecosystems on the planet. Since ancient times, camels have been exploited as beasts of burden and sources of milk and meat and for riding; today, they are tremendously important as a sustainable livestock species. During the past 10 years, the camel farming system has evolved rapidly and improved substantially (*13*). The emergence of a prion disease in a farmed animal species of such importance requires a thorough risk assessment for implementing evidence-based policies to control the disease in animals and minimize human exposure.

## Materials and Methods

### Animals and Tissue Samples

The Ouargla abattoir is one of the largest slaughterhouses in slaughtered volume for cattle, camels, and small ruminants in Algeria. In the past 5 years, neurologic symptoms have been observed more often in adult dromedaries at antemortem examination. The signs include weight loss; behavioral abnormalities; and neurologic signs, such as tremors, aggressiveness, hyperreactivity, typical down and upward movements of the head, hesitant and uncertain gait, ataxia of the hind limbs, occasional falls, and difficulty getting up (Video 1, https://wwwnc.cdc.gov/EID/article/24/6/17-2007-V1.htm; Video 2, https://wwwnc.cdc.gov/EID/article/24/6/17-2007-V2.htm).

According to breeders' descriptions, the early stage of the disease was mainly characterized by behavioral signs, such as loss of appetite and irritability. Separation from the herd at pastures along with aggressiveness and tendency to kick and bite when handled were usually observed. With disease progression, neurologic signs became obvious; animals showed ataxia that eventually led to recumbency and death.

Breeders reported that signs progressed slowly and that the duration of disease varied from 3 to 8 months. Although it has not been possible to date back the first cases of illness, information gathered from breeders and slaughterhouse personnel suggests the illness has been present since the 1980s.

Prion disease was suspected in dromedaries brought to the abattoir on the basis of clinical signs.

We collected brain samples from 3 dromedaries (nos. 3, 4, and 8) showing neurologic symptoms and from 1 clinically healthy animal (no. 5), as well as cervical, prescapular, and lumbar aortic lymph nodes from 1 animal (no. 8). The animals were all females, belonging to the Sahraoui population, 10, 11, 13, and 14 years of age, respectively.

We fixed samples in formalin for histologic and immunohistochemical examination. We also collected frozen brain samples from animals 4 and 8 for Western blot and genetic analysis and sampled formalin-fixed brain tissue from a clinically healthy animal (no. 5). We obtained brain samples from BSE-infected cattle and from ARQ/ARQ sheep, either naturally affected by scrapie or experimentally infected with BSE, from the surveillance system in Italy or from previous studies (*14*).

### Neuropathologic, Immunohistochemical, and Paraffin-Embedded Tissue Blot Analyses

We embedded brain and lymph node samples in paraffin wax, sectioned at 5 μm, and stained with hematoxylin and eosin or subjected to immunohistochemical or paraffin-embedded tissue blot analysis. We pretreated sections for immunohistochemistry with 98% formic acid for 5 min, followed by autoclaving in citrate buffer for 5 min at 121°C. We then treated sections with 6% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in phosphate-buffered saline for 60 min. We performed immunohistochemical detection of $PrP^{Sc}$ with L42 monoclonal antibody (mAb) (R-Biopharm, Darmstadt, Germany) at 0.01 μg/mL in phosphate-buffered saline overnight at 4°C. We treated sections with secondary biotinylated mouse antibody (Vector Laboratories), ABC Complex (Vector Laboratories) for 45 min, and diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) for 3 min. We used Mayer's hematoxylin for counterstaining. Each run comprised positive- and negative-control sections. We analyzed 3 sections from each lymph node sample.

We collected sections for paraffin-embedded blot on prewetted 0.45-μm–pore nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and dried membranes for 24 h at 55°C. We performed membrane treatments, proteinase K (PK) (Sigma-Aldrich) digestion (50 μg/mL), and immunodetection as described (*15*). We used mAb L42 (0.01 μg/mL) as the primary antibody.

### Western Blot Analysis

We performed Western blot analysis of $PrP^{Sc}$ from brain homogenates as previously described (*16*) and performed preliminary diagnosis with a final concentration of PK at 50 μg/mL. To compare dromedary $PrP^{Sc}$ with $PrP^{Sc}$ from sheep and cattle prion diseases, we performed molecular typing of their protease-resistant cores ($PrP^{res}$) by discriminatory immunoblotting, conducted according to the ISS (Istituto

Superiore di Sanità) discriminatory Western blot method (*17*) with minor modifications. The principle of discrimination is based on the differential N terminal cleavage by PK (200 μg/mL), revealed by using N terminal mAb with an epitope that is partially lost after PK digestion of BSE samples (*14,18*). As an additional discriminatory parameter, we measured the relative proportions of diglycosylated, monoglycosylated, and unglycosylated PrP fragments in L42 blots.

We performed deglycosylation by adding 18 μL of 0.2 M sodium phosphate buffer (pH 7.4) containing 0.8% Nonidet P40 (Roche. Penzberg, Germany) and 2 μL (80 U/mL) di N-Glycosidase F (Roche) to 5 μL of denaturated samples and incubating overnight at 37°C with gentle shaking. The mAbs used and their epitope on ovine PrP were as follows: L42 (148–153), 12B2 (93–97), SAF32 (octarepeat).

### PrP Gene Sequence Analysis

We extracted DNA from 100 mg of frozen brain tissue with DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. We amplified the PrP gene (*PRNP*) coding sequence in a 50-μL final volume using 5 μL of extracted DNA, 1× AmpliTaq Gold 360 PCR Buffer (Applied Biosystems, Foster City, CA, USA), 2.5 mmol/L MgCl2, 1× 360 GC Enhancer, 200 μmol/L dNTPs, 0.25 μmol/L of forward (5′-GCTGACACCCTCTTTATTTTGCAG-3′) and reverse (5′-GATTAAGAAGATAATGAAAACAGGAAG-3′) primers (*19*), and 0.5 μL of AmpliTaq Gold 360 (Applied Biosystems), according to the following amplification protocol: 5 min at 96°C; 30 s at 96°C, 15 s at 57°C, 90 s at 72°C for 40 cycles, and 4 min at 72°C.

We purified amplicons by using an Illustra ExoProStar 1-Step clean-up kit (GE Healthcare Life Sciences, Little Chalfont, UK). We conducted sequencing reactions by using the BigDye Terminator v1.1 Cycle Sequencing Kit, purified using BigDye XTerminator Purification Kit, and detected with the ABI PRISM 3130 apparatus (all Applied Biosystems). We analyzed sequences by using Seq Scape version 2.5 (Applied Biosystems).

## Results

Histopathologic examination showed spongiform change, gliosis, and neuronal loss in several brain areas of the 3 symptomatic animals (Figure 1, panels A, B) but not in the asymptomatic dromedary. We observed vacuoles preferentially in the neuropil (Figure 1, panel A) but also frequently involving the neuronal bodies (Figure 1, panel B). Confluent vacuoles were rarely observed. These neurodegenerative changes consistently occurred in gray matter of subcortical brain areas, such as striatum, thalamus (Figure 1, panel A), midbrain, and pons (Figure 1, panel B) of all 3 animals; white matter was rarely affected. We observed moderate vacuolation in medulla oblongata, particularly in the vestibular and the olivary nucleus; nucleus of solitary tract and hypoglossal nucleus were less often affected. Cervical medulla, available only for animal 8, showed no spongiform changes. Cortical brain areas were variably involved. Animals 3 and 8 showed dispersed vacuolation in cingulate, piriform, and frontal cortices. In contrast, cerebral cortices were more heavily affected in animal 4. Cerebellum was collected from animals 4 and 8, and vacuoles were observed only in the molecular layer of animal 4.

By immunohistochemical analysis, we detected $PrP^{Sc}$ in the brain of all symptomatic dromedaries. Overall, $PrP^{Sc}$ deposition was invariably observed in brain areas with spongiform degeneration (Figure 1, panels C, D). In addition, $PrP^{Sc}$ deposits also involved areas less often affected or not affected by spongiosis, such as the nucleus of the solitary tract (Figure 1, panel E); the hypoglossal nucleus; pyramidal cells of hippocampus; the granular layer of cerebellum, including Purkinje cells (Figure 1, panel F); and several white matter areas.

$PrP^{Sc}$ deposition patterns involving neuropil, neurons, and glia differed. Patterns included synaptic/punctate (Figure 1, panel G), intraneuronal (Figure 1, panel H), perineuronal and linear (Figure 1, panel I), intraglial (Figure 1, panels J–L), and perivascular (Figure 1, panel M). In pons and medulla oblongata, we frequently observed an atypical intracellular pattern (Figure 1, panel N) in which $PrP^{Sc}$ filled the whole cytoplasm. $PrP^{Sc}$ was absent in the brain of the asymptomatic dromedary (Figure 1, panel O). Prominent protease-resistant $PrP^{Sc}$ deposition was easily detected by paraffin-embedded blot in the same brain areas found positive by immunohistochemical analysis, such as the deep layers of cortices (Figure 1, panel P), the pyramidal layer and fimbria of hippocampus (Figure 1, panel Q), the granular layer of cerebellum and the associated white matter (Figure 1, panel R), and the gray matter of pons (Figure 1, panel S).

We detected $PrP^{Sc}$ deposits in cervical, prescapular, and lumbar aortic lymph nodes from animal 8 (Figure 2) that involved >80% of primary and secondary follicles in the 3 sections analyzed. $PrP^{Sc}$ deposits consisted of a reticular network at the center of the lymphoid follicles, which varied in staining intensity, accompanied by fine to coarse granules of $PrP^{Sc}$ in the cytoplasm of nonlymphoid cells within the follicle. We also observed additional granular or intracellular $PrP^{Sc}$ immunolabeling in the interfollicular areas.

Western blot analysis of brain homogenates from dromedaries 4 and 8 revealed $PrP^{Sc}$ with a $PrP^{res}$ showing the classical electrophoretic profile, characterized by 3 main bands representing diglycosylated, monoglycosylated, and unglycosylated $PrP^{res}$ (Figure 3, panel A). Accordingly, the 3 bands were resolved in a single band of ≈18 kDa after enzymatic deglycosylation (Figure 3, panel B).

The apparent molecular weight of $PrP^{res}$ from both animals was slightly higher than classical scrapie and clearly higher than BSE and sheep-passaged BSE (Figure 3, panels A, C, left side). This finding prompted us to investigate the N terminal PK cleavage under stringent PK conditions by discriminatory immunoblotting, which enables the molecular discrimination of the most common ruminant TSE strains from classical BSE (*14,18,20*). Epitope mapping of

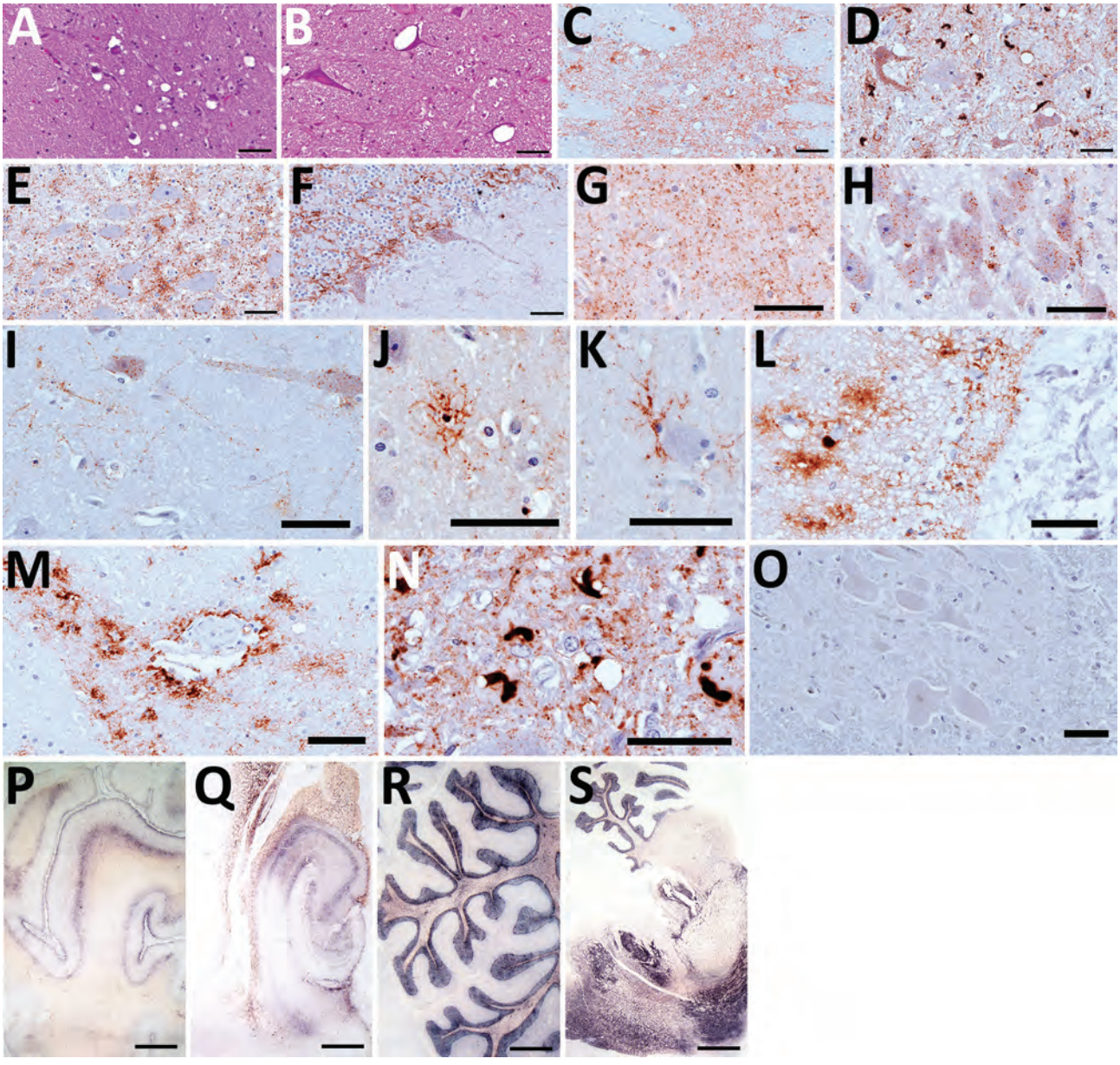


**Figure 1.** Hematoxylin and eosin staining (A, B), immunohistochemistry (C–O), and paraffin-embedded tissue blot analysis (P–S) of brains of dromedary camels brought for slaughter to the Ouargla abattoir, Algeria, 2016–2017. Spongiform change of neuropil, gliosis, and neuronal loss in thalamus (A) and intraneuronal vacuolation in pons (B) (scale bar = 50 μm). Immunohistochemistry for prion protein ($PrP^{Sc}$) with L42 monoclonal antibody evidenced dense synaptic/punctate deposition in thalamus (C) and intraneuronal and extraneuronal $PrP^{Sc}$ deposits in pons (D), accompanied by spongiform change. Perineuronal, diffused in neuropil, and glial-associated $PrP^{Sc}$ staining were also observed in the nucleus of the solitary tract (E) and cerebellum (F), which showed rare vacuoles (scale bars = 50 μm). Immunohistochemical analysis performed on brains of symptomatic dromedaries revealed several $PrP^{Sc}$ deposition patterns, such as synaptic/punctate pattern diffused in the neuropil (G); intraneuronal deposition in pyramidal cells of hippocampus (H); perineuronal and linear staining in frontal cortex (I); intraglial $PrP^{Sc}$ deposition (J–L); perivascular deposition (M); atypical intracellular $PrP^{Sc}$ deposition pattern in pons (N). $PrP^{Sc}$ was absent in asymptomatic dromedary used as negative control (O) (scale bars = 50 μm). $PrP^{Sc}$ distribution, by paraffin-embedded tissue blot analysis, was observed in several brain areas, such as prefrontal cortex (P), hippocampus (Q), cerebellum (R), and a sagittal section of pons (S) (scale bar = 3 mm).

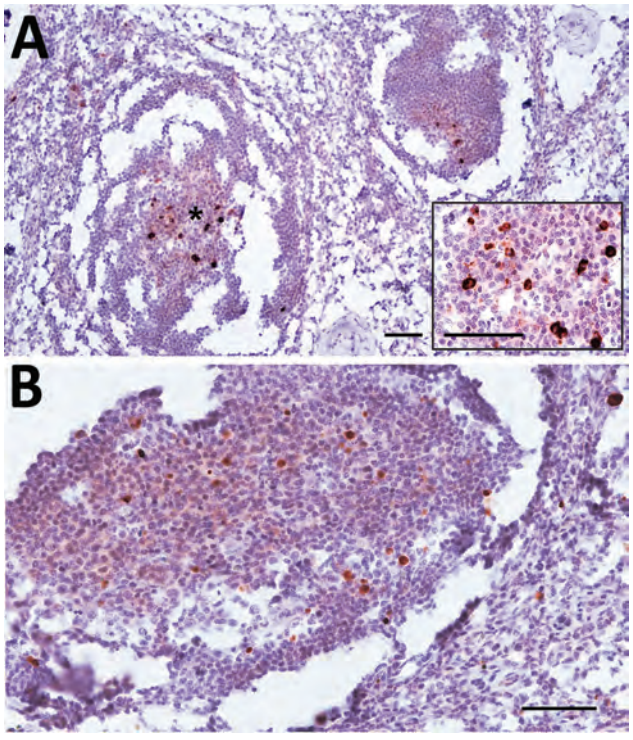


**Figure 2.** Prion protein immunolabeling in the germinal center of lymphoid follicles of cervical (A) and prescapular (B) lymph nodes of dromedary camel no. 8, Ouargla abattoir, Algeria. The architecture of lymph nodes appears moderately compromised by the partial freezing of samples that accidentally occurred before fixation. Scale bars = 50 μm. Inset in panel A: higher magnification showing the germinal center marked with asterisk; scale bar = 25 mm.

$PrP^{res}$ showed that the higher apparent molecular weight in dromedary $PrP^{res}$ reflects a more N terminal cleavage site than with BSEs and scrapie samples. Indeed, upon treatment with PK, dromedary $PrP^{res}$ preserved the N terminal 12B2 and SAF32 mAb epitopes, whereas classical scrapie lost the SAF32 mAb epitope while preserving the 12B2 mAb epitope, and BSE samples lost both epitopes, being negative with SAF32 and 12B2 mAbs (Figure 3, panel C). We have previously shown that, with the ISS discriminatory Western blot, BSE and scrapie are both characterized by a diglycosylated dominant $PrP^{res}$ pattern, although BSE is more heavily glycosylated than scrapie (*18*). Our data confirm this difference and show that $PrP^{res}$ from dromedary camels is further less glycosylated than classical scrapie, being characterized by a monoglycosylated dominant $PrP^{res}$ (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/6/17-2007-Techapp1.pdf). Sequencing revealed the same *PRNP* sequence in animals 4 and 8 (GenBank accession nos. MF990558–9), which, in turn, showed 100% nt identity with the *PRNP* sequence already reported for dromedary camels (*19*).

In parallel to the laboratory analyses, we undertook a retrospective investigation of neurologic signs in dromedaries at the Ouargla slaughterhouse. Twenty of 937 animals in 2015 and 51 of 1,322 in 2016 showed the previously described neurologic signs (Table); the overall prevalence was 3.1% in dromedaries brought for slaughter. All slaughtered animals derived from the area surrounding Ouargla, and the disease was observed only in animals >8 years of age.

## Discussion

We describe a prion disease in dromedary camels, designated as camel prion disease (CPD), that we detected during routine antemortem inspection at the Ouargla slaughterhouse in Algeria. Retrospective analysis indicated a 3.1% prevalence of animals with neurologic signs suggestive of the disease in dromedaries brought for slaughter. That figure appears to be reliable given that clinical suspicion was confirmed in all 3 animals undergoing laboratory analysis. However, because prion diseases are characterized by long incubation periods and the age at which the disease becomes apparent (>8 years) is more advanced than the age at which most dromedaries are slaughtered (<5 years), the prevalence found in the older animals is probably higher than the actual prevalence (excluding younger animals).

The spectrum of animal species susceptible to prion disease is large. However, only in ruminants belonging to the *Bovidae* and *Cervidae* families do prion diseases behave as infectious and naturally occurring conditions. Dromedaries are not ruminants (suborder *Ruminantia*) but rather are *Tylopoda*, a suborder of *Artiodactyla*, which also includes the 2-humped camel (*Camelus bactrianus*), wild Bactrian camel (*C. ferus*), llamas (*Lama glama*), alpacas (*Vicugna pacos*), and vicuñas (*V. vicugna*) (*21*). The presence of a prion disease in dromedaries extends the spectrum of animal species naturally susceptible to prion diseases to taxa different from those already known and opens up new research areas on the ecology and the host–pathogen relationship of prion diseases.

Whether CPD is an infectious disease in natural conditions is a key question. In scrapie and CWD, in which lymphoid tissues are extensively involved, the horizontal transmission in natural conditions is efficient. In contrast, when the peripheral lymphoid tissues are not substantially involved, as in cattle BSE, atypical/Nor98 scrapie, and most human prion diseases, the horizontal transmission appears to be inefficient. This inefficiency usually is explained by assuming the in vivo dissemination of $PrP^{Sc}$ to the periphery as a prerequisite to facilitate prion shedding into the environment (*22*). Although we obtained samples from a single animal, our detection of $PrP^{Sc}$ in all lymph nodes available suggests an abundant extraneural pathogenesis and, along with the notable prevalence of clinical cases at the slaughterhouse, concurs to suggest the infectious nature of CPD. These observations also suggest that the disease has an acquired rather than spontaneous onset.

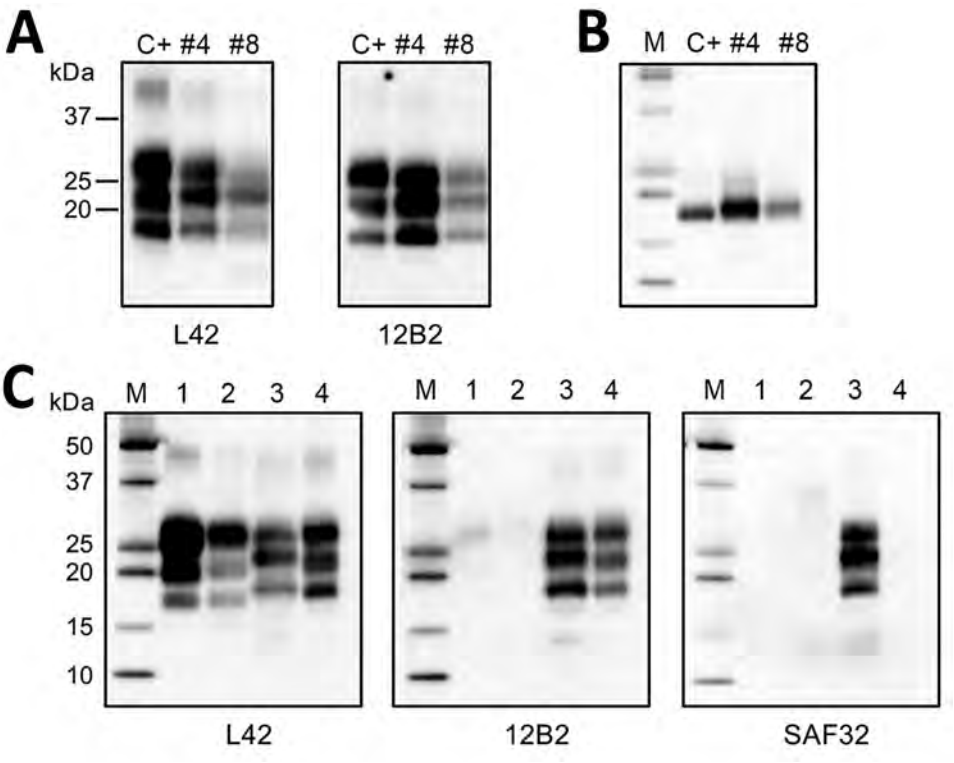


**Figure 3.** Western blot analysis of protein-resistant core ($PrP^{res}$) of pathological dromedary prion protein. A) Western blot analysis of proteinase K (PK)–treated $PrP^{Sc}$ in brain homogenates from dromedary camels with neurologic symptoms (nos. 4 and 8), Algeria. A sample of sheep scrapie was loaded as control (indicated as C+). Membranes were probed with L42 (left) and 12B2 monoclonal antibody (mAb) (right). Molecular weights (kDa) are indicated on the left. Tissue equivalents loaded per lane were 2 mg for camel samples and 0.1 mg for sheep scrapie. B) Samples after deglycosylation. Membrane was probed with L42 mAb. C) Comparison of dromedary $PrP^{res}$ (from camel no. 4) with sheep bovine spongiform encephalopathy (BSE), bovine BSE, and sheep scrapie samples by ISS (Istituto Superiore di Sanità) discriminatory Western blot (17). Tissue equivalents loaded per lane were 2 mg for dromedary camel and bovine samples and 0.1 mg for sheep samples. In each blot, samples were loaded as follows: lane 1, ovine BSE; lane 2, bovine BSE; lane 3, dromedary camel no. 4; lane 4, ovine scrapie. Membranes were probed with L42, 12B2, and SAF32 mAbs, as indicated. For the analyses in panels B and C, protein standards were loaded and are indicated as M.

The origin of CPD is unknown. It might be a disease unique to dromedaries or a malady deriving from transmission of a prion disease from another species. It is worth noting that meat and bone meal has been exported from the United Kingdom worldwide, and after the ban on feeding animals with ruminant protein in 1988, export to the Third World had soared to 30,000 tons (*23*) in 1991. Thus, the possibility that BSE-infected feed could have reached North Africa cannot be ruled out. However, even if the risk for BSE has not been formally assessed in Algeria and an official surveillance system for animal prion diseases is lacking, BSE is unlikely to appear in dromedaries without evidence in cattle populations. Moreover, dromedaries are mostly raised with no use of feedstuff. Lastly, the $PrP^{Sc}$ biochemical signature in CPD clearly differs from that of BSE or sheep-passaged BSE. Although host factors are known to be able to alter the $PrP^{Sc}$ signature during interspecies transmission, the BSE profile generally has been preserved in species accidentally or experimentally affected. In principle, CPD also might have derived from scrapie. Dromedaries often are raised along with sheep and goats, sharing common pastures. However, although the absence of an effective surveillance system prevents drawing any conclusions, scrapie has never been reported in Algeria, and a field survey in northeastern Algeria could not provide evidence of the disease (*24*). Moreover, the $PrP^{Sc}$ signature of CPD differed from the classical scrapie case used for comparison (Figure 3). To help clarify the origin and nature of CPD, bioassays in a panel of rodent models are ongoing for a thorough prion strain characterization.

Future investigations of the geographic distribution of CPD will help clarify its origin. If the disease is confined to the dromedary populations of the Ouargla region, a localized event of transmission could be hypothesized. Common-source scrapie epidemics in sheep and goats occurred in the United Kingdom and Italy as a consequence of the use of accidentally contaminated vaccines (*25*,*26*). However, in the Ouargla region, no vaccination program has been implemented for infectious disease prophylaxis in dromedaries. Intriguingly, dromedary breeders indicate that the only food source other than pasture available to dromedaries in the Ouargla region are the waste dumps widespread in the desert near the oil extraction plants, where dromedaries and small ruminants gather and scavenge (Video 3, https://wwwnc.cdc.gov/EID/article/24/6/17-2007-V3.htm). The possibility that dromedaries acquired the disease from eating prion-contaminated waste needs to be considered.

**Table.** Suspected prion disease in dromedary camels at antemortem inspection at the Ouargla slaughterhouse, Algeria

| Month | 2015 | | 2016 | |
|---|---|---|---|---|
| | No. animals presented at abattoir | No. with clinically suspected prion disease | No. animals presented at abattoir | No. with clinically suspected prion disease |
| Jan | 63 | 0 | 67 | 3 |
| Feb | 70 | 2 | 83 | 4 |
| Mar | 86 | 1 | 73 | 3 |
| Apr | 79 | 2 | 85 | 3 |
| May | 97 | 3 | 93 | 4 |
| Jun | 81 | 1 | 117 | 5 |
| Jul | 92 | 2 | 135 | 6 |
| Aug | 121 | 4= | 145 | 7 |
| Sep | 31 | 1 | 44 | 5 |
| Oct | 42 | 1 | 110 | 4 |
| Nov | 89 | 2 | 164 | 4 |
| Dec | 86 | 1 | 206 | 3 |
| Total | 937 | 20 | 1,322 | 51 |

Tracing the origin of prion diseases is challenging. In the case of CPD, the traditional extensive and nomadic herding practices of dromedaries represent a formidable factor for accelerating the spread of the disease at long distances, making the path of its diffusion difficult to determine. Finally, the major import flows of live animals to Algeria from Niger, Mali, and Mauritania (*27*) should be investigated to trace the possible origin of CPD from other countries.

Camels are a vital animal species for millions of persons globally. The world camel population has a yearly growth rate of 2.1% (*28*). In 2014, the population was estimated at ≈28 million animals, but this number is probably underestimated. Approximately 88% of camels are found in Africa, especially eastern Africa, and 12% are found in Asia. Official data reported 350,000 dromedaries in Algeria in 2014 (*28*).

On the basis of phenotypic traits and sociogeographic criteria, several dromedary populations have been suggested to exist in Algeria (*29*). However, recent genetic studies in Algeria and Egypt point to a weak differentiation of the dromedary population as a consequence of historical use as a cross-continental beast of burden along trans-Saharan caravan routes, coupled with traditional extensive/nomadic herding practices (*30*).

Such genetic homogeneity also might be reflected in *PRNP*. Studies on *PRNP* variability in camels are therefore warranted to explore the existence of genotypes resistant to CPD, which could represent an important tool for CPD management as it was for breeding programs for scrapie eradication in sheep.

In the past 10 years, the camel farming system has changed rapidly, with increasing setup of periurban dairy farms and dairy plants and diversification of camel products and market penetration (*13*). This evolution requires improved health standards for infectious diseases and, in light of CPD, for prion diseases.

The emergence of another prion disease in an animal species of crucial importance for millions of persons worldwide makes it necessary to assess the risk for humans and develop evidence-based policies to control and limit the spread of the disease in animals and minimize human exposure. The implementation of a surveillance system for prion diseases would be a first step to enable disease control and minimize human and animal exposure. Finally, the diagnostic capacity of prion diseases needs to be improved in all countries in Africa where dromedaries are part of the domestic livestock.

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# Frequent Implication of Multistress-Tolerant *Campylobacter jejuni* in Human Infections

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*Campylobacter jejuni*, a major cause of bacterial foodborne illnesses, is considered highly susceptible to environmental stresses. In this study, we extensively investigated the stress tolerance of 121 clinical strains of *C. jejuni* against 5 stress conditions (aerobic stress, disinfectant exposure, freeze-thaw, heat treatment, and osmotic stress) that this pathogenic bacterium might encounter during foodborne transmission to humans. In contrast to our current perception about high stress sensitivity of *C. jejuni*, a number of clinical strains of *C. jejuni* were highly tolerant to multiple stresses. We performed population genetics analysis by using comparative genomic fingerprinting and showed that multistress-tolerant strains of *C. jejuni* constituted distinct clades. The comparative genomic fingerprinting subtypes belonging to multistress-tolerant clades were more frequently implicated in human infections than those in stress-sensitive clades. We identified unique stress-tolerant *C. jejuni* clones and showed the role of stress tolerance in human campylobacteriosis.

*Campylobacter* spp., particularly *Campylobacter jejuni*, are a leading bacterial cause of gastroenteritis and cause ≈166 million cases of infection worldwide per year (*1*). Human exposure to *C. jejuni* occurs through various routes, including foodborne and waterborne transmission and direct contact with farm and companion animals (*2*). However, foodborne transmission accounts for most cases of human campylobacteriosis, mainly through consumption of contaminated poultry (*1*,*3*). *C. jejuni* inhabits chicken intestines as a commensal microorganism at a level $\geq 10^6$–$10^8$ CFU/g of feces (*4*). Thus, release of fecal contents from chicken might contaminate poultry carcasses at multiple steps during poultry processing to finished product (*5*). To reduce *C. jejuni* contamination in poultry meat, various mitigation strategies are used in poultry processing, including chemical treatment with decontamination agents (*6*), physical treatment with hot water and steam, and chilling and freezing of carcasses (*7*).

Although *C. jejuni* is transmitted to humans through the food chain or by other routes in the environment, *C. jejuni* encounters a wide range of stress conditions before human exposure (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/6/17-1587-Techapp1.pdf). Stress tolerance plays a major role in transmission of foodborne pathogens to humans by enhancing bacterial survival during food processing, preservation, and cooking (*8*). For example, the capability of *Salmonella* spp. to survive in low-moisture environments enables this pathogen to contaminate dry foods (*9*), and the psychrotrophic nature of *Listeria monocytogenes* enables it to survive in ice cream (*10*).

Unlike these robust enteric pathogens, *C. jejuni* is considered highly susceptible to stresses primarily because of the lack of many stress response factors (*11*). Because of its stress sensitivity, *C. jejuni* is considered unlikely to survive effectively outside animal hosts. However, regarding food safety, it is still an enigma how this stress-sensitive bacterium survives under hostile conditions in various transmission routes and is increasingly responsible for human illnesses worldwide. Despite the role of stress tolerance during transmission of pathogens to humans, the role of stress tolerance in human infections has not yet been elucidated for *C. jejuni*. To fill this major knowledge gap, in this study, we extensively examined stress tolerance in 121 clinical strains of *C. jejuni* and detected stress-tolerant *C. jejuni* populations that are frequently involved in human infections.

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## Materials and Methods

### Strains and Culture Conditions

Clinical strains of *C. jejuni* were provided by the Provincial Laboratory for Public Health in Alberta, Canada. These strains were obtained from 5 health zones (North, Edmonton, Central, Calgary, and South) that cover the entire province

of Alberta. A total of 121 selected clinical strains of *C. jejuni* from human stool samples were tested: 24 from the North Zone, 48 from the Edmonton Zone, 13 from the Central Zone, 15 from the Calgary Zone, 11 from the South Zone, and 10 from unknown locations. We routinely grew strains in Mueller-Hinton medium at 42°C microaerobically (5% $O_2$, 10% $CO_2$, and 85% $N_2$).

### Aerotolerance Test

The aerotolerance test was performed according to a method described in our previous study (*12*). We grew *C. jejuni* strains on Mueller-Hinton agar at 42°C overnight under microaerobic conditions. Strains were harvested, placed in fresh Mueller-Hinton broth, and diluted to an optical density at 600 nm ($OD_{600}$) of 0.1. We incubated bacterial suspensions at 42°C aerobically with shaking at 200 rpm. Aliquots were taken at 0, 12, and 24 h for serial dilution and enumeration.

### Aerobic Survival of Strains on Refrigerated Raw Chicken

Survival of *C. jejuni* was determined as described (*13*). We prepared pieces of raw chicken skin (≈0.2 g/piece) from chicken thigh by cutting with a sterile razor and placed them into 96-well microtiter plates. The *C. jejuni* suspension prepared from overnight cultures was diluted to an $OD_{600}$ of 0.07 in phosphate-buffered saline, and 100 μL of the *C. jejuni* suspension was applied to the chicken skin. The chicken skin pieces spiked with *C. jejuni* were stored at 4°C. We placed sterile needles under both sides of the lid of a microtiter plate to prevent blockage of air circulation. We placed a container filled with water near the plate to prevent samples from being dried during incubation. After incubation, chicken skin pieces were transferred to 15-mL tubes containing 1 mL of fresh Mueller-Hinton broth. After we vigorously vortexed samples for 2 min, we collected supernatants to enumerate bacteria by using Preston *Campylobacter*-selective agar.

### Stress Tolerance Tests

We grew the 121 clinical strains of *C. jejuni* on Mueller-Hinton agar overnight at 42°C under microaerobic conditions and resuspended them in Mueller-Hinton broth for stress tolerance tests as follows. Each assay was performed with negative controls without artificial contamination of *C. jejuni* and was repeated 3 times.

#### Resistance to Peracetic Acid, a Chemical Decontaminant

This experiment was performed on the basis of a previous study with slight modifications (*14*). We prepared a piece of raw chicken (≈0.2 g/piece) containing skin and muscle by using a sterile razor. We spiked each chicken piece with ≈$10^8$ CFU of *C. jejuni* and incubated each piece at 4°C for 1 h under microaerobic conditions. The chicken piece was dipped in 750 ppm peracetic acid (PAA) (Sigma Aldrich, St. Louis, MO, USA) for 15 s and transferred into a 15-mL tube containing 1 mL fresh Mueller-Hinton broth. After we vortexed supernatants for 2 min, we serially diluted and spread them on Preston *Campylobacter*-selective agar to enumerate *C. jejuni*.

#### Tolerance to Freeze-Thaw

We diluted *C. jejuni* suspensions to an $OD_{600}$ of 0.1 (≈$10^8$ CFU/mL). Aliquots (≈100 μL) were applied to 0.2 g of chicken skin and placed in 96-well microtiter plates. After incubation at –20°C, samples were defrosted at 4°C for 2 h and transferred to a 15-mL tube containing 1 mL of Mueller-Hinton broth. After we vortexed supernatants for 2 min, we serially diluted them in Mueller-Hinton broth for enumeration by plating on Preston *Campylobacter*-selective agar.

#### Thermotolerance Test

The *C. jejuni* suspension from overnight cultures was 100-fold diluted in whole milk (3.25% milk fat). We transferred milk contaminated with *C. jejuni* to 96-well microtiter plates and subjected them to heat treatment by using a thermocycler (Eppendorf, Hamburg, Germany) at 72°C for 15 s or 30 s. After a serial dilution, samples were plated on Preston *Campylobacter*-selective agar.

#### Osmotolerance Test

After a 10-fold serial dilution, 5 μL of *C. jejuni* suspension was spotted on Mueller-Hinton agar (negative control) or Mueller-Hinton agar plates supplemented with 2% and 4% NaCl. We measured viability after overnight incubation at 42°C under microaerobic conditions.

### Comparative Genomic Fingerprinting Analysis

We performed the comparative genomic fingerprinting 40 (CGF40) assay as described (*15*) and stored data in a secured database at the Provincial Laboratory for Public Health in Alberta. We performed cluster analysis by using BioNumerics software version 7.6 (Applied Maths NV, Sint-Martens-Latem, Belgium) with the unweighted pair group method with arithmetic mean clustering algorithm and the simple matching coefficient.

### Multilocus Sequence Typing

We performed multilocus sequence typing (MLST) analysis of *C. jejuni* strains as described (*16*). We conducted PCR amplification of 7 housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA*) by using ExTaq polymerase (Takara, Tokyo, Japan). PCR products were sequenced by Macrogen Inc. (Seoul, South Korea), and sequences were analyzed by using the pubMLST database (https://pubmlst.org/).

### Statistical Analysis

We performed statistical analysis for aerotolerance and stress tolerance tests by using 2-way analysis of variance. We calculated Pearson correlation coefficients on the basis of correlations between level of stress tolerance and prevalence of *C. jejuni* in the CGF collection of human clinical isolates in Alberta. Statistical analyses were performed by using SPSS version 21 (IBM, Armonk, NY, USA).

## Results

### Prevalence of Hyperaerotolerant Strains in Human Clinical Cases

Because aerotolerance plays a major role in survival of *C. jejuni* under aerobic conditions, we determined the aerotolerance of the 121 clinical strains. Strains that did not survive aerobic shaking at 200 rpm for 12 h were considered oxygen sensitive; those that survived for ≈12–24 h were classified as aerotolerant, and those that survived for >24 h were classified as hyperaerotolerant (Figure 1). Most clinical strains of *C. jejuni* were hyperaerotolerant (65/121, 53.7%) and aerotolerant (46/121, 38.0%); only 8.3% (10/121) of strains were oxygen sensitive (Figure 1). Results showed that hyperaerotolerant and aerotolerant strains of *C. jejuni* are highly prevalent in cases of human infection.

### Survival of Hyperaerotolerant Strains in Refrigerated Chickens under Aerobic Conditions

We determined the viability of the 121 clinical strains of *C. jejuni* on refrigerated raw chicken. When we compared oxygen-sensitive *C. jejuni* strains with aerotolerant and hyperaerotolerant strains, the aerotolerant and hyperaerotolerant strains survived for longer periods on chicken at refrigeration temperatures in the air. Both aerotolerant and hyperaerotolerant strains were recovered after 7 days; however, only 1 oxygen-sensitive strain was recovered after 3 days (Figure 2). Some hyperaerotolerant strains survived on poultry meat after 2 weeks under aerobic conditions with only a marginal reduction in CFU (<1 log CFU/g meat) (Figure 2). Results showed that aerotolerant and hyperaerotolerant strains survived on refrigerated chicken longer than oxygen-sensitive strains.

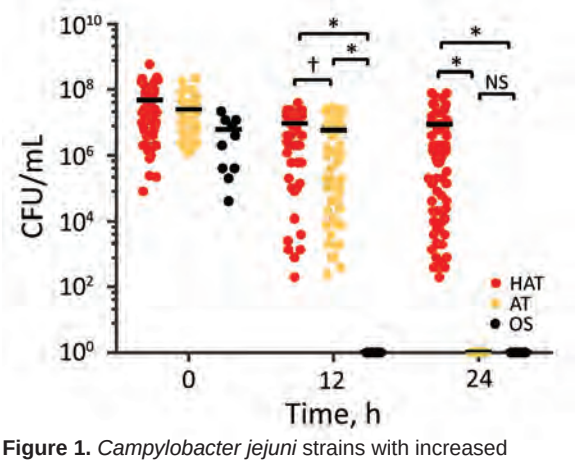


**Figure 1.** *Campylobacter jejuni* strains with increased aerotolerance in human clinical cases of campylobacteriosis. Results show levels of aerotolerance in 121 *C. jejuni* strains, including HAT (n = 65), AT (n = 46), and OS strains (n = 10). Clinical strains of *C. jejuni* were mostly AT and HAT. Results are representative of 3 independent experiments, and similar results were observed in all repeated experiments. Solid horizontal lines indicate average CFU. AT, aerotolerant; HAT, hyperaerotolerant; NS, not significant; OS, oxygen sensitive. *p<0.0001; †p<0.001.

### Prevalence of Clinical Strains Tolerant to Environmental Stresses

#### Disinfectant Exposure

Decontamination with antimicrobial agents is a common harsh stressor that *C. jejuni* encounters on poultry carcasses during processing. Among the decontamination agents that are used during poultry processing (*6*), PAA is highly effective in reducing *C. jejuni* load on poultry carcasses and is widely used (*14*). PAA is a mixture of acetic acid and hydrogen peroxide, and spontaneously decomposes to acetic acid, oxygen, and water (*17*). Most hyperaerotolerant (59/65, 90.8%) and aerotolerant (39/46, 84.8%) strains survived exposure to PAA; however, only 1 oxygen-sensitive strain survived with a major reduction in CFU (Figure 3, panels A, E). Results show that aerotolerant and hyperaerotolerant *C. jejuni* strains are highly tolerant to PAA.

#### Freezing

Because freezing has been reported to decrease the prevalence of *Campylobacter* spp. in chicken, freezing is considered a major measure in controlling *C. jejuni*

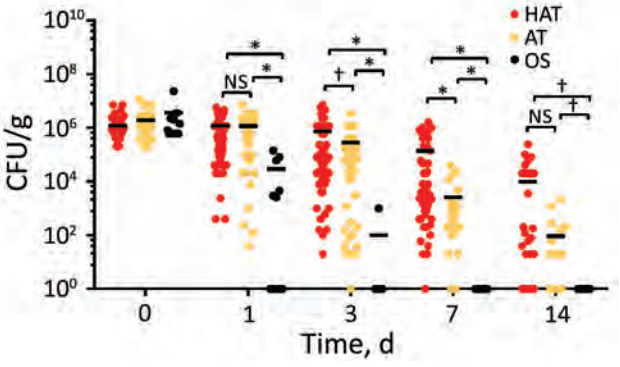


**Figure 2.** Extended survival of HAT *Campylobacter jejuni* strains on refrigerated raw chicken under aerobic conditions. Results show CFU of 121 *C. jejuni* strains, including HAT (n = 65), AT (n = 46), and OS (n = 10). Results are representative of 3 independent experiments, and similar results were observed in repeated experiments. Solid horizontal lines indicate average CFU. AT, aerotolerant; HAT, hyperaerotolerant; NS, not significant; OS, oxygen sensitive. *p<0.0001; †p<0.05.

contamination in poultry products (*18*,*19*). We inoculated the 121 clinical strains onto raw chicken pieces and subjected the pieces spiked with *C. jejuni* to freezing at –20°C. After 3 days of freezing, none of the oxygen-sensitive strains survived. However, 93.8% (61/65) of the hyperaerotolerant strains and 91.3% (42/46) of the aerotolerant strains survived, even after 7 days of freezing (Figure 3, panels B, E), which demonstrated that hyperaerotolerant and aerotolerant strains were highly tolerant to freezing.

### Heat Treatment

Heat treatment during pasteurization and cooking processes might kill pathogenic bacteria in foods. Although *C. jejuni* is a thermophile and grows optimally at 42°C, it is more sensitive to heat treatments than other enteropathogenic bacteria (*11*). We examined heat tolerance by exposing *C. jejuni* in milk to high-temperature short-time (HTST) pasteurization conditions (72°C for 15 s). A total of 83.5% (101/121) of clinical strains were tolerant to HTST pasteurization conditions, and 76.0% (92/121) of strains survived even after an extended heat treatment (72°C for 30 s) (Figure 3, panel C). Although no oxygen-sensitive strains survived HTST pasteurization conditions, 86.2% (56/65) of hyperaerotolerant strains and 78.3% (36/46) of aerotolerant strains survived after heat treatment at 72°C for 30 s (Figure 3, panels C, E). Results showed that the level of thermotolerance was also different depending on the strain.

### Osmotic Stress

Compared with other enteric pathogens, *C. jejuni* is considered highly sensitive to osmotic stress and easily inactivated by >2% NaCl (*20*). However, 44.6% (54/121) of the 121 clinical strains survived 2% NaCl, and 35.5% (43/121) of the strains were tolerant, even in 4% NaCl (Figure 3, panels D, E; online Technical Appendix Table). In contrast to our knowledge about *C. jejuni*, most clinical strains of *C. jejuni* were highly tolerant to high salt concentrations.

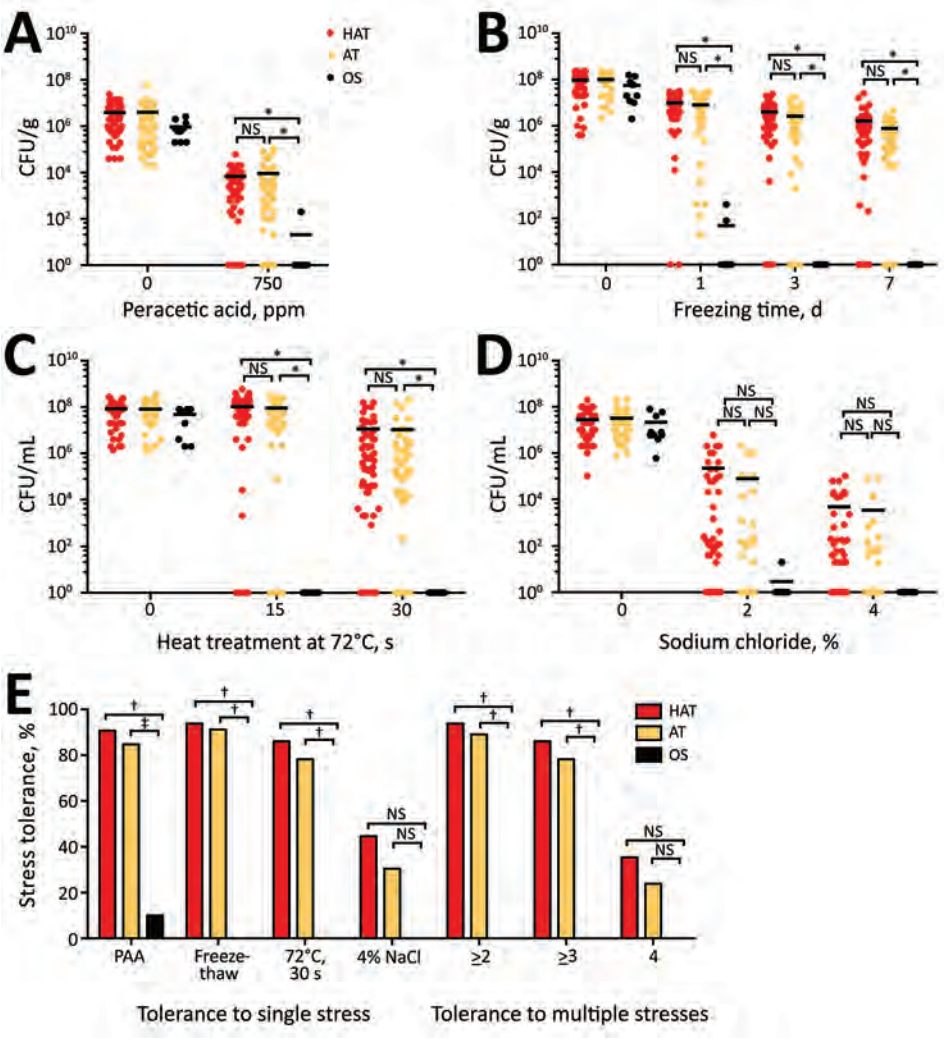


**Figure 3.** Tolerance to multiple stresses in 121 clinical strains of *Camplyobacter jejuni*. A) Resistance of *C. jejuni* to disinfectant (PAA) on chicken. B) Freeze-thaw tolerance of human clinical strains of *C. jejuni* on chicken. C) Tolerance to heat treatment of clinical *C. jejuni* strains in milk. D) Osmotolerance of clinical strains of *C. jejuni*. E) Percentage of stress-tolerant strains in each aerotolerance group: HAT (n = 65), AT (n = 46), and OS (n = 10). Results are representative of 3 independent experiments, and similar results were observed in all repeated experiments. Solid horizontal lines indicate average CFU. AT, aerotolerant; HAT, hyperaerotolerant; NS, not significant; OS, oxygen sensitive. *p<0.00001; †p<0.01; ‡p<0.05.

### Identification of Clades Tolerant to Multiple Stresses

On the basis of the high prevalence of *C. jejuni* in human clinical isolates tolerant to multiple stresses, we hypothesized that the multistress-tolerant (MST) strains are more likely to cause human illnesses because they can overcome various stresses in food processing, preservation, and cooking. This feature might enable MST *C. jejuni* strains to establish unique clones by outcompeting stress-sensitive *C. jejuni*. To examine this hypothesis, we conducted a population genetics analysis by using the CGF40 method, which is based on allelic assessment of ≈40 different genes in *C. jejuni* (*15*). Using a 90% similarity cutoff, we found that 7 clades containing ≥5 strains were identified in the CGF40 dendrogram (Figure 4). Three additional clades were also found to be highly populated with hyperaerotolerant strains (clades a, b, and c) (Figure 4), but these strains were excluded from further analysis because they did not meet the analysis criteria (i.e., 90% cutoff and ≥5 strains/clade). Each clade consisted of strains belonging to different MLST clonal complexes (CCs). For example, all strains in clades I and II belonged to MLST CC 21, whereas strains in clade IV belonged to several different MLST CCs (Figure 4). Nevertheless, hyperaerotolerant *C. jejuni* strains clustered in the CGF40 dendrogram (Figure 4), and hyperaerotolerant *C. jejuni* strains were found predominantly in clades I–IV (Figure 4). These results suggest that hyperaerotolerance might be associated with unique genetic backgrounds in *C. jejuni*.

### Association of Stress Tolerance with Human Infections

We analyzed stress tolerance levels of the strains in each clade. Strains in clades II, III, and IV were highly tolerant to PAA, freeze-thaw, and heat treatment (Figure 5, panel A). When we compared strains in clades II, III, and IV with strains in clade I, strains in clade I were relatively less tolerant to PAA, freeze-thaw, and heat treatment, but highly tolerant to osmotic stress (Figure 5, panel A). Clades V, VI, and VII, which included mostly oxygen-sensitive and aerotolerant strains, showed relatively reduced stress tolerance in comparison with clades I–IV. Strains in clade V were highly sensitive to all stresses tested (Figure 5, panel A). Each clade showed differential levels of tolerance to different stresses. Because of high levels of tolerance to multiple stresses for clades I–IV, we now call them MST clades.

To investigate effects of stress tolerance on strains that cause human campylobacteriosis, we examined how frequently CGF subtypes of MST clades were involved in cases of human infection. The 3 most frequent CGF subtypes (0169.001.002, 0044.003.001, and 0083.001.002) in the CGF collection of human clinical *C. jejuni* strains in Alberta isolated during 2006–2008 belonged to MST clades (Figure 5, panel B). In addition, these 3 CGF subtypes showed the highest prevalence in the recent (2016–May 2017) collection of *C. jejuni* clinical strains in Alberta (Figure 5, panel B). The total prevalence of all CGF subtypes in each clade indicates that MST clades have been a persistent cause of human campylobacteriosis in Alberta (Figure 5, panel C). Furthermore, the prevalence of *C. jejuni* in human clinical cases showed a statistical correlation with level of hyperaerotolerance (Pearson correlation coefficient [r] = 0.797; $p<0.05$), disinfectant (i.e., PAA) resistance ($r = 0.771$; $p<0.05$), freeze-thaw tolerance ($r = 0.773$; $p<0.05$), and heat tolerance ($r = 0.825$; $p<0.05$), whereas the level of osmotolerance was not associated with prevalence ($r = 0.18$; $p = 0.699$). These results strongly suggest that stress tolerance of *C. jejuni* plays a critical role in development of human infections, and that the MST clades include the primary *C. jejuni* strains causing human campylobacteriosis.

## Discussion

*C. jejuni* is considered a fastidious and highly stress-sensitive bacterium. In contrast to this perception, most clinical strains of *C. jejuni* tested in this study showed increased tolerance to multiple stresses. MST *C. jejuni* clones were identified in this study by combining extensive stress tolerance testing with a population genetics analysis (Figure 4; Figure 5, panel A). Furthermore, we demonstrated that MST *C. jejuni* clones are the primary cause of human campylobacteriosis in Alberta (Figure 5, panels B, C). The findings in this study strongly suggest that stress tolerance in *C. jejuni* is a critical determinant affecting human campylobacteriosis. These findings would help to answer the unresolved issue about how this fastidious, stress-sensitive bacterial pathogen is increasingly responsible for an increase in human illnesses worldwide; some *C. jejuni* strains are highly stress tolerant.

On the basis of findings in this study, we can speculate that MST *C. jejuni* strains can overcome and survive in hostile stress conditions in food processing and various environmental niches during transmission to humans more effectively than stress-sensitive strains. Consequently, MST *C. jejuni* strains are more likely to be transmitted to humans than stress-sensitive strains because these strains might be enriched and form unique clones by repeating the cycle of environmental survival, transmission, and human infection. The predominant CGF subtypes in MST clades (0169.001.002, 0044.003.001, and 0083.001.002) are not only prevalent in the CGF collection of human clinical *C. jejuni* strains in Alberta but also in the Canadian *Campylobacter* CGF database, which contains CGF information for >25,000 *C. jejuni* strains from foods, animals, humans, and environmental samples across Canada (*21*). Presumably, the level of stress tolerance for clinical strains will be different from that for nonclinical strains because clinical strains have already undergone a range of stress conditions

during transmission and infection. We are currently investigating stress tolerance in nonclinical strains of *C. jejuni* from foods.

Strains in CGF subtype 0044.003.001 in MST clade III were highly tolerant to multiple stresses, including PAA, freezing, and heat treatment (Figure 4). The CGF subtype

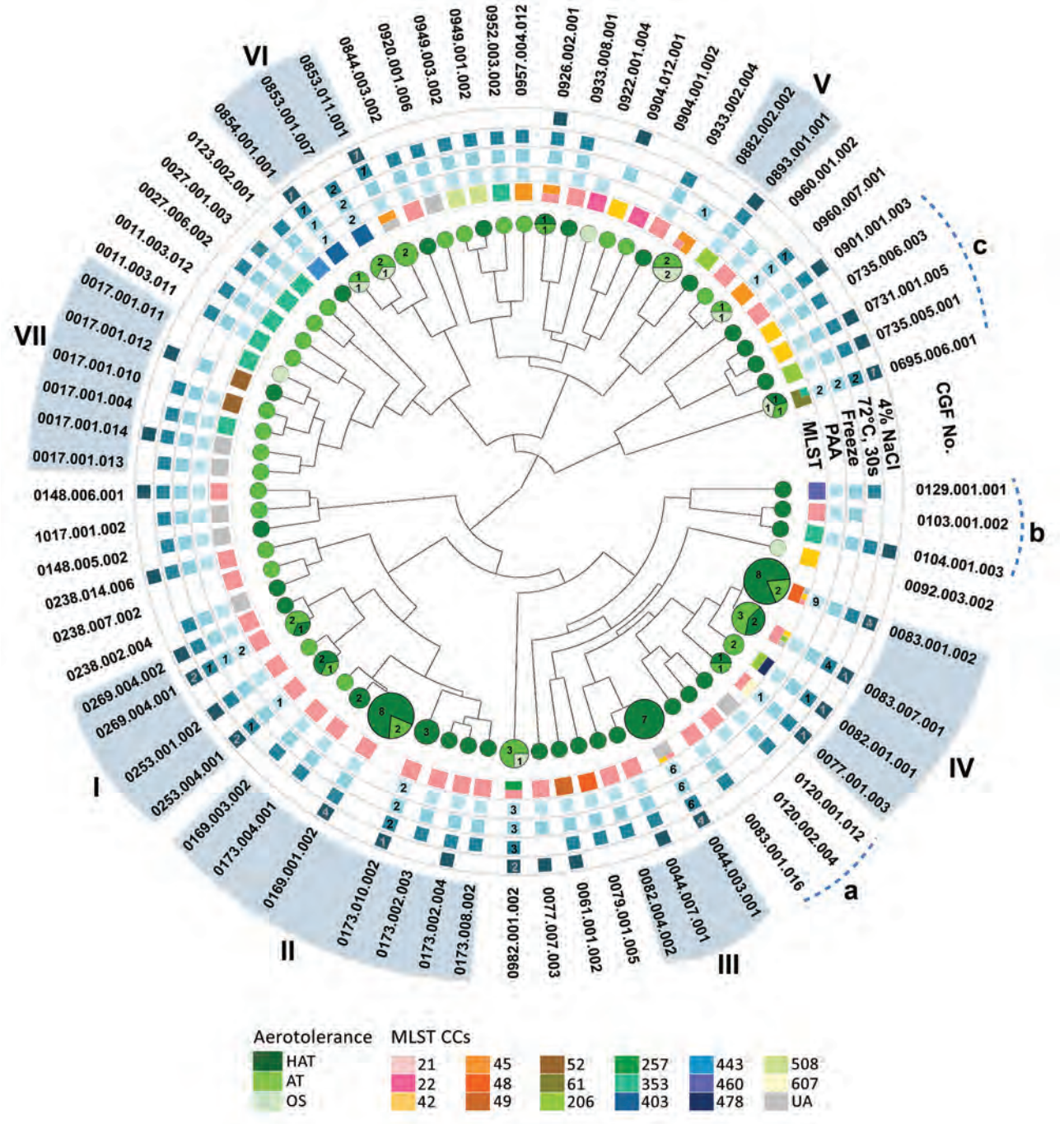


**Figure 4.** Clonal distribution of MST *Campylobacter jejuni* strains from human clinical cases of campylobacteriosis. The phylogenetic tree was generated from CGF40 profiles. Circles indicate aerotolerant strains, and squares indicate for stress-tolerant strains. A square without a number indicates all tested strains in the subtype were stress tolerant. Clades I–VII were identified on the basis of the analysis criteria (90% similarity cutoff and ≥5 strains/clade). Additional clades that consisted predominantly of HAT strains, but did not meet the criteria, were named a, b, and c. AT, aerotolerant; CCs, clonal complexes; CGF, comparative genomic fingerprinting; HAT, hyperaerotolerant; MLST, multilocus sequence typing; OS, oxygen sensitive; PAA, peracetic acid; UA, unassigned.

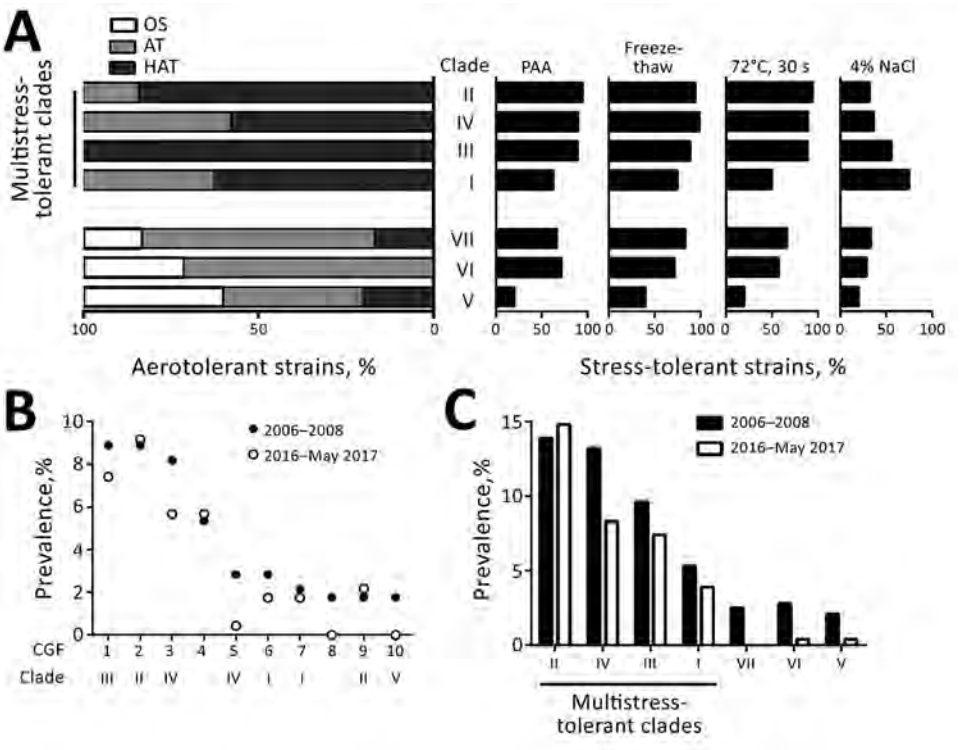


**Figure 5.** Increased stress tolerance and prevalence of multistress-tolerant (MST) clades of *Camplyobacter jejuni*. A) Augmented tolerance of MST clades to 5 stressors (aeration, disinfectant [PAA], freeze-thaw, heat, and salt). Clades are arranged in the order of decreasing levels of overall stress tolerance. B) Top 10 most prevalent CGF subtypes in the CGF collection of human clinical strains of *C. jejuni* in Alberta, Canada, during 2006–2008 (n = 281) and 2016–May 2017 (n = 229). 1, 0044.003.001; 2, 0169.001.002; 3, 0083.001.002; 4, 0695.006.001; 5, 0083.007.001; 6, 0269.004.001; 7, 0253.004.001; 8. 0123.002.001; 9, 0173.004.001; 10, 0882.002.002. C) Cumulative prevalence of the CGF subtypes belonging to MST clades in the CGF collection of human clinical strains of *C. jejuni* in Alberta, Canada, during 2006–2008 (n = 281) and 2016–May 2017 (n = 229). AT, aerotolerant; CGF, comparative genomic fingerprinting; HAT, hyperaerotolerant; OS, oxygen sensitive; PAA, peracetic acid.

0044.003.001 was commonly found in human clinical cases in Alberta (Figure 5, panel B) and Canada (*21*). This CGF subtype is detected more frequently in human clinical cases (59.0%) than in animals (40.5%) in the Canadian *Campylobacter* CGF database (*21*). According to a recent study from Nova Scotia, a province geographically distant from Alberta, the predominant CGF subtype 0083.001.002 in MST clade IV is also the most common CGF subtype found in human clinical isolates of *C. jejuni* in this province (*22*). CGF subtype 0083.001.002 is primarily associated with chicken (*22*), and strains in 0083.001.002 were highly tolerant to PAA, freezing, and heat treatment (Figure 4). This finding suggests that multistress tolerance may facilitate *C. jejuni* survival in poultry processing, preservation, and cooking, and consequently might increase chances of food contamination and human exposure.

Because of the common understanding about high-stress sensitivity in *C. jejuni*, food contamination by *C. jejuni* has been relatively underestimated when compared with that for other robust foodborne pathogens. For other enteric pathogens, high-risk strains (e.g., *Escherichia coli* O157:H7) are strictly monitored and controlled during food inspection. However, such risk-based strain differentiation is not performed for *C. jejuni*, and current food safety policies regarding *Campylobacter* spp. are based on total *Campylobacter* spp. count in foods. The findings in this study suggest that the MST *C. jejuni* clones might be the primary target to monitor and control to address the major public health issue of *Campylobacter* infections.

Most of the stress conditions tested in this study, such as freezing, refrigeration, heat treatment, and high salt concentrations, are commonly used in poultry processing and cooking in many countries. Thus, findings of this study might not be limited only to strains of *C. jejuni* from Canada. However, tolerance to antimicrobial disinfectants in *C. jejuni* might vary because each jurisdiction has a different policy on use of disinfectants and this might affect the possibility of *C. jejuni* exposure to specific disinfecting substances. For example, PAA is used widely to decontaminate poultry carcasses in Canada and the United States. However, the European Union recently performed safety and efficacy analysis of PAA application on poultry carcasses (*23*). Given the well-known genetic diversity in *C. jejuni*, MST *C. jejuni* clones might have different genotypes in different geographic locations. Future studies should aim to compare multistress tolerance in historical and recent strains of *C. jejuni* from different countries. In addition, further investigations are required to elucidate the molecular mechanisms of multistress tolerance in *C. jejuni*.

## Acknowledgments

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# Bioclinical Test to Predict Nephropathia Epidemica Severity at Hospital Admission

**Maxime Hentzien, Stéphanie Mestrallet, Pascale Halin, Laure-Anne Pannet, Delphine Lebrun, Moustapha Dramé, Firouzé Bani-Sadr, Jean-Marc Galempoix, Christophe Strady, Jean-Marc Reynes, Christian Penalba, Amélie Servettaz**

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**Release date: May 17, 2018; Expiration date: May 17, 2019**

**Learning Objectives**

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

1. Describe clinical and laboratory characteristics of nephropathia epidemica (NE), based on a multicenter, retrospective cohort study
2. Assess bioclinical factors predictive of severe NE
3. Determine clinical performance and other clinical implications of a bioclinical score predictive of severe NE.

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We conducted a multicenter, retrospective cohort study of hospitalized patients with serologically proven nephropathia epidemica (NE) living in Ardennes Department, France, during 2000–2014 to develop a bioclinical test predictive of severe disease. Among 205 patients, 45 (22.0%) had severe NE. We found the following factors predictive of severe NE: nephrotoxic drug exposure (p = 0.005, point value 10); visual disorders (p = 0.02, point value 8); microscopic or macroscopic hematuria (p = 0.04, point value 7); leukocyte count >10 × $10^9$ cells/L (p = 0.01, point value 9); and thrombocytopenia ≤90 × $10^9$/L (p = 0.003, point value 11). When point values for each factor were summed, we found a score of ≤10 identified low-risk patients (3.3% had severe disease), and a score ≥20 identified high-risk patients (45.3% had severe disease). If validated in future studies, this test could be used to stratify patients by severity in research studies and in clinical practice.

Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) are enveloped viruses with negative, trisegmented, single-stranded RNA genomes that can induce hemorrhagic fever with renal syndrome (HFRS) or hantavirus pulmonary syndrome (*1*). The viruses that cause HFRS include Hantaan virus, Dobrava-Belgrade virus, Seoul virus, Tula virus, and Puumala virus (PUUV). PUUV, predominantly transmitted by the bank vole (*Myodes glareolus*), is the most common species of hantavirus in central and north Europe and frequently causes a mild form of HFRS, nephropathia epidemica (NE) (*1*–*3*). NE is endemic in the northeast of France; the Champagne-Ardenne and Picardie regions are most affected. In 2015, the incidence was 12.02 cases/100,000 inhabitants in the Ardennes Department (*4*).

The clinical presentation of NE is the same throughout Europe (*5*); typically, the signs and symptoms of NE are sudden onset high-grade fever, headache, visual disorders, gastrointestinal irregularities, and low back pain. The biological profile of NE is characterized by acute kidney injury (AKI) associated with proteinuria, thrombocytopenia, and biological inflammatory syndrome, including elevated leukocyte count and C-reactive protein level (*3*).

NE is a benign disease with a low case-fatality rate (<1%) (*3*,*6*) and favorable early and long-term outcomes (*7*–*9*). Nevertheless, patients with mild disease are frequently held in hospitals for continued renal observation and treatment. Moreover, a severe form of the disease can develop, although infrequently, additionally contributing to the high frequency of prolonged hospitalization for this disease (*10*,*11*). Severe forms of NE have been defined according to varying criteria in the literature but are generally defined by using AKI severity criteria (*11*–*14*), such as the RIFLE (risk for renal dysfunction, kidney injury, failure or loss of kidney function, and end-stage renal disease) (*15*), KDIGO (Kidney Disease: Improving Global Outcomes) (*16*), or AKIN (Acute Kidney Injury Network) (*17*) classifications. Renal replacement therapy is required in ≈5% of patients with NE (*3*).

The predictive factors of severe NE are not well known, and consequently, identifying the patients at low and high risk for severe NE is not possible. Patients are, therefore, frequently kept hospitalized for observation (*10*,*11*). Improved knowledge of the predictors of severe forms could help in the identification of patients at low and high risk for severe NE in routine care and, therefore, reduce the prolonged hospitalization of patients at low risk. The main objective of this study was to identify the predictive factors for severe disease among patients with serologically proven NE in the Champagne-Ardenne region in France and to derive a bioclinical score that enables identification of patients more likely to develop severe NE.

## Materials and Methods

### Study Design and Patients

In this multicenter, retrospective cohort study, we included all patients living in Ardennes Department, France, who were hospitalized for serologically proven NE during January 2000–December 2014. We identified hospitalized patients fulfilling the inclusion criteria by searching through microbiology laboratory databases of the following centers: Reims University Hospital (Reims, France); Charleville-Mézières Hospital (Charleville-Mézières, France); Sedan Hospital (Sedan, France); and the National Reference Center for Hantaviruses (Lyon, France). We included only patients positive for hantavirus IgM and IgG by the Hantavirus IgM and IgG DxSelect ELISA kits (Focus Diagnostics, Cypress, CA, USA) that were confirmed by the National Reference Center for Hantavirus. We excluded patients who sought treatment at an emergency department who were not admitted and patients who had a severe form (defined later) at admission. The study was conducted in accordance with French Jarde's law on retrospective data studies and the Declaration of Helsinki.

### Data Collection and Definitions

We extracted all data retrospectively from patients' medical records using a standardized case report form. Data were deidentified, then extracted and stored for analysis. We collected the following variables: sociodemographic data, updated Charlson comorbidity index (not adjusted for age) (*18*), center, clinical presentation, intake of nephrotoxic treatments, results of HFRS laboratory diagnosis, date of first symptoms, date and duration of hospitalization, results of standard biological data at baseline and during hospitalization, and the occurrence of severe disease.

The clinical characteristics collected at admission were fever (temperature >38°C); myalgia; low back pain; visual disorders (myopic shift, blurred vision); chest symptoms

(cough, dyspnea, chest auscultation abnormalities, pathologic chest radiograph); digestive symptoms (nausea, diarrhea, abdominal pain); oliguria (urine output <500 mL/d); neurologic signs (meningism, headache without meningism); and nonsevere hemorrhagic signs (hematuria, mucosal bleeding, purpura, petechiae, conjunctival hemorrhage). We considered the following nephrotoxic drug exposures during hospitalization, including during the first 24 h: nonsteroidal antiinflammatory drugs, iodinated contrast media, diuretics, renin angiotensin aldosterone system inhibitors, and nephrotoxic antimicrobial drugs (aminoglycosides, glycopeptides). Biological data collected at admission included hemoglobin level, leukocyte count, neutrophil count, platelet count, C-reactive protein, plasma creatinine, aspartate aminotransferase, alanine aminotransferase, hematuria (>10 cells/mm$^3$ in urine sediment or macroscopic hematuria), and proteinuria. If multiple plasma creatinine values were available between 2 weeks and 1 year after discharge, we also collected the first of these readings. We defined severe NE as the occurrence of ≥1 of the following criteria during hospitalization: hypovolemic, hemorrhagic, or septic shock; plasma creatinine level >353.6 μmol/L (*9*,*16*,*19*); anuria (urine output <300 mL/d); need for dialysis; hemorrhage requiring blood transfusion; admission to the intensive care unit; or death.

### Statistical Analysis

Quantitative variables are presented as mean ± SD or median (interquartile range [IQR]), as appropriate, and qualitative variables as number (percentage). We assessed the differences between groups using the $\chi^2$ test or Fisher exact test for categorical variables and Student *t*-test or the Mann-Whitney U-test for continuous variables, as appropriate.

We performed univariable and multivariable logistic regression to develop the prognostic model and generate unadjusted odds ratios (OR) and adjusted ORs (aOR) and the associated 95% CIs. Occurrence of a severe form of NE was the primary endpoint. We considered baseline characteristics potential explanatory variables and decided not to include urine dipstick proteinuria at admission as a predictor of severe NE because of the amount of missing data for this variable. We used a manual stepwise method to identify variables independently associated with the occurrence of severe NE and systematically adjusted the multivariable model for the time since symptom onset. We also performed bootstrap analysis to evaluate the internal validity of the model performance. Replication on 2,000 different samples drawn with replacement was the bootstrap method performed. We used the C statistic and the Hosmer-Lemeshow goodness-of-fit test to assess model performances (discrimination and calibration).

For the development of the bioclinical score, we assigned a point value for each independent factor according to the aOR of the final model. The aORs were multiplied by 3, rounded to the nearest integer, and then summed. We constructed a receiver operating characteristic curve and obtained C statistic 95% CIs using bootstrap methods. We performed statistical analyses with SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) and considered p values <0.05 significant.

## Results

### Population Characteristics

Among the 272 patients with NE during the study period, 227 (83.5%) were hospitalized. Of these, 22 (9.7%) had a severe form of NE at admission and were excluded from the study (Figure 1). The patients with severe NE at admission were hospitalized significantly later after symptom onset (7.8 ± 3.1 days) than the patients included in the study (5.3 ± 2.7 days; p = 0.0003). These excluded patients were all men; were older than included patients; and had higher nephrotoxic drug exposure, more frequent low back pain, more frequent oliguria during the clinical course, and higher leukocyte counts than included patients (M. Hentzien, unpub. data).

Among the 205 patients included in the analysis, the mean length of hospital stay was 7.2 ± 3.5 days, their mean age was 38.6 ± 14.3 years, 74.6% were men, and the median Charlson comorbidity index was 0 (IQR 0–0) (Table 1). Only 24 (11.7%) patients had a Charlson comorbidity index >0, and 60 (29.3%) patients had taken nephrotoxic drugs, predominantly nonsteroidal antiinflammatory drugs (17.1%), around the time of admission.

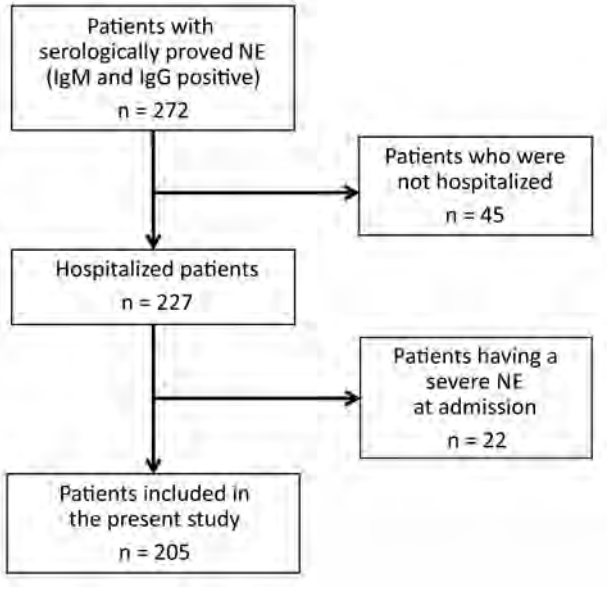


**Figure 1.** Determination of study population used to derive a bioclinical score that enables identification of patients more likely to develop severe NE. Patients were those living in Ardennes Department, France, who were hospitalized for serologically proven NE during January 2000–December 2014. NE, nephropathia epidemica.

**Table 1.** Clinical characteristics of 205 hospitalized patients with nephropathia epidemica, Ardennes Department, France, January 2000–December 2014*

| Characteristic | Value | Missing |
|---|---|---|
| Sex | | |
| M | 153 (74.6) | 0 |
| F | 52 (25.4) | 0 |
| Age, y, mean ± SD | 38.6 ± 14.3 | 0 |
| Charlson comorbidity index score, median (IQR) | 0 (0–0) | 0 |
| >0 | 24 (11.7) | |
| Chronic renal disease | 4 (2.0) | 0 |
| Time from onset of symptoms to hospitalization, d, mean ± SD | 5.3 ± 2.7 | 0 |
| Nephrotoxic drug intake† | 60 (29.3) | 0 |
| Nonsteroidal anti-inflammatory drugs | 35 (17.1) | 0 |
| Iodinated contrast media | 9 (4.4) | 0 |
| Diuretics | 14 (6.8) | 0 |
| Renin angiotensin aldosterone system inhibitors | 12 (5.9) | 0 |
| Nephrotoxic antimicrobial drugs | 5 (2.4) | 0 |
| Clinical signs and symptoms at admission | | |
| Fever | 203 (99.5) | 1 |
| Arthromyalgia | 167 (81.5) | 0 |
| Low back pain | 92 (44.9) | 0 |
| Visual disorders | 68 (33.2) | 0 |
| Chest symptoms | 96 (46.8) | 0 |
| Cough | 65 (31.7) | 0 |
| Dyspnea | 11 (5.4) | 0 |
| Chest auscultation abnormalities | 34 (16.6) | 0 |
| Pathological chest radiograph | 41 (23.0) | 27 |
| Digestive symptoms | 144 (70.2) | 0 |
| Nausea | 107 (52.2) | 0 |
| Diarrhea | 38 (18.5) | 0 |
| Abdominal pain | 104 (50.7) | 0 |
| Oliguria during clinical course | 32 (15.6) | 0 |
| Neurologic signs | 149 (72.7) | 0 |
| Meningism | 21 (10.2) | 0 |
| Headache without meningism | 147 (71.7) | 0 |
| Nonsevere hemorrhagic signs‡ | 28 (13.7) | 0 |
| Laboratory findings at admission | | |
| Hemoglobin, g/L, mean ± SD | 146 ± 15 | 10 |
| Platelet count, $\times 10^9$/L, median (IQR) | 92 (66–123) | 1 |
| >90 | 104 (50.9) | |
| 50–≤90 | 78 (38.2) | |
| ≤50 | 22 (10.8) | |
| Leukocyte count, $\times 10^9$ cells/L, mean ± SD | 8.0 ± 3.2 | 5 |
| >10 | 45 (22.5) | 5 |
| Neutrophil count, $\times 10^9$ cells/L, mean ± SD | 5.5 ± 2.4 | 7 |
| Lymphocyte count, $\times 10^9$ cells/L, mean ± SD | 1.3 ± 0.7 | 11 |
| <1 | 78 (40.2) | 11 |
| Creatinine, µmol/L, median (IQR) | 98 (79–130) | 6 |
| Alanine aminotransferase, × ULN, median (IQR) | 1.0 (1.0–1.5) | 28 |
| Aspartate aminotransferase, × ULN, median (IQR) | 1.0 (1.0–1.5) | 26 |
| C-reactive protein, median (IQR) | 79 (46–117) | 2 |
| >100 mg/L, >952 nmol/L | 70 (34.5) | 2 |
| Hematuria | | 6 |
| Absent | 103 (51.8) | |
| Microscopic | 90 (45.2) | |
| Macroscopic | 6 (3.0) | |
| Urine dipstick proteinuria§ | | 128 |
| 0 | 15 (17.9) | |
| + | 15 (17.9) | |
| ++ | 15 (17.9) | |
| +++ | 20 (23.8) | |
| ++++ | 12 (14.3) | |

*Vales are n (%) unless otherwise specified. IQR, interquartile range; ULN, upper limit of normal.
†More than 1 factor is possible.
‡Defined as hemorrhagic signs not requiring blood transfusion.
§For urine dipstick readings, 0 indicates no proteinuria, + indicates ≈30 mg/dL, ++ indicates ≈100 mg/dL, +++ indicates ≈300 mg/dL, and ++++ indicates ≈2,000 mg/dL.

### Clinical Course

Creatinine plasma level peaked at a median of 198 (IQR 110–318) μmol/L at a median of 8 (IQR 6–10) days after symptom onset. Proteinuria also peaked at a median of 8 (IQR 6–10) days after symptom onset at a median of 2.3 (IQR 0.9–4.9) g/d. Patients who were exposed to nephrotoxic drugs had a higher median peak creatinine plasma (277 [IQR 148–389] μmol/L) than patients who were not (180 [IQR 106–264] μmol/L; p = 0.002; reference range 53–106 μmol/L; Figure 2), although their median creatinine plasma levels at admission were similar (exposed 107 [IQR 77–161] μmol/L vs. not exposed 97 [IQR 80–123] μmol/L; p = 0.58).

### Occurrence of Severe NE

During hospitalization, NE progressed in severity in 45 (22.0%) patients. Among these patients, 41 (91.1%) had plasma creatinine >353.6 μmol/L, 6 (13.3%) had anuria, 3 (6.7%) required dialysis, 2 (4.4%) experienced shock, and 2 (4.4%) required admission to the intensive care unit. Of the 4 patients with severe NE without elevated plasma creatinine levels of >353.6 μmol/L, 2 experienced shock and 3 had ≥1 d of anuria. No patient had hemorrhaging requiring a blood transfusion, and no patient died.

The time from onset of symptoms to hospitalization in patients who had severe NE (6.0 ± 2.8 d) was not significantly different from those who did not (5.4 ± 2.8 d; p = 0.11), enabling baseline characteristic comparison. Patients who had severe NE had a significantly longer hospital stay (8.5 ± 3.5 d) than did patients who did not (6.9 ± 3.4 d; p = 0.0005). Among patients whose biological data was available between day 15 and 1 year after the end of the hospitalization (60.0%, n = 123), the median value of the first observed plasma creatinine level was 80 (IQR 68–87, range 40–128) μmol/L. This variable was not significantly different between patients who had severe NE (81 [IQR 76–88] μmol/L) and those who did not (78 [IQR 67–87] μmol/L; p = 0.21).

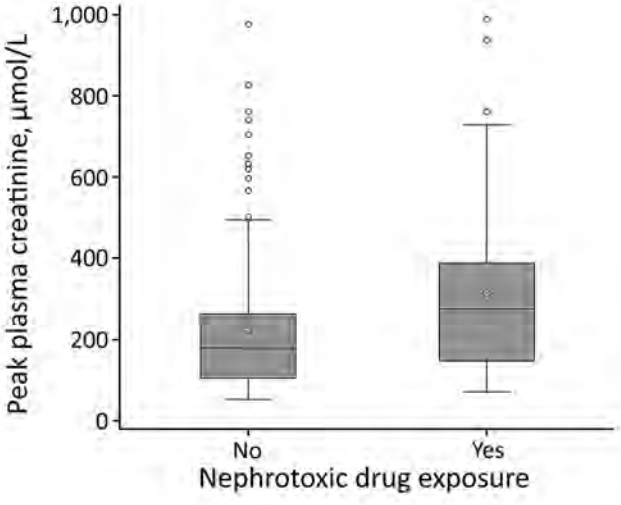


**Figure 2.** Peak creatinine plasma levels in patients hospitalized for nephropathia epidemica by nephrotoxic drug exposure, Ardennes Department, France, January 2000–December 2014. Top and bottom borders of boxes indicate interquartile ranges (IQRs), horizontal lines within boxes indicate medians, diamonds indicate means, and circles represent outliers. A whisker is drawn from the upper edge of the box to the largest observed value within the upper fence (located at 1.5 × IQR above the 75th percentile), and another is drawn from the lower edge of the box to the smallest observed value within the lower fence (located at 1.5 × IQR below the 25th percentile). Peak plasma creatinine levels were higher in the patients exposed to nephrotoxic drugs (p = 0.002).

### Predictors of Severe NE and Derived Predictive Score

By univariable analysis (Table 2), the factors significantly associated with the occurrence of severe NE were nephrotoxic drug intake, visual disorders, hematuria, leukocyte count >10 × $10^9$ cells/L, C-reactive protein >100 mg/L (>952 nmol/L), and thrombocytopenia ≤90 × $10^9$/L. By multivariable analyses (Table 2), the factors that remained significantly associated with the occurrence of severe NE after adjustment were nephrotoxic drug intake (aOR 3.25, 95% CI 1.42–7.46; p = 0.005), visual disorders (aOR 2.64, 95% CI 1.17–5.96; p = 0.02), microscopic or macroscopic hematuria (aOR 2.37, 95% CI 1.03–5.43; p = 0.04), leukocyte count >10 × $10^9$ cells/L (aOR 3.03, 95% CI 1.25–7.39; p = 0.01), and thrombocytopenia ≤90 × $10^9$/L (aOR 3.74, 95% CI 1.59–8.81; p = 0.003). Because of the collinearity between the leukocyte count and C-reactive protein level and the fact that the predictive ability of the leukocyte count was better, we decided to include only the leukocyte count in the score. ORs obtained by bootstrap analysis of the final multivariable model were similar to those in the final multivariate model, suggesting good internal validity.

With this statistical analysis, we derived a test to predict the occurrence of severe NE. In this test, point values for each of the predictive factors we identified (Table 3) are added up for a possible total of 45, with 0 indicating the lowest risk and 45 indicating the highest. The mean observed score for our patient population was 16.4 ± 10.0 (range 0–45). The uncorrected C statistic of the receiver operating characteristic curve for our test (Figure 3) was the same as the C statistic obtained from bootstrap analysis (0.80, 95% CI 0.72–0.87). Using this test for our patient population, we found that a score of ≤10 identified patients at low risk for severe NE (3.3% of patients in this group had severe disease) and a score ≥20 identified patients at high risk for severe NE (45.3% of patients in this group had severe disease) (Table 4).

## Discussion

In this study, which included a large representative cohort of patients hospitalized for NE during January 2000–December 2014, we identified nephrotoxic drug intake, visual

**Table 2.** Univariable and multivariable analysis of factors predictive of severe nephropathia epidemica, Ardennes Department, France, January 2000–December 2014*

| Category | Univariable analysis, n = 205 | | | Multivariable analysis,† n = 194 | |
|---|---|---|---|---|---|
| | OR‡ (95% CI) | p value | Missing | aOR (95% CI) | p value |
| Age ≥40 y | 0.63 (0.31–1.27) | 0.19 | 0 | | |
| Female sex | 0.80 (0.37–1.76) | 0.58 | 0 | | |
| Charlson comorbidity index score ≥1 | 0.68 (0.22–2.11) | 0.51 | 0 | | |
| Nephrotoxic drug intake | 2.12 (1.06–4.23) | 0.03 | 0 | 3.25 (1.42–7.46) | 0.005 |
| Chronic renal disease | 1.19 (0.12–11.72) | 0.88 | 0 | | |
| Low back pain | 1.38 (0.71–2.68) | 0.34 | 0 | | |
| Visual disorders | 3.01 (1.53–5.20) | 0.002 | 0 | 2.64 (1.17–5.96) | 0.02 |
| Microscopic or macroscopic hematuria | 2.54 (1.26–5.11) | 0.009 | 6 | 2.37 (1.03–5.43) | 0.04 |
| Platelet count ≤90 × $10^9$/L | 3.15 (1.53–6.46) | 0.002 | 1 | 3.74 (1.59–8.81) | 0.003 |
| Leukocyte count >10 × $10^9$ cells/L | 2.87 (1.36–6.05) | 0.006 | 5 | 3.03 (1.25–7.39) | 0.01 |
| C-reactive protein >100 mg/L, >952 nmol/L | 2.19 (1.12–4.31) | 0.02 | 2 | | |
| Alanine aminotransferase >1 × ULN | 1.66 (0.76–3.59) | 0.20 | 28 | | |
| Aspartate aminotransferase >1 × ULN | 1.18 (0.53–2.61) | 0.69 | 26 | | |

*aOR, adjusted odds ratio; OR, odds ratio; ULN, upper limit of normal.
†The multivariable model was systematically adjusted for the time between onset of symptoms and hospitalization. The C statistic for the multivariable model was 0.81. Hosmer-Lemeshow goodness-of-fit test: p = 0.87.
‡Logarithm for odds of severe nephropathia epidemica = −3.0830 − (0.1214 × days between first symptoms and hospitalization) + (0.9693 × visual disorder) + (0.8621 × hematuria) + (1.1099 × elevated leukocyte count) + (1.3185 × thrombocytopenia) + (1.1787 × nephrotoxic drug exposure).
§Nephrotoxic drugs included nonsteroidal antiinflammatory drugs, iodinated contrast media, diuretics, renin angiotensin aldosterone system inhibitors, and nephrotoxic antimicrobial drugs (aminoglycosides, glycopeptides).

disorder, microscopic or macroscopic hematuria, leukocyte count >10 × $10^9$ cells/L, and thrombocytopenia (≤90 × $10^9$/L) as independent predictive factors of severe NE. We derived a simple bioclinical test that can be calculated on the day of admission that makes it possible for clinicians to distinguish between patients at low, intermediate, and high risk of developing severe NE.

Our patients were comparable to patients described in other studies. Infected patients were young (*9*,*19*), predominantly men (*9*,*11*,*19*–*21*), with no or few concurrent conditions (*9*,*11*,*19*). The clinical presentation was also similar to other reports (*11*). The time elapsed between first symptoms and hospitalization (≈5 days) was the same as that reported in the literature (*9*,*11*,*20*,*22*). The clinical course observed in our patients was classical, with a peak of plasma creatinine and proteinuria around 8 days after onset of symptoms (*9*,*21*) and a mean duration of hospitalization of 7 days (*11*).

According to the definition we used, severe NE developed during hospitalization in 22% of patients, and 9.7% of patients had a severe form at admission. The total number of severe forms we observed was similar to that found by Outinen et al. (34%), who used similar severity criteria (but excluded anuria, hemorrhage requiring blood transfusion, admission to the intensive care unit, and death from their definition) (*9*). Median peak of plasma creatinine was similar and renal replacement therapy was low, as in other studies (*9*,*11*).

Contrary to other studies (*11*–*14*), we decided not to use strict RIFLE (*15*), KDIGO (*16*), or AKIN criteria (*17*) to define severe NE because, in our experience, these criteria are too sensitive yet not specific enough to reflect severe NE. In particular, a 3-fold increase in plasma creatinine over baseline does not seem adequate for this disease. First, in this young population with few, if any, concurrent conditions, baseline plasma creatinine level is rarely available in clinical practice (*9*). Second, in this population, a 3-fold increase would include patients with a peak creatinine plasma level <200–300 μmol/L, which is very common in NE (*13*). Consequently, severe forms might be overrepresented in studies involving such criteria. By this definition, severe NE could represent as many as 65% of the total cases during the course of disease (*13*), whereas NE is usually a benign disease with favorable short- and long-term outcomes. In fact, a reversible form of AKI is frequent, and severe complications are rare.

Most severe NE patients had plasma creatinine >353.6 μmol/L (as in our definition of severe disease), which is expected according to the bioclinical course of the disease. Elevations in plasma creatinine are linked to other criteria, such as anuria and the need for dialysis, which reflect the severity of AKI and generally the severity of NE. However, some defining elements of severe NE, such as death, shock, hemorrhage requiring transfusion, or admission to the intensive care unit, are not necessarily linked to AKI.

**Table 3.** Point value assigned for each predictive factor of severe nephropathia epidemica to be used for bioclinical assay to access risk for nephropathia epidemica severity*

| Predictive factor | Point value |
|---|---|
| Hematuria | 7 |
| Visual disorders | 8 |
| Leukocyte count >10 × $10^9$ cells/L | 9 |
| Nephrotoxic drug exposure† | 10 |
| Thrombocytopenia ≤90 × $10^9$/L | 11 |

*Predictive factors were assessed at admission.
†Nephrotoxic drugs included nonsteroidal antiinflammatory drugs, iodinated contrast media, diuretics, renin angiotensin aldosterone system inhibitors, and nephrotoxic antimicrobial drugs (aminoglycosides, glycopeptides).

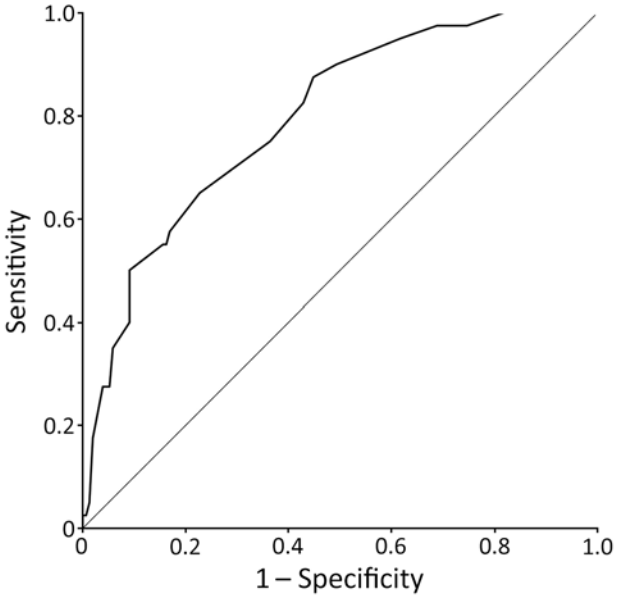


**Figure 3.** Receiver operating characteristic curve of test to predict development of severe nephropathia epidemica among patients hospitalized for nephropathia epidemica, Ardennes Department, January 2000–December 2014. Severe nephropathia epidemica was defined as the occurrence of ≥1 of the following criteria: hypovolemic, hemorrhagic, or septic shock; plasma creatinine level >353.6 μmol/L; anuria (urine output <300 mL/d); acute kidney injury or hydroelectrolytic disorders requiring dialysis; hemorrhage requiring blood transfusion; admission to the intensive care unit; or death. Area under the curve is 0.80.

**Table 4.** Events observed in patients hospitalized with NE by risk group as determined by bioclinical test score, Ardennes Department, France, January 2000–December 2014*

| Risk group | Score | No. NE patients | Observed severe NE, no. (%) |
|---|---|---|---|
| Low | 0–10 | 61 | 2 (3.3) |
| Intermediate | 11–19 | 80 | 14 (17.5) |
| High | 20–45 | 53 | 24 (45.3) |

*Data were missing for 11 patients, who were excluded in this analysis. However, similar results for observed severe NE were obtained when assuming a score of zero for missing data: low (4.7%, 3/64), intermediate (20.1%, 18/87), and high (44.4%, 24/54). The leukocyte count and platelet count at admission were missing from the 1 additional patient with severe NE categorized as low risk in this analysis. NE, nephropathia epidemica.

In our study, a nonnegligible portion (9%) of severe NE forms were not related to plasma creatinine elevations; not classifying the patients with these disease forms as severe would have been damaging to the validity of this study, as these patients were in need of urgent inpatient care.

All of the factors we found independently predictive of severe NE have already been mentioned in the literature. Nephrotoxic drug intake was frequent in our study; about one third of patients had taken this type of drug, probably to treat the pain and fever that are common in NE. Nephrotoxic drug use is common and should be systematically recorded at initial examination and considered when assessing the risk for severe NE. In 1 study, patients exposed to ibuprofen or diclofenac were found to have higher initial and peak creatinine levels, even after adjustment for confounders (*23*). Physicians should be aware of this association when assessing patients with possible NE and avoid administering nephrotoxic drugs to these patients because often other drugs may be administered in their place, potentially modifying patient outcomes for the better.

We found visual disorders in 68 of the 205 patients in our cohort, and this variable was found to be independently associated with the occurrence of severe NE. The association between visual disorders and NE severity has previously been investigated, with conflicting results. Hautala et al. found an association between the change in anterior chamber depth and creatinine plasma level (*24*). Conversely, Theiler et al. failed to find any significant association between blurred vision or myopic shift, as assessed by an ophthalmologist, and NE severity, although the number of patients included was low (n = 18) (*25*). Other studies in which patients were examined by an ophthalmologist (*25*,*26*) showed increased visual disorders (e.g., blurred vision, myopic shift) compared with other studies, probably because of selection bias or higher sensitivity of the ophthalmologic examination. In this study, we found a strong association between patient's reported ocular involvement and the risk for severe NE, probably because only symptomatic ocular disorders, which are the most severe, were taken into account. Loss of visual acuity in patients with NE might reflect higher tissue permeability, which could explain an association with greater renal damage (*26*).

Hematuria is frequently observed in NE. We found a prevalence of 50% hematuria at admission, whereas other studies have reported prevalences of 25%–58% (*11*,*23*,*27*). At least 1 study found an association between hematuria (but not thrombocytopenia) and progression to severe AKI (*28*). Hematuria is also considered a marker of NE severity predictive of polyuria (*29*). In Hantaan virus infections, hematuria has been associated with the occurrence of severe HFRS (*30*,*31*). Leukocyte count has previously been identified as a predictive marker of severe NE (*32*) and a predictive marker of death from HFRS among populations in China, where Hantaan virus and Seoul virus are the 2 major circulating species (*22*,*30*,*33*,*34*). Leukocyte count was collinear with C-reactive protein level, which has also been found associated with severe NE (*13*,*35*). Thrombocytopenia $\leq 90 \times 10^9$/L has been found associated with a more severe course of disease (*13*,*27*,*31*,*34*,*36*,*37*).

Proteinuria, especially urine sample proteinuria: creatinuria ratio, or dipstick proteinuria at admission would have been good candidate predictors of severe NE (*12*,*13*,*38*,*39*), as would have been urine output during the first 24 h and data on tobacco use (*19*). However, because

of the retrospective design, these factors were not reliably assessed on admission in our centers, contrary to during follow-up. Another good candidate predictor might be albumin level (or proteinemia) at admission, as this parameter could reflect severe vascular leakage or an increased degree of systemic inflammation (*9*). Unfortunately, albumin level was not routinely assessed on admission in our centers. Other independent risk factors, such as elevated urokinase-type plasminogen activator receptor plasma level, interleukin 6, pentraxin-3, indoleamine 2,3-dioxygenase, cell-free DNA, Mac-2 binding protein, cerebrospinal fluid neopterin concentration, and urine GATA-3 mRNA level, have been reported in the literature (*14*,*32*,*35*,*40*–*42*). However, tests of these parameters are not typically available in clinical practice.

The simple test proposed here could be calculated on admission to evaluate the risk for severe NE (using the more stringent definition of severe NE) and is applicable for patients for whom the need for hospitalization is being considered. This bioclinical test could help physicians avoid prolonged hospitalizations of low-risk patients and better treat high-risk patients, keeping them hospitalized and monitored. The discriminatory ability of the test score was satisfactory, with an area under the curve (C statistic) of 0.80. The model also showed good internal validity, as parameter estimates were stable after bootstrapping. In another study, a predictive test was proposed to identify patients at high risk for severe AKI in acute NE (*13*). In a retrospective study in Germany, Latus et al. studied 137 patients who had normal kidney function at hospital admission during 2001–2012 and identified 3 predictive factors (thrombocytopenia, proteinuria, 12-fold elevated C-reactive protein) of severe AKI (defined as kidney injury and failure of kidney function, according to the RIFLE criteria) (*15*). Patients without these factors had a relatively high probability (18%) of developing severe AKI, as defined by the authors, probably because of the definition used to characterize the severe form.

Our study has several limitations that should be acknowledged. Atypical NE cases might have been underdiagnosed, especially in younger patients with mild disease. However, these patients might not be those who would most benefit from the use of the test we developed. In addition, the retrospective design of our study incurs a high risk for misclassification and missing data. Also, our study was not performed nationwide, although it was conducted in the more NE-endemic regions and in centers experienced with treating NE. Finally, external validation of our test is needed before recommending wider use. Despite these limitations, we believe that the bioclinical test proposed could be helpful in the initial evaluation of patients and subsequent management of NE, given that this test is easy to use in routine practice. This scoring system could also be useful in clinical research, allowing for stratification and evaluation of patients by risk group.

In conclusion, we developed a simple bioclinical test assessing the presence of visual disorders, nephrotoxic drug exposure, leukocytosis, hematuria, and thrombocytopenia at hospital admission to discriminate patients at low, intermediate, and high risk for severe NE. This test could be helpful in identifying patients at high risk for severe NE in clinical practice, pending external validation with other potentially larger-scale studies.

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## About the Author

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# Hepatitis E in Long-Term Travelers from the Netherlands to Subtropical and Tropical Countries, 2008–2011

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Hepatitis E virus (HEV) is a common cause of acute viral hepatitis. Virus genotypes 1 and 2 infect humans in developing countries by the fecal–oral route. To assess attack rates and disease incidence for travelers, we prospectively studied 604 long-term travelers to subtropical and tropical countries. Participants donated blood samples pretravel and posttravel and kept a diary. A total of 89/604 (15%) pretravel samples were positive for HEV IgG by ELISA, suggesting previous HEV infection. Seroconversion for HEV was found for 19/515 travelers (attack rate 3.7%, incidence 1.8 cases/1,000 person-weeks). We believe there is a substantial risk for acquiring HEV infection among long-term travelers. Although HEV infection does not seem to be a major problem in this healthy cohort, hygienic measures should be stressed in all pretravel health advice, particularly for pregnant women and immunocompromised travelers who are at risk for severe disease.

Hepatitis E virus (HEV) is a common cause of acute viral hepatitis worldwide (*1*). There are 4 genotypes of HEV. Genotypes 1 and 2 infect humans in developing countries in areas with poor sanitation; transmission occurs through the fecal–oral route, causing occasional large outbreaks or frequent sporadic cases. Genotype 1 is found in Asia and Africa, and genotype 2 is found in Mexico and Africa. Genotypes 3 and 4 are transmitted zoonotically from animal reservoirs in industrialized and developing countries, mainly through consumption of uncooked or undercooked meat, and are responsible for sporadic cases of disease (*1*). In the Netherlands, genotype 3 is endemic in pigs and responsible for cases in humans.

The mean incubation period for hepatitis E is 40 days (range 15–60 days). Symptoms range from subclinical to fulminant and include fever, fatigue, loss of appetite, nausea, vomiting, abdominal pain, jaundice, joint pain, and hepatomegaly (*1*).

Hepatitis E is usually a self-limiting disease. The mortality rate for fulminant hepatitis is 0.5%–4%. Pregnant women, immunosuppressed persons, and persons with preexisting liver disease are at risk for severe hepatitis E. However, fulminant liver disease in immunocompetent persons has also been reported (*2*). Mortality rates for hepatitis E caused by genotype 1 for pregnant women are 20%–25% (*3*,*4*).

A previous HEV infection is characterized by the presence of specific IgG and is assumed to protect against reinfection. There is cross-neutralization among all genotypes (*5*).

Travelers from industrialized countries to developing countries are assumed to be at risk for acquiring an HEV infection through the fecal–oral route (genotypes 1 and 2). A vaccine against HEV is available only in China (*4*).

A study that included data in the GeoSentinel surveillance network for returned travelers with infectious gastrointestinal diseases during 1996–2005 reported a proportionate HEV illness rate of 1.2 cases/1,000 ill returned travelers (*6*). A recent case report identified a nonpregnant immunocompetent traveler who returned to Canada from India and was given a diagnosis of HEV infection, in whom fulminant liver failure developed (*2*). A study in Israel of 4,970 ill returning travelers during 1997–2012 reported 49 (1%) with acute hepatitis (32 cases were enterically transmitted): 19 travelers were given a diagnosis of hepatitis E, of whom 16 contracted their cases on the Indian subcontinent (*7*). The estimated risk for acquiring HEV for this study was 3.2 cases/100,000 travelers.

A prospective study of 1,206 short-term travelers from the Netherlands to subtropical and tropical countries (*8*), a prospective study of 105 long-term backpackers in Israel (*9*), and a prospective study of American missionaries (*10*) showed no seroconversions for HEV. Another prospective study of 356 short-term travelers from the United States reported 4 (1.7%) seroconversions (*11*). However, because

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seroconversions were found only for samples obtained 6 months after return of travelers and not in samples obtained 6 weeks posttravel, HEV might have been contracted after their return.

To our knowledge, no recent prospective studies of long-term travelers have been conducted. Because the risk for hepatitis E in subtropical and tropical countries might have increased, and the sensitivity of ELISAs for diagnosing HEV infection has improved over the past decade (*3*), we determined the incidence and risk factors of acquiring hepatitis E among long-term travelers (12–52 weeks) from the Netherlands to subtropical and tropical countries.

## Methods

### Study Population and Design

This study was conducted as part of a larger, prospective, monocenter study of immunocompetent travelers ≥18 years of age who visited the Public Health Service travel clinic in Amsterdam, the Netherlands, during December 2008–September 2011. All clients planning to travel to subtropical or tropical countries for 12–52 weeks were invited to participate. Subtropical and tropical countries were defined as those with moderate to high risk for hepatitis A according to the World Health Organization (*12*). All participants consulted a nurse or physician specialized in travel medicine, and oral and written information was provided about how to avoid travel-related diseases. The study was approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam.

At their return, travelers were asked additional questions regarding behavior during travel, including drinking unboiled tap water or water from natural sources. Pretravel written informed consent was obtained, and travelers were interviewed by a nurse or physician about travel purpose, travel duration, planned destination(s), and demographic details.

Participants were given a digital thermometer (Huikeshoven Medical, Tiel, the Netherlands) and asked to record their temperatures if they felt feverish while traveling. Travelers kept a structured, weekly travel diary until 2 weeks after return and recorded their itinerary, symptoms, and physician visits while ill. Predefined symptoms that could be related to hepatitis E were fever and vomiting. It was also possible to report other complaints. Diaries were completed on paper or digitally. Travelers received a weekly email reminder and were seen 2–6 weeks after return. Blood samples were obtained before and after travel. The pretravel sample was tested only if the posttravel sample tested was positive for antibodies against HEV.

Primary regions visited were grouped into regions according to the classification of the United Nations Country Classification with some modifications. For our study, Oceania included only Melanesia, Micronesia, and Polynesia and was merged with Southeast Asia because New Zealand and Australia matched exclusion criteria for participating. Latin America was divided into South America and the Caribbean/Central America.

### Laboratory Methods

Blood samples were immediately stored at 6°C, centrifuged, and frozen at −80°C. We tested serum samples for HEV IgG by using an ELISA (Wantai Biologic Pharmacy Enterprise, Beijing, China), according to the manufacturer's instructions. This assay had a reported sensitivity of 98% but does not discriminate between different virus genotypes. If HEV IgG was detected in a posttravel sample, pretreatment samples were also tested. Presence of HEV IgG in a pretravel sample was regarded as evidence of previous HEV infection. A recent infection was defined as a positive posttravel sample and a negative pretravel sample.

### Data Analysis

We calculated risk factors for previous HEV infection by using SPSS version 19.0 (IBM, Armonk, NY, USA) to obtain prevalence, univariable and multivariable prevalence ratios (PRs), and 95% CIs by means of logistic regression modeling. A p value <0.05 was considered statistically significant. All variables with p values <0.10 by univariable analysis were included in a multivariable model.

We calculated the attack rate of a recent HEV infection by dividing the number of seroconversions by the total number of participants still at risk for infection (i.e., all travelers who did not have HEV antibodies pretravel). We also calculated incidence rates by dividing the number of seroconversions by the total number of travel weeks of travelers still at risk. Person-time denominators for seroconversion were divided in half, assuming that the infection occurred halfway through travel.

We used univariable Poisson regression models to examine the effect of covariates (sex, age, travel purpose, primary destination, hospital admission) on seroconversion. Variables with p values <0.10 in univariable analysis were included in multivariable analysis. Outcomes were expressed as incidence rate ratios with 95% CIs. A p value <0.05 was considered statistically significant.

## Results

### Study Population

During December 2008–September 2011, a total of 685 persons who intended to travel to subtropical and tropical countries for 12–52 weeks provided informed consent. Of

these persons, 81 (12%) were excluded after completion of the study: 42 had their travel arrangements changed and no longer met the study criteria, 38 were lost to follow-up, and 1 did not provide a posttravel blood sample. The remaining 604 persons formed the study population.

Median age of the study population was 25 years (interquartile range [IQR] 23–29 years), ≈66.6% were female, and 20% had never been to subtropical or tropical regions. Tourism was the main purpose for traveling (62.9%). Median interval between the first sample and departure was 38 days (IQR 20–55 days). Median interval between return and the second blood sample was 25 days (IQR 21–33 days).

### Previous HEV Infection

A total of 89 of 604 persons were positive for HEV pretravel and posttravel, which indicated previous HEV infection, for a pretravel seroprevalence rate of 14.7% (Table 1). Univariate analysis indicated that previous HEV infection showed a positive correlation with older age, a nonwestern origin, and a history of travel to subtropical or tropical regions. Multivariate analysis showed that age, travel history, and nonwestern origin remained major predictors for previous HEV infection.

### HEV Infection Acquired during Current Travel

IgG seroconversion was found for 19/515 travelers, resulting in an attack rate of 3.7% and an incidence of 1.8 (95% CI 1.1–2.8) per 1,000 person-weeks. We obtained characteristics, attack rates, and incidence for recent HEV infections (seroconversions) (Table 2). At return, 32% (163/510) of participants reported they had used unboiled or untreated tap water for consumption, 19 did not remember, and 5 had missing results. The remaining 328 travelers did not consume unboiled or untreated water. Six persons who showed seroconversion reported having drunk unboiled or untreated tap water; 1 person had a missing result. Logistic regression of characteristics tested did not identify any major risk factors for acquiring HEV infection during travel.

### Signs and Symptoms in Travelers Showing Seroconversion

A total of 215 (42%) of 515 travelers reported vomiting during their trip, and 35% (180/515) reported fever at least once. Nine of 19 travelers showing seroconversion reported ≥1 nonspecific symptoms possibly associated with HEV infection: 2 participants reported fever, 3 reported vomiting, 1 reported vomiting and fatigue, 2 reported vomiting and fever, and 1 reported abdominal pain and nausea.

A total of 31 (6%) of 515 participants were admitted to a hospital while abroad, of whom 1 person who showed seroconversion was admitted because of symptoms of fever and dehydration caused by diarrhea. Jaundice, dark-colored urine, and light-colored stool were not reported as other complaints in the diary.

## Discussion

In this prospective study of long-term travelers from the Netherlands to subtropical and tropical countries, we found a substantial hepatitis E attack rate of 3.7% and an incidence of 1.8 cases/1,000 person-weeks. Results were obtained by using an HEV IgG ELISA and were higher than those in the 4 previous prospective studies of travelers (*8*–*11*).

The relatively high HEV seroconversion rate we found compared with those for previous prospective studies could be explained by an increase of hepatitis E incidence in developing countries over time. However, our results probably reflect improved sensitivity of currently available tests compared with those used in these previous studies. A combination of these factors is possible. Therefore, comparison of results of previous prospective and seroprevalence studies with those of our study should be interpreted with caution. We also found no major risk factors for acquiring HEV infection during travel.

Two nonprospective studies reported that hepatitis E is associated with travel to southern Asia (*7*,*13*). We found higher attack rates and incidences for southern Asia, other regions of Asia, and Central America than for Africa,

**Table 1.** Characteristics of 604 travelers who visited a travel clinic for pretravel advice and prevalence of previous HEV infection, the Netherlands, December 2008–September 2011*

| Characteristic | Travelers | HEV IgG positive pretravel | Univariable analysis PR (95% CI) | p value | Multivariable analysis PR (95% CI) | p value |
|---|---|---|---|---|---|---|
| Total | 604 | 89 (14.7) | | | | |
| Median age, y (IQR) | 25 (23–29) | 26 (23–30) | 1.0 (1.01–1.07) | **0.002** | 1.0 (1.01–1.06) | **0.01** |
| Sex | | | | | | |
| F | 389 (64.4) | 54 (13.9) | 1.0 | 0.43 | | |
| M | 215 (35.6) | 35 (16.3) | 1.2 (0.76–1.92) | | | |
| Region of birth | | | | | | |
| Western (the Netherlands), n = 563 | 590 (97.7) | 84 (14.2) | 1.0 | **0.034** | 1.0 | **0.03** |
| Nonwestern | 14 (2.3) | 5 (35.7) | 3.4 (1.10–10.23) | | 3.6 (1.5–11.28) | |
| Previous travel to subtropical region | | | | | | |
| No | 122 (20.2) | 7 (5.7) | 1.0 | **0.003** | 1.0 | **0.01** |
| Yes | 482 (79.8) | 82 (17.0) | 3.4 (1.5–7.5) | | 2.9 (1.27–6.45) | |

*Values are no. (%) except as indicated. Bold indicates statistical significance. HEV, hepatitis E virus; IQR, interquartile range; PR, prevalence ratio.

**Table 2.** Attack rates and incidence of seroconversions in HEV antibody levels for 515 long-term travelers to subtropical and tropical countries, the Netherlands, December 2008–September 2011*

| Characteristic | Travelers at risk | HEV seroconversions | Attack rate, % (95% CI) | Person-weeks of travel | Incidence/1,000 person-weeks (95% CI) | Univariable analysis | |
|---|---|---|---|---|---|---|---|
| | | | | | | IRR (95% CI) | p value |
| Total | 515 | 19 | 3.7 (2.4–5.7) | 10,715 | 1.8 (1.1–2.8) | | |
| Median age, y (IQR) | 25 (23–29) | 26 (22–31) | | | | | |
| Sex | | | | | | | |
| F | 335 (65) | 9 | 2.7 (1.4–5.0) | 6,795 | 1.3 (0.7–2.5) | 1.0 | 0.16 |
| M | 180 (35) | 10 | 5.6 (3.0–9.9) | 3,920 | 2.6 (1.4-4.7) | 1.9 (0.8–4.7) | |
| Region of birth | | | | | | | |
| Western | 506 (98.3) | 19 | 3.8 (2.4–5.8) | | | | |
| Nonwestern | 9 (1.7) | 0 | NA | | | | |
| Purpose of travel | | | | | | | |
| Holiday | 324 (62.9) | 10 | 3.1 (1.7–5.6) | 6,556 | 1.5 (0.8–2.8) | 1.0 | 0.54 |
| Work or study | 184 (35.7) | 8 | 4.3 (2.2–8.3) | 3,970 | 2.0 (1.0–4.0) | 1.3 (0.5–3.3) | |
| VFR | 7 (1.4) | 1 | 14.3 (2.6–51.3) | 1,885 | 5.3 (0.9–29.4) | 3.5 (0.4–27.0) | |
| Primary region of travel | | | | | | | |
| Southeast Asia and Oceania | 172 (33.4) | 5 | 2.9 (1.2–6.6) | 3,543 | 1.4 (0.6–3.3) | 1.0 | 0.57 |
| South America | 148 (28.7) | 5 | 3.4 (1.5–7.7) | 3,211 | 1.6 (0.7–3.6) | 1.1 (0.3–3.8) | |
| Sub-Saharan Africa | 94 (18.3) | 2 | 2.1 (0.6–7.4) | 1,972 | 1.0 (0.3-3.7) | 0.7 (0.1–3.7) | |
| Southern Asia | 47 (9.1) | 3 | 6.4 (2.2–17.2) | 898 | 3.3 (1.1–9.8) | 2.4 (0.6–9.9) | |
| Central America and Caribbean | 37 (7.2) | 3 | 8.1 (2.8–21.3) | 754 | 4.0 (1.4–11.6) | 2.8 (0.7–11.8) | |
| Asia, other | 17 (3.3) | 1 | 5.9 (1.0–27.0) | 336 | 3.0 (0.5–16.7) | 2.1 (0.2–18.1) | |
| Travel duration, wk | | | | | | | |
| 12–16 | 189 (36.7) | 6 | 3.2 (1.5–6.8) | NA | | | |
| 17–24 | 190 (36.9) | 7 | 3.7 (1.8–7.4) | NA | | | |
| 25–52 | 136 (26.4) | 6 | 4.4 (2.0–9.3) | NA | | | |
| Hospital admission | | | | | | | |
| No | 484 (94) | 18 | 3.7 (2.4–5.8) | 10,032.5 | 1.8 (1.1–2.8) | 1.0 | 0.84 |
| Yes | 31 (6) | 1 | 3.2 (0.6–16.2) | 682.5 | 1.5 (0.3–8.2) | 0.8 (0.11–6.11) | |

*Values are no. (%) except as indicated. HEV, hepatitis E virus; IQR, interquartile range; IRR, incidence rate ratio; NA, not applicable; VFR, visiting friends and relatives.

Southeast Asia, and South America, but this finding did show a major difference.

In 2 other studies, travelers visiting friends and relatives were found to be at greater risk than tourist travelers for infectious diseases such as typhoid fever (*6*) and hepatitis E (*14*). Our study showed a higher attack rate (14%) and incidence (5.3 cases/1,000 person-weeks) for travelers visiting friends and relatives than for persons traveling for tourism or work/study, but this association was not strong. This finding could be caused by the small numbers of travelers visiting friends and relatives.

The pretravel seroprevalence of 15% we found was higher than the 2% found in the study of travelers from the Netherlands conducted during 2006–2007 (*8*) and the 6% found in the study of Boston, Massachusetts, USA, area travelers conducted during 2009–2010 (*15*). However, seroprevalence in this study was lower than the 27% found in the study of blood donors from the Netherlands conducted during 2011 (*16*) and in the population of Amsterdam during 2004 (*17*). Although these differences should also be interpreted with caution, there are several possible explanations for the differences in prevalence between studies.

The major difference in sensitivity between different assays could be an explanation for the higher prevalence we found than the prevalence of 2% found in the previous prospective study among travelers from the Netherlands. The test we used in our study was the same test used in the study of blood donors from the Netherlands (*16*) and for the population of Amsterdam (*17*). However, because HEV immunity increases with age and depends on ethnicity, the difference in characteristics between the different study groups could explain why we found a prevalence of 15% rather than 27%. Our study population was composed of mostly young persons of western origin.

Independent risk factors for previous HEV infection were being born in a nonwestern country and previous travel to subtropical and tropical regions, which can be explained by higher endemicity in nonwestern countries. Also, older age was a major risk factor for previous HEV infection, as observed by Sadik et al. (*17*).

A total of 9 of the 19 persons who showed seroconversion reported nonspecific symptoms possibly related to HEV infection. Only 1 of 31 hospitalized travelers showed seroconversion for antibodies against HEV, but hospitalization was probably not related to HEV infection (self-reported diagnosis was dehydration caused by diarrhea). None of the persons who showed seroconversion were given a diagnosis of HEV infection during the study. Because many cases of

hepatitis E are subclinical in otherwise healthy persons and only immunocompetent travelers were included in our study, it is not surprising that so many persons who showed seroconversion did not report specific symptoms.

The strength of our study is that it is a prospective study in which blood samples before and after travel and diaries kept during travel were available for all 604 long-term travelers. However, our study also had limitations. Because this study was part of a larger study, the travel diary contained general clinical symptoms instead of hepatitis E–specific symptoms. Thus, we could have missed signs of a mild clinical HEV infection. Also, the median interval between obtaining a posttravel blood sample and return from travel was only 25 days. Because the incubation period for hepatitis E is 15–60 days, this period could have led to an underestimation of cases. However, because this study involved long-term travelers who traveled for 12–52 weeks, it is unlikely that many infections were contracted in the last weeks of travel. Therefore, we assume the short interval between return from travel and obtaining a blood sample had limited consequences for the final results. In addition, the median interval between obtaining the first blood sample and travel departure was 38 days. Therefore, persons who showed seroconversion might have contracted the virus before travel, which could have led to overestimation of travel-related attack rates and incidences, but we assume this had limited effect on the final results.

We assumed that persons with HEV IgG were protected against reinfection and did not include them in additional analyses. However, reinfection is possible, even in immunocompetent persons (*1*,*4*). We compared pretravel and posttravel sample titers and found only 1 person with a high posttravel titer and a much lower but still above the positive threshold pretravel titer. This result could have been a reinfection, but a low false-positive value for the pretravel sample is also possible. We considered this traveler immune in our additional analysis; this person also did not report any symptoms of HEV infection. A 4-fold increase in titer between pretravel and posttravel samples was not found for other persons.

Our study could have had a selection bias because all participants sought pretravel health advice in which advice on personal hygiene was stressed. Because genotypes 1 and 2 of HEV are contracted through the fecal–oral route, this finding could have led to an underestimation of HEV incidence. However, most (82%) travelers in our cohort experienced travelers' diarrhea, which could also be contracted through the same route. Therefore, we believe that this selection bias had limited consequences on the outcome.

Finally, seroprevalence research most often lacks a diagnostic standard because it resembles a postinfection status in which conformatory tests using PCR are not feasible. In previous studies, the HEV IgG ELISA appeared to be one of the most sensitive tests available (*18*–*21*). However, in the absence of World Health Organization HEV-negative reference material, studies investigating the specificity of the test are scarce. Although results from a study in France were promising (specificity 97.8%) (*21*), possible false-positive test results cannot be excluded.

Using the HEV IgG ELISA, we found an attack rate for HEV infection of 3.7% and an incidence of 1.8 cases/1,000 person-weeks, which are higher than values from previous prospective studies. This finding could be a reflection of an increasing risk for travelers, but it could also (partially) reflect improved sensitivity of the available test. Almost half of persons who showed seroconversion had mild, nonspecific clinical symptoms possibly associated with HEV infection. Therefore, HEV infection does not seem to be a major problem in healthy immunocompetent travelers. However, rare fulminant liver failure in immunocompetent travelers has been reported (*2*), and in pregnant women and immunocompromised travelers, the risk for severe or fatal disease is much higher. Because travel has increased during the past few decades, at-risk groups also travel more (*22*). Good sanitation and clean drinking water should be discussed in all travel health advice. If an HEV vaccine were approved and found to be safe and effective for pregnant women and immunocompromised travelers, these vulnerable travelers could especially benefit from its protection.

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# Novel Parvovirus Related to Primate Bufaviruses in Dogs

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A novel protoparvovirus species, related genetically to human bufaviruses, was identified in dogs with respiratory signs. The canine bufavirus was distantly related to the well-known canine protoparvovirus, canine parvovirus type 2, sharing low amino acid identities in the nonstructural protein 1 (40.6%) and in the capsid protein 1 (33.4%). By screening collections of fecal, nasal, and oropharyngeal samples obtained from juvenile dogs (≤1 year of age), canine bufavirus DNA appeared as a common component of canine virome. The virus was common in the stool samples of dogs with or without enteric disease and in the nasal and oropharyngeal swab samples of dogs with respiratory signs. However, the virus was not detected in nasal and oropharyngeal swab samples from animals without clinical signs.

Parvoviruses (family *Parvoviridae*) are small, nonenveloped viruses of 25–30 nm in diameter, with an icosahedral capsid. The genome is a single-strand DNA of 4.5–5.5 kb (*1*) with complex hairpin structures at the 5′ and 3′ ends. The genome is predicted to encode 3 or 4 proteins: nonstructural (NS) 1, nucleoprotein (NP) 1, and viral protein (VP) 1 and 2.

Parvoviruses have long been known in dogs, since the identification of canine minute virus, or canine parvovirus (CPV) type 1 (CPV-1; genus *Bocaparvovirus*), in 1967 from the fecal samples of healthy dogs (*2*). CPV-1 infection is responsible for reproductive disorders and occasionally for respiratory and gastrointestinal signs in young puppies (*3*). A second CPV (CPV-2; genus *Protoparvovirus*) was reported in the 1970s in Europe and North America in puppies with signs of hemorrhagic gastroenteritis and myocarditis (*4*). CPV-2 is currently regarded as the major causative agent of severe gastroenteritis in puppies and is included in canine core vaccination schedules globally (*5*). In 2011, a second canine bocaparvovirus (CBoV) was identified from nasal swabs of healthy and sick dogs (*6*); a third species of CBoV was identified in 2013 in the liver of a dog with multiorgan failure (*7*) (Table). Whether the newly identified parvoviruses play a role as canine pathogens has not yet been assessed.

We report the identification of a novel CPV. We determined the genome sequence of the CPV and designed specific primers and probes useful for laboratory diagnosis. Screening of enteric and respiratory samples from dogs with either gastroenteric or respiratory disease and from animals without clinical signs suggested a possible association between the novel virus and respiratory disease in young dogs.

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## Material and Methods

### Identification of the DNA of a Novel Parvovirus

In 2011, an outbreak of canine infectious respiratory disease (CIRD) occurred in a litter of 3 mixed-breed 5-month-old puppies in Italy. The animals' clinical signs were nasal discharge, coughing, and respiratory distress, but they completely recovered from the disease after 2 weeks. Nasal and oropharyngeal swab specimens tested negative to a panel of molecular assays for CIRD-associated common and emerging viral agents: canine adenovirus (CAV) types 1 and 2, canine distemper virus, canid herpesvirus 1 (CHV-1), canine respiratory coronavirus, influenza virus, canine parainfluenza virus, canine pneumovirus, nonprimate canine hepacivirus, *B. bronchiseptica*, *Streptococcus equi* subspecies *zooepidemicus*, and *Mycoplasma cynos* (*12*). Because the etiology of the outbreak was unknown, the case was considered eligible for metagenomic investigation.

We performed random primed reverse transcription PCR and PCR assays on pooled samples (the nasal and oropharyngeal swab specimens of the 3 puppies) to amplify nucleic acids and used them as templates for next-generation sequencing (NGS) experiments on the Ion Torrent platform (New England Biolabs, Ipswich, MA, USA) (*13*). We evaluated sequence data using CLC Genomic Workbench (http://www.clcbio.com). NGS revealed the

**Table.** Parvoviruses identified in dogs and their classification and proposed classification of canine bufaviruses*

| Genus and species | Common/used names in literature | Year identified | Place identified | Reference | GenBank accession no. |
|---|---|---|---|---|---|
| *Bocaparvovirus* | | | | | |
| Carnivore bocaparvovirus 1 | CPV-1, minute virus of canines (MVC) or CBoV-1 | 1970 | United States | (*2*) | FJ214110 |
| Carnivore bocaparvovirus 2 | CBoV-1 or CBoV-2 | 2011 | United States | (*6*) | JN648103 |
| Carnivore bocaparvovirus 3 | Feline bocaparvovirus | 2009 | United States | (*8*) | JQ692585 |
| Carnivore bocaparvovirus 4† | CBoV-3 | 2011 | United States | (*7*) | KC580640 |
| *Protoparvovirus* | | | | | |
| Carnivore protoparvovirus 1 | CPV-2 | 1978 | United States | (*4*) | |
| | CPV-2a | 1983 | United States | (*9*) | M24000 |
| | CPV-2b | 1984 | United States | (*10*) | M74849 |
| | CPV-2c | 2000 | Italy | (*11*) | AY380577 |
| Carnivore protoparvovirus 2† | Canine bufavirus | 2012–2016 | Italy and Hungary | This study | MF198244–46 |

*CBoV, canine bocavirus; CPV, canine parvovirus.
†Candidate novel species.

presence of parvovirus-related sequence reads that mapped to human bufaviruses. We mapped a total of 3,530 reads to human bufavirus and assembled them into 3 contigs, 422-, 416-, and 191-nt long. We reconstructed the nearly complete genomic sequence of the new CPV (canine bufavirus [CBuV]), strain ITA/2011/297-15, by combining 5′ rapid amplification of cDNA ends (RACE) protocols (*14*) with minor modifications, using the kit 5′ RACE System for Rapid Amplification of cDNA Ends version 2.0 (Life Technologies, Paisley, UK) and a primer-walking strategy with specific primers designed to close the gaps among noncontiguous sequences. We purified and cloned the amplicons using a TOPO XL Cloning Kit (Life Technologies) and generated consensus sequences by sequencing ≥3 clones for each PCR fragment.

### Screening of Samples in Conventional and Quantitative PCR

We designed specific primers on the VP2 genomic region of strain ITA/2011/297-15 for quantitative detection in real-time PCR (qPCR) (CPPV-L3-for 5′ TGAACAAGAAATAGACAACATTGTCAT 3′, CPPV-L3-rev 5′ AAAGAGCAGTTAGGTCATTGTTGT 3′, and CPPV-L3 Pb 5′ Fam CCAAACAAGGTACAGGACAGGAAGAAACAACACAA BHQ1 3′). We calculated CBuV DNA copy numbers on the basis of standard curves generated by 10-fold dilutions of a plasmid standard TOPO XL PCR containing a 500-nt fragment of the VP2 region of strain ITA/2011/297-15. We added 10 μL of sample DNA or plasmid standard to the 15-μL reaction master mix (IQ Supermix; Bio-Rad Laboratories SRL, Segrate, Italy) containing 0.6 μmol/L of each primer and 0.2 μmol/L of probe. Thermal cycling consisted of activation of iTaq DNA polymerase at 95°C for 3 min, 42 cycles of denaturation at 95°C for 10 s, and annealing extension at 60°C for 30 s. We evaluated the specificity of the assay with a panel of canine DNA viruses (CPV-1, CPV-2, CHV-1, circovirus, CAV-1, and CAV-2). The qPCR detected $\geq 10^1$ DNA copies/10 μL of standard DNA and $8.78 \times 10^0$ DNA copies/10 μL of DNA template extracted from clinical samples. CBuV quantification had an acceptable level of repeatability over various magnitudes of target DNA concentrations, when calculating (*15*) the intraassay and interassay coefficients of variation within and between runs, respectively.

In addition, we designed specific primers (CPPV 165F 5′ CTGGTTTAATCCAGCAGACT 3′ and CPPV 371R 5′ TGAAGACCAAGGTAGTAGGT 3′) to amplify and sequence a 207-nt fragment of VP2. We used the AccuPrime Taq DNA polymerase (Life Technologies) for PCR amplification. Cycling thermal conditions included initial activation of the polymerase at 94°C for 2 min, 45 cycles at 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s, followed by final extension at 72°C for 10 min.

### Respiratory Samples

During 2011–2015, we obtained nasal and oropharyngeal swab (NOP) samples from 58 pups and young dogs (≤1 year of age) with CIRD (collection RIS); the animals were early acute clinically ill CIRD dogs with onset of respiratory signs at 0–3 days at the time of sample collection. The RIS samples were a subset of a larger collection used for surveillance of traditional and emerging pathogens associated with CIRD (*12*). We collected samples from the nasal and oral cavities in parallel and stored them separately. We also screened NOP swabs obtained from 90 dogs <1 year of age without clinical signs (collection RIA) as controls. We collected all samples with dry swabs and immediately stored them at –20°C.

### Enteric Samples

We screened archived enteric samples (stools and rectal swabs) collected at the Department of Veterinary Medicine, University of Bari, Italy, during 2010–2015 for CBuV. The samples had been obtained from pups and young dogs (<1 year of age). We screened 81 samples from animals with

signs of gastroenteritis (collection EIS) and 78 samples of animals without clinical signs (collection EIA).

We also searched for CBuV DNA in a collection of enteric samples from pups and young dogs (<1 year of age) either with gastroenteritis (collection EHS) or without clinical signs of gastroenteritis (collection EHA), obtained in Hungary in 2012 and available at the Institute of Veterinary Medical Research, Hungarian Academy of Science, Budapest. We screened 40 samples from healthy animals and 20 samples from animals with clinical signs.

### Statistical Analysis

We evaluated the associations among clinical signs, age, and presence of the virus in the respiratory and enteric samples by the $\chi^2$ test using a web-based software program (R version 3.3.0; http://www.r-project.org/). We set statistical significance at $p<0.05$.

### Genome Analysis of Hungarian CBuV Strains

We selected samples positive for CBuV on the basis of the genome copies as revealed by quantitative PCR. We also identified the genomes of 2 additional CBuV strains, HUN/2012/22 and HUN/2012/126.

### Sequence and Phylogenetic Analyses

We retrieved genome sequences of protoparvovirus strains from GenBank and aligned them using the Clustal Omega tool from the European Molecular Biology Laboratory (https://www.ebi.ac.uk/Tools/msa/clustalo/). We conducted sequence and phylogenetic analyses in Geneious version 9.1.8 (Biomatters Ltd., Auckland, New Zealand); we used the neighbor-joining method, Jukes–Cantor genetic distance model, and bootstrapping for <1,000 replicates.

### Virus Cultivation

We homogenized PCR-positive respiratory and fecal samples in 10% Dulbecco's modified Eagle's medium and then centrifuged them at 10,000 × *g*. We filtered the supernatant with 0.22-μm filters and inoculated it onto freshly seeded canine fibroblastic tumor (A-72) cells, incubated at 37°C in 5% $CO_2$. We also inoculated Madin-Darby canine kidney cell (MDCK) and Walter Reed canine cell (WRCC) lines. We evaluated viral growth through 5 serial passages, monitoring the onset of cellular cytopathic effect and testing the cell supernatant by qPCR.

## Results

### Genome Analysis of Canine Bufaviruses

We determined the nearly complete genome (4,537 nt) of the CBuV strain ITA/2011/297-15, including a partial 5′ untranslated region (UTR) (310 nt), the complete NS1 sequence (638 aa), the complete VP1 (710 aa) and VP2 (568 aa) sequences, and a partial 3′ UTR (8 nt). The genome coding sequence, excluding the terminal UTR regions, was 4,219 nt (GenBank accession no. MF198244). The genome contained 2 major open reading frames (ORFs); the left ORF, coding for NS1, was 1,917 nt and the right ORF, encoding VP1 and VP2, was 2,316 nt (Figure 1, panel A). Full-genome sequence alignment showed a high degree of sequence divergence, up to 58% overall nucleotide identity to most parvoviruses but not bufaviruses. CBuV was more closely related to bufaviruses identified in primates (61.6%–63.2% nt similarity), pigs (59.6% nt), and bats (58% nt) (*16*–*20*) and more distantly related to CPV-2 (45% nt) (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/6/17-1965-Techapp1.pdf). The putative bufavirus NS1 start codon was located in a strong Kozak sequence, ACCATGG. The ATP- or GTP-binding Walker loop motif (GXXXXGK[T/S]) was found in NS1 (405-GPASTGKS-412) (*21*). In addition, the NS1 contained 2 conserved replication initiator motifs, GL**H**F-**H**VLLQ and IVR**Y**FLTKQP (boldface type indicates conserved amino acids) (*22*). We generated an nt and aa sequence identity matrix. NS1 showed <69.4% nt and 51.4% aa identity with other parvovirus NS1 sequences, including its closest relatives in the *Parvovirus* genus (online Technical Appendix Table).

The termination of ORF1 overlapped the start of ORF2 by 14 nt. From sequence alignment and comparison with other BuVs, we detected 2 potential splice sites in the ORF1/ORF2 junction, a potential donor site (AG¯GT) at nt 1931 and an acceptor site (AG¯G) at nt 2115. The putative VP1 sequence started at the end of ORF1 at nt 1906, upstream of the splice donor site at nt 1931 (Figure 1, panel B). We found the phospholipase A2 (PLA2) motif (Figure 1, panel A), with its highly conserved calcium binding site (YLGPG), in the main ORF of VP1. The phospholipase catalytic residues (HD and D) were present at amino acid positions 41–42 and 63 (Figure 1, panel A). The VP1 showed <67.2% aa identity to other genera of the family *Parvovirinae*, including its closest relative in the *Parvovirus* genus (online Technical Appendix Table). The N terminus of the CBuV VP2 protein contained a glycine-rich sequence (GGGGGGGSGVG) that was also present in other parvoviral VP2 proteins (Figure 1, panel A).

We successfully determined the complete coding genome sequence of 2 additional CBuV strains of enteric origin, HUN/2012/22 (GenBank accession no. MF198245) and HUN/2012/126 (GenBank accession no. MF198246); the sequence was 4,219 nt (4,463 and 4,308 nt with the partial UTRs, respectively). Overall, the 3 CBuV strains displayed 99.8%–99.9% nt identity to each other, with only 1 nonsilent mutation in the NS1 protein and 1 nonsilent mutation in the VP2 protein.

Upon phylogenetic analysis (Figure 2), the CBuV strains segregated into a well-defined group (bootstrap value 100), encompassing parvoviruses identified in rats, bats, pigs, and primates. The closest relatives within this group were parvoviruses from humans, commonly called human bufaviruses (*16,17*), and parvoviruses detected in monkeys (*Macaca mulatta*) (*18*) and pigs (*19*).

### Screening in PCR of Canine Samples

#### Respiratory Samples

Molecular screening by qPCR detected CBuV DNA in 10/58 (17.2%) oropharyngeal swabs and 15/58 (25.8%) nasal swabs from collection RIS. In total, 31% (18/58) of the animals tested positive for CBuV in either the pharyngeal or nasal sample, whereas 12.1% (7/58) were positive in both the oral and nasal swab sample. We did not detect CBuV DNA in the NOP samples of the control group (0/90) (collection RIA). The viral loads of collection RIS ranged from $4.91 \times 10^1$ to $8.78 \times 10^3$ DNA copies/10 μL of template (mean $8.73 \times 10^2$ DNA copies; median $2.91 \times 10^2$ DNA copies). We found a statistically significant difference for CBuV prevalence in oropharyngeal (odds ratio [OR] 6, 95% CI 1.6–23) and nasal (OR 10.1, 95% CI 2.7–36.8) swab specimens when we compared animals with and without clinical signs ($p<0.05$ for both comparisons).

We reanalyzed the results by age-based cohorts of animals (0–6 and 7–12 months). In the 0–6-month group, only 1/17 (5.8%) animals were positive; 17/41 (41.4%) tested positive in the 7–12-month group. However, this difference was not statistically significant ($p>0.05$).

We also screened the NOP swabs of the litter infected by strain ITA/2011/297-15 in qPCR. We detected CBuV DNA in all 3 puppies, with viral titers as high as $2.79 \times 10^2$, $1.01 \times 10^3$ DNA, and $3.77 \times 10^3$ copies/10 μL of template.

#### Enteric Samples

Molecular screening by qPCR revealed CBuV DNA in 26/81 (32.1%) stools or rectal swabs from collection EIS

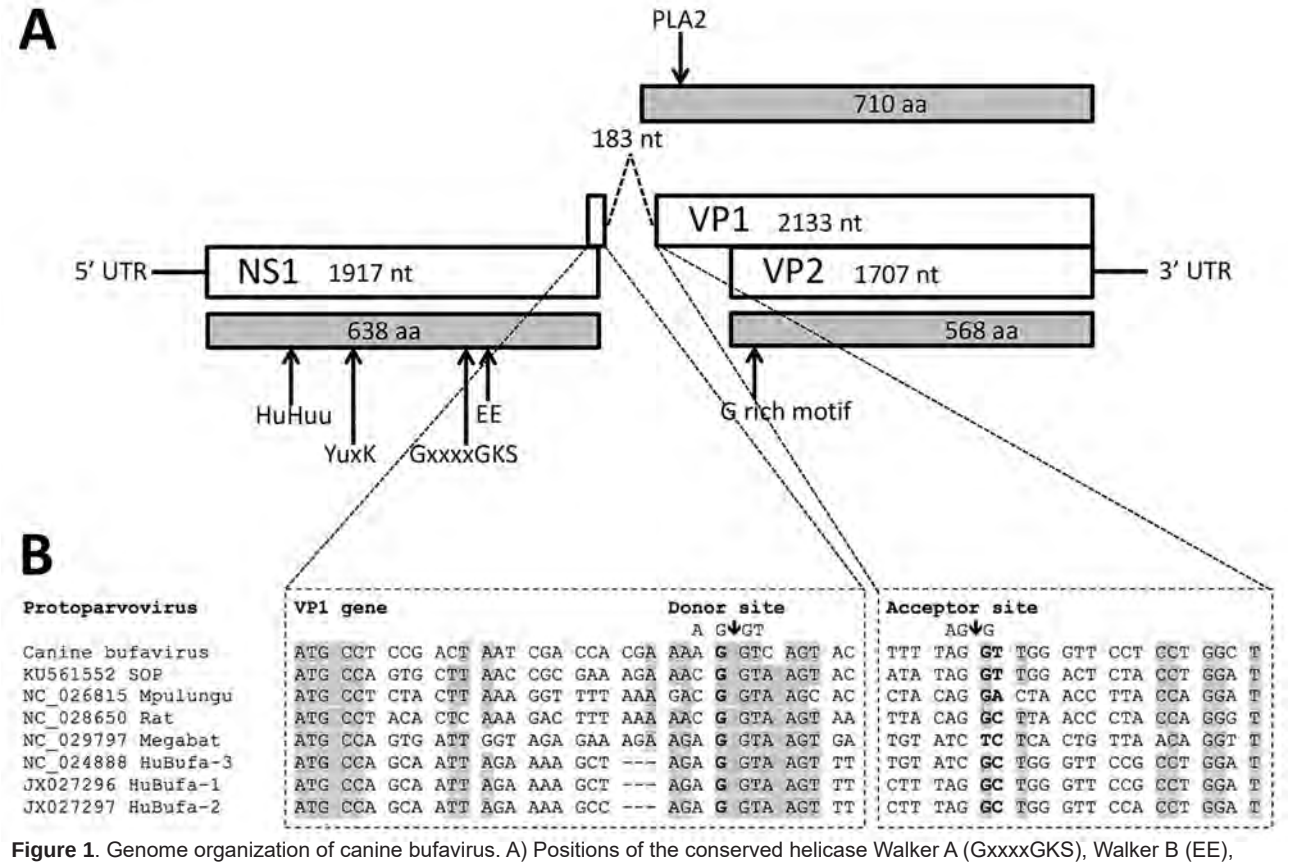


**Figure 1**. Genome organization of canine bufavirus. A) Positions of the conserved helicase Walker A (GxxxxGKS), Walker B (EE), and replication initiator motifs (HuHuu and YuxK) in NS1 and of the phospholipase A2 (PLA2) and glycine-rich region (G-rich) in VP1 and VP2. B) Putative splicing mechanism in the VP1 gene of canine bufavirus, human bufaviruses, and other protoparvoviruses. Two potential splice sites are a potential donor site (AG↓GT) at nt 1931 and an acceptor site (AG↓G) at nt 2115. The putative VP1 sequence starts with ATG at the end of ORF1 at nt 1906 upstream of the splice donor site at nt 1931. Gray shading indicates strictly and highly conserved bases. GenBank accession numbers are provided for reference sequences. NS, nonstructural; UTR, untranslated region; VP, viral capsid protein.

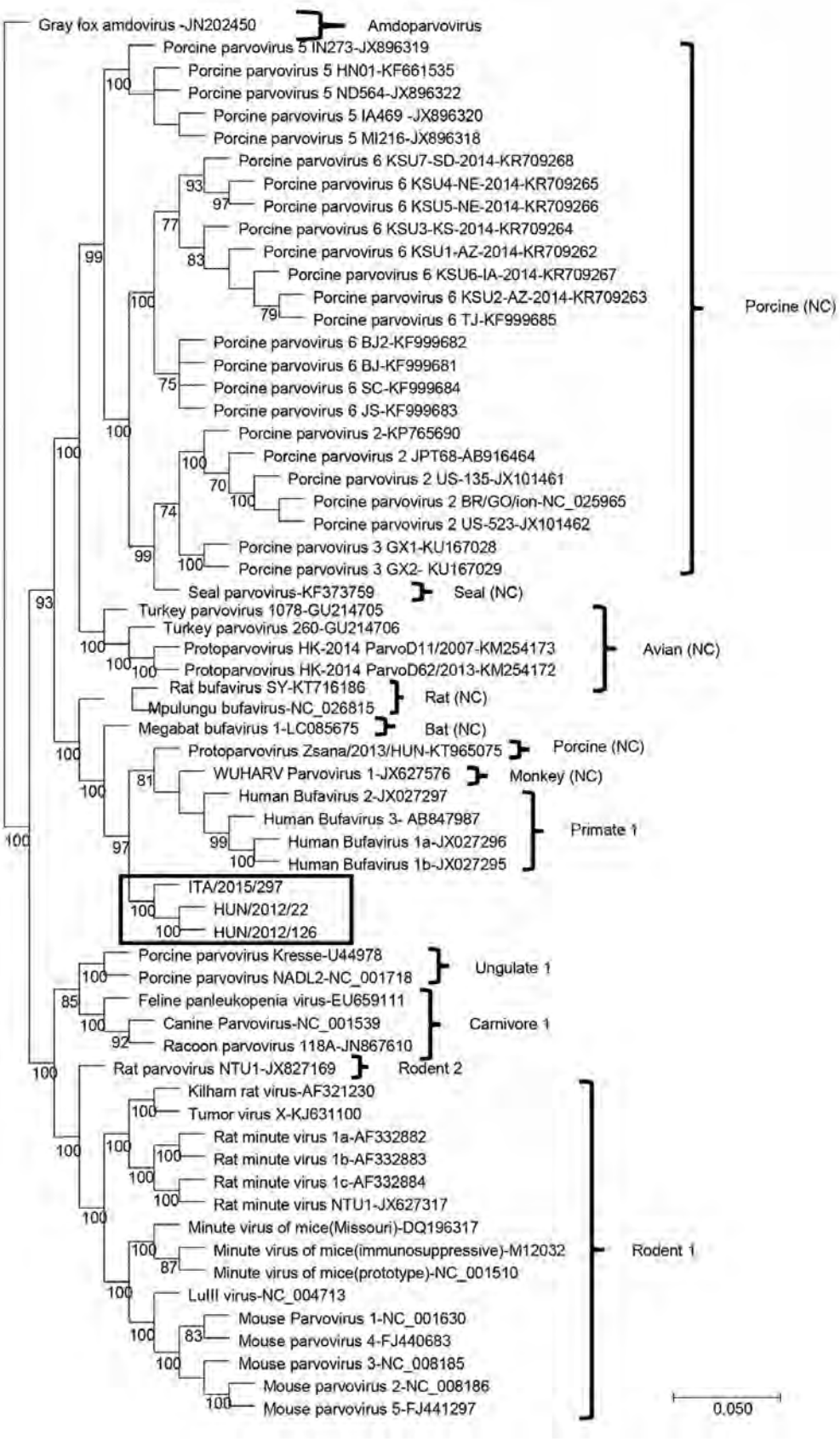


**Figure 2**. Capsid-based phylogenetic tree displaying the diversity of protoparvoviruses. The protoparvoviruses officially recognized by the International Committee on Taxonomy of Viruses are included, along with nonclassified (NC) protoparvoviruses. The tree was generated using the neighbor-joining method with the Jukes-Cantor algorithm of distance correction, with bootstrapping over 1,000 replicates. Box indicates canine bufavirus strains. GenBank accession numbers are provided for reference isolates; gray fox amdovirus (GenBank accession no. JN202450) is used as outgroup. Scale bar indicates nucleotide substitutions per site.

and 15/78 (19.2%) samples of collection EIA. The viral loads of collection EIS ranged from $4.91 \times 10^1$ to $8.78 \times 10^3$ DNA copies/10 μL of template (mean $4.21 \times 10^2$ DNA copies; median $1.38 \times 10^2$ DNA copies). The viral loads of collection EIA ranged from $5.10 \times 10^1$ to $4.34 \times 10^2$ DNA copies per 10 μL of template (mean $1.76 \times 10^2$ DNA copies; median $1.48 \times 10^2$ DNA copies). We found no statistically significant difference between enteric signs and the presence of CBuV DNA in stools or rectal swabs.

We detected CBuV DNA in 8/20 (40%) enteric samples from collection EHS and 19/40 (47.5%) samples from collection EHA. The viral loads of collection EHS ranged from $1.31 \times 10^1$ to $5.42 \times 10^3$ DNA copies/10 μL of template (mean $6.55 \times 10^2$ DNA copies; median $3.99 \times 10^1$ DNA copies). The viral loads of collection EHA ranged from $1.21 \times 10^1$ to $2.57 \times 10^{10}$ DNA copies/10 μL of template (mean $1.35 \times 10^9$ DNA copies; median $2.92 \times 10^2$ DNA copies). We found no statistically significant difference between enteric signs and the presence of CBuV DNA in the samples from Hungary.

We subjected samples containing genome copies $>10^3$ DNA copies/10 μL of template to PCR amplification with primers CPPV 165F and CPPV 371R, which amplify a 207-nt fragment of VP2. We successfully sequenced 12 samples, yielding amplicons of the expected size in PCR and confirming the specificity of the qPCR.

#### Virus Cultivation

We visually inspected the inoculated monolayers of A-72, MDCK, and WRCC cells after 5 serial passages and monitored virus titer in cellular supernatant by qPCR. We did not observe viral growth in any of the cells.

## Discussion

We report the detection and genomic characterization of CBuV, a novel canine protoparvovirus, from a small outbreak of CIRD. In the NS1 gene, CBuV displayed low nt (24.1%–69.4%) and aa (19.3%–51.4%) identities compared with other protoparvoviruses. Current International Committee on Taxonomy of Viruses criteria for classification of parvoviruses into the same species require >85% aa identity in the NS1 protein; on this basis, CBuV could be classified as a new parvovirus species. The closest relatives to CBuV were protoparvoviruses identified in primates and other mammals (*16*–*19*), commonly termed as bufaviruses. Bufaviruses were first identified in 2012 in Burkina Faso in fecal samples from a child with enteric signs (*16*). Similar bufaviruses have been subsequently identified in different species of domestic and wild animals (*18*–*20*). CBuV exhibited the most similarity in NS1 (66.9%–69.4% nt and 47.2%–51.4% aa) and VP1 (66%–68.2% nt and 62.5%–67.2% aa) to bufaviruses identified in primates. In addition, the genetic organization of CBuV resembled that of primate bufaviruses, with the conservation of a potential splicing mechanism regulating VP1 translation. On the other hand, CBuV was genetically more distantly related to CPV-2, showing only 56% nt and 40.6% aa identity in the NS1 and 42.6% nt and 33.4% aa identity in the VP1 between the 2 canine protoparvoviruses. Because we were not able to adapt the virus to in vitro growth in different canine cell lines, we could not assess whether there is an antigenic relationship between the 2 canine protoparvoviruses, CBuV and CPV-2, in cross-neutralization studies.

A small case–control study on samples of enteric and respiratory origin in puppies and young dogs (<1 year of age) revealed that CBuV appeared significantly more common in NOP swab samples from dogs with acute CIRD. We detected CBuV DNA in 17.2% (10/58) of oropharyngeal swab specimens and 25.8% (15/58) of nasal swab specimens from collection RIS. In total, 31% (18/58) of the animals with CIRD tested positive for CBuV, whereas the virus was not detectable in respiratory samples of 90 animals without clinical signs. When we analyzed the results of collection RIS by age-based cohorts, we observed an increased prevalence (41.4%, 17/41) in the 7- to 12-month age group, whereas only 1/17 animals (5.8%) in the 0- to 6-month age group tested positive for CBuV. We also found a high prevalence of CBuV DNA in canine stool samples, although we observed no substantial difference between dogs with enteric disease and clinically healthy dogs. These findings indicate that CBuVs are a common component of the canine fecal virome.

With certain exceptions, it has been difficult to demonstrate a clear association of many potential pathogens with CIRD in either epidemiologic studies or experimental infections. Our study could not provide conclusive evidence for a role of this novel virus in CIRD.

CIRD or kennel cough has a multiagent etiology, with >1 agent (viruses or bacteria) involved sequentially or synergistically to cause disease (*23*). Pathogens commonly associated with CIRD include CAV-2, canine parainfluenza virus, and *Bordetella bronchiseptica* (*12*,*24*,*25*). Less commonly, CHV-1 can cause respiratory disease (*26*). CAV-1 and canine distemper virus infections are also associated with respiratory disease but are usually responsible for systemic disease (*26*,*27*).

In recent years, other emerging agents have been associated with CIRD, including canine respiratory coronavirus (*27*,*28*), canine pneumovirus (*29*), nonprimate canine hepacivirus (*30*), CBoVs (*6*), *Mycoplasma cynos* (*31*), and *Streptococcus equi* subsp. *zooepidemicus* (*32*,*33*). In addition, thus far, ≥5 strains of influenza virus have been identified in dogs: the equine-derived H3N8 virus, the human-derived H1N1 virus, and the avian-like H3N2, H3N1, and H5N2 viruses (*34*,*35*).

Of interest, the titer of CBuV in the NOP samples was not high, with a median value of $2.91 \times 10^2$ DNA copies/10 μL. This low level of virus shedding in NOP secretions was difficult to interpret, and intrinsic properties of the virus, or the dynamics of virus shedding at the time of sampling, or both could account for it. For comparison, a human parvovirus associated with respiratory disease, human bocaparvovirus 1, can be shed at titers as high as $3.9 \times 10^{11}$ copies/mL in NOP samples (*36*). It is worth noting that we also found high loads of CBuV ($\leq 2.57 \times 10^{10}$ DNA copies/10 μL of template) in 4 fecal samples that substantially exceeded the median value ($2.92 \times 10^2$ DNA copies/10 μL) of collection EHA.

To date, scientists have searched for bufaviruses almost exclusively in fecal samples and have detected them in diarrheal stools of patients of all ages worldwide. The prevalence of bufaviruses in human patients ranges from 0.3% to 4%, and their etiologic role in enteric or extraenteric diseases remains uncertain (*16*,*17*,*37*–*41*). Bufaviruses have also been found in other mammalian hosts, including wild and captive nonhuman primates, swine, shrews, rats, bats, and fur seals (*18*–*20*,*42*–*46*). Of interest, bufaviruses have also been detected in the serum and spleens of monkeys and in the spleens of shrews (*18*,*44*) and in a unique NOP sample of 955 human patients with lower respiratory tract signs (*47*), suggesting the possibility of extraintestinal or systemic infections. In this study, we have also reconstructed the genome sequence of 2 CBuV strains detected in canine fecal samples. By genome comparison, we observed only 2 aa differences between the CBuV strains of respiratory and enteric origin, although the viruses were identified from animals of different geographic origin (i.e., Italy and Hungary). Although our findings corroborate earlier evidence that bufaviruses can target extraintestinal tissues and organs, only animal experiments or detailed observational studies can fully address this issue.

In conclusion, the advancement of techniques available for pathogen discovery is quickly broadening the list of potential canine infectious agents. Understanding in more depth the effects of those agents on canine health will be pivotal to implementing future strategies for prophylaxis, chiefly for complex diseases like CIRD.

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# Novel Poxvirus in Proliferative Lesions of Wild Rodents in East Central Texas, USA

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Northern pygmy mice from 2 localities in east central Texas, USA, had proliferative epidermal lesions on the tail and feet. Electron microscopy of lesion tissue revealed poxvirus. Phylogenetic analyses indicated the virus differed 35% from its closest relatives, the *Chordopoxvirinae*. Future research is needed to determine whether this virus could affect human health.

*Chordopoxvirinae* is a diverse subfamily of viruses within *Poxviridae*. These geographically widespread viruses infect birds, reptiles, and mammals, and many are zoonotic (*1*). The increasing use of molecular methods has resulted in the identification of several novel poxviruses from humans and animals, many of which probably represent new genera (*2*–*9*); often, the reported host range of these viruses is limited to the species of the index case. Novel human poxvirus infections identified in the 21st century have often been presumed to have animal origins; for example, an investigation of a novel poxvirus isolated from 2 men in the country of Georgia revealed serologic evidence of orthopoxvirus exposure in cows in their herd and in captured rodents (*5*). We report a novel poxvirus infection characterized by proliferative epidermal lesions in wild northern pygmy mice (*Baiomys taylori*) found at 2 localities in east central Texas, USA, and further characterize the virus through genetic analysis.

## The Study

In August 2014, we captured a severely affected adult male *B. taylori* mouse (mouse 1) at the Attwater Prairie Chicken National Wildlife Refuge in Colorado County, Texas. The mouse had large (4–8-mm diameter) proliferative lesions on the hind feet and tail (Figure 1, panel A) but otherwise appeared healthy. In April 2017, at the Biodiversity Research and Teaching Collections at Texas A&M University in College Station, Texas (160 km north of the first locality), we captured an additional adult male *B. taylori* mouse (mouse 2) with mild 1–2-mm proliferative lesions on the left hind foot and tail. Both animals were euthanized in accordance with Texas Parks and Wildlife Department scientific collections permit (SPR-0512-917) and Texas A&M University Institutional Animal Care and Use Committee's animal use protocol (2015-0088). These 2 *B. taylori* specimens are housed at Biodiversity Research and Teaching Collections (mammal voucher nos. TCWC 65223 and TCWC 65224; http://portal.vertnet.org/search).

We subjected mouse 1 to a full necropsy and found the mouse to be in good body condition. Extending from the skin of the dorsal aspect of the left hind foot, plantar aspect of the right hind foot, and the dorsal tail were several firm, pedunculated, irregular masses 0.4–0.8 cm in diameter (Figure 1, panel A). When the masses were sectioned, the cut surface was light tan with a papillated appearance, and the masses did not appear to invade the underlying tissues. The tail was partially amputated but was healed and apparently unrelated to the lesions. We did not observe any other lesions. We froze a section of a mass at −80°C for molecular work and fixed the remaining tissues in 10% neutral-buffered formalin; the fixed tissues were processed for histology and stained with hematoxylin and eosin.

On microscopic examination, necrotizing and proliferative dermatitis was observed. The epidermis of the affected area had multiple large, exophytic, pedunculated masses composed of markedly hyperplastic epithelial cells forming papillary projections with abundant orthokeratotic and parakeratotic hyperkeratosis (Figure 1, panel B). The epithelial surface was multifocally eroded to ulcerated, and the stratum corneum contained aggregates of coccoid bacteria. The stratum spinosum and stratum granulosum were markedly thickened with swollen keratinocytes (ballooning degeneration) frequently containing intracytoplasmic eosinophilic viral inclusions (Figure 1, panels B, C). These inclusions frequently extended extracellularly, forming large lakes ≤50 μm in diameter. The dermis and stratum basale were infiltrated by lymphocytes, plasma cells, and macrophages and a large number of viable and degenerate neutrophils. We examined sections of the spleen, liver, lungs,

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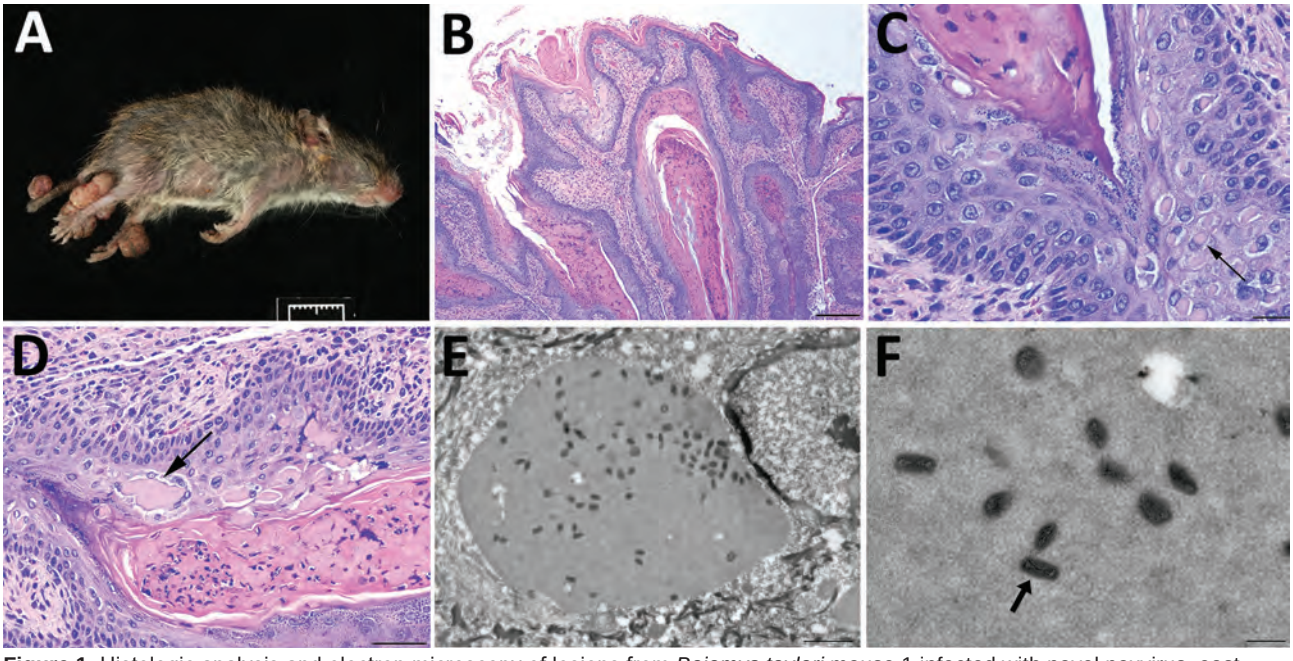


**Figure 1.** Histologic analysis and electron microscopy of lesions from *Baiomys taylori* mouse 1 infected with novel poxvirus, east central Texas, USA, 2014. A) Large epidermal masses arose from skin of both hind limbs and tail. B–D) Skin mass. Hematoxylin and eosin stain. B) The proliferative epidermis forms papillary projections with abundant hyperkeratosis and thickening of the stratum spinosum and stratum granulosum. Scale bar indicates 200 µm. C, D) Keratinocytes contain intracytoplasmic eosinophilic viral inclusions (C, arrow) that occasionally form extracellular lakes (D, arrow). Panel C scale bar indicates 20 µm; panel D scale bar indicates 50 µm. E, F) Electron microscopy. E) Cytoplasmic inclusions containing granular, electron-dense material and numerous brick-shaped virions. Scale bar indicates 1.5 µm. F) Virions have dense cores and shells of inner and outer membranes consistent with poxvirus (arrow). Scale bar indicates 300 nm.

heart, kidneys, and intestines and identified no substantial lesions. A viral etiology was suspected, so formalin-fixed paraffin-embedded sections of the mass were processed for transmission electron microscopy. Ultra-thin sections were examined with a Morgagni 268 transmission electron microscope (FEI, Hillsboro, OR, USA) at an accelerating voltage of 80 kV. Cytoplasmic inclusions contained granular, electron-dense material and numerous brick-shaped virions with dense cores and inner and outer membrane shell consistent with poxvirus (Figure 1, panel D).

We extracted DNA from skin masses of both mouse 1 and mouse 2 and subjected the DNA to PCR with low–GC content poxvirus primers targeting a region of the putative metalloproteinase gene (*10*) and Sanger sequencing. The virus sequences (220 bp) from mouse 1 (BtTX2014) and mouse 2 (BtTX2017) were identical to each other (GenBank accession no. MG367479), and the top 10 matches to this sequence in GenBank were all poxviruses with only 76%–78% shared identity. In addition, we extracted DNA from formalin-fixed paraffin-embedded sections of liver, lung, kidney, and spleen from mouse 1, and all were PCR negative for poxvirus DNA. A DNA aliquot of the mass from mouse 1 was sent to Otogenetics (Atlanta, GA, USA) for whole-genome sequencing with an Illumina HiSeq platform (Illumina Inc., San Diego, CA, USA). From these data, we extracted 9 core genes of BtTX2014 located within the conserved coding portion of the poxvirus genome used in previous studies (*5*) (GenBank accession nos. MG367480–8). In Geneious version 8.1.4 (https://www.geneious.com/), we converted the gene sequences into amino acid sequences for the purpose of alignment to those of 49 other chordopoxviruses (*Chordopoxvirinae*) and 2 entomopoxviruses (*Entomopxvirinae*), which were used as outgroup taxa. We then concatenated the gene sequences (total alignment 27,674 bp) and conducted partitioned phylogenetic analyses using Bayesian approaches in MrBayes version 3.2.2 (http://mrbayes.sourceforge.net/download.php). The phylogenetic position of this virus revealed it to be a divergent member of the *Chordopoxvirinae* (Figure 2), divergent from other *Chordopoxvirinae* poxviruses by an average of 35% (uncorrected p distances for the 27,674 bp of the concatenated alignment; online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/6/17-2057-Techapp1.pdf).

## Conclusions

The poxvirus sequenced from *B. taylori* in east central Texas is distinct from previously identified viruses, with

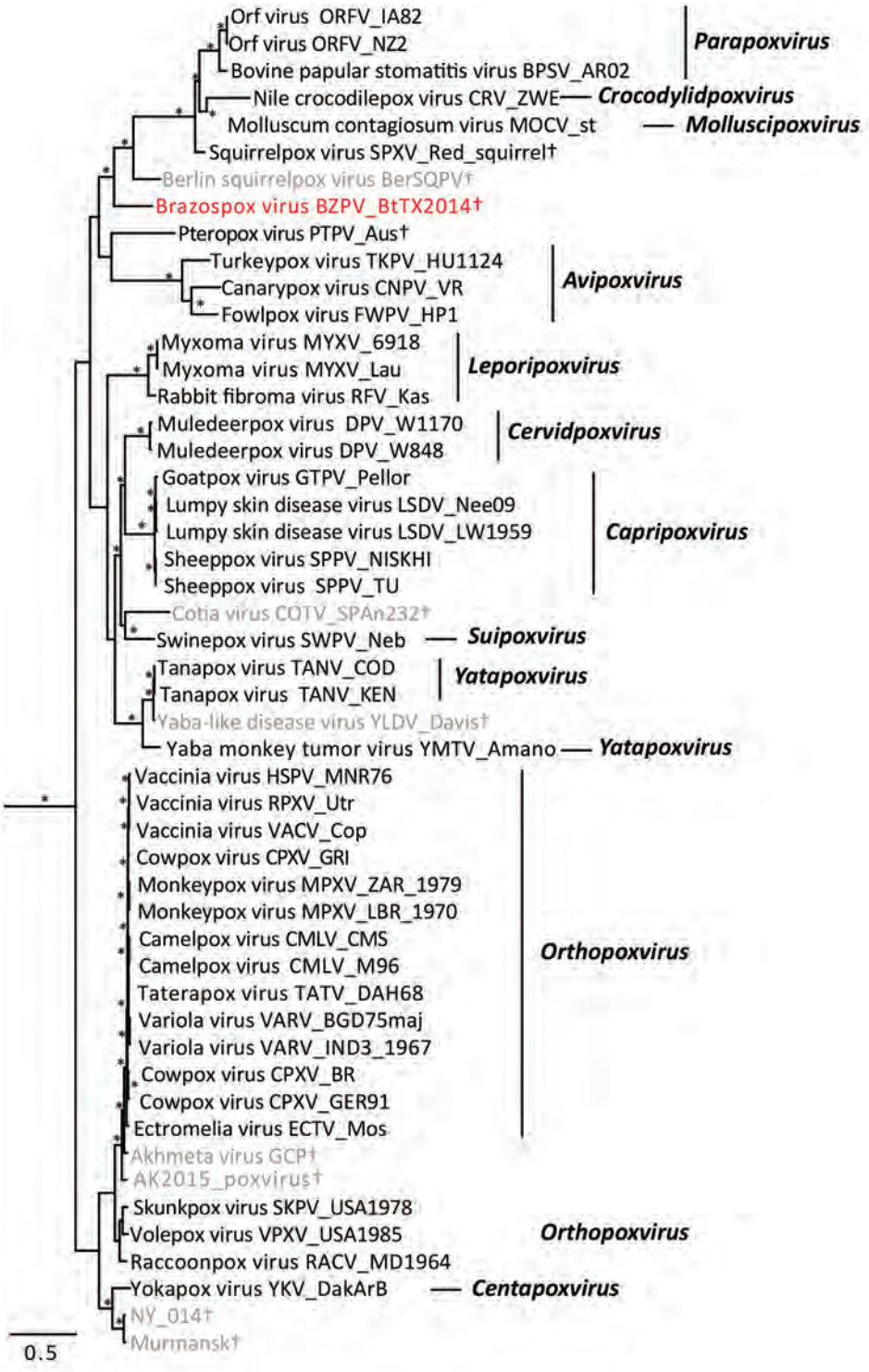


**Figure 2.** Bayesian phylogram of poxvirus isolates constructed by using a concatenated 27,674-bp alignment of 9 conserved open reading frames (Copenhagen homologs A7L, A10L, A24R, D1R, D5R, E6R, E9L, H4L, and J6R). Genera (if assigned) are listed. Brazospox virus (red; GenBank accession nos. MG367480–8) and sequences from other species not recognized by the International Committee on Taxonomy of Viruses (gray) are indicated. *Nodes with posterior probabilities >0.95; †species not assigned to a genus. Scale bar corresponds to the number of nucleotide substitutions per site.

genetic distances similar to those observed between genera (online Technical Appendix Table). Although support for several phylogenetic relationships is low (possibly due to high genetic variation within *Chordopoxvirinae*), the genetic data strongly suggest this poxvirus does not belong to any recognized genus as of March 2018. We propose the tentative species name Brazospox virus in reference to the proximity of both field sites to the Brazos River.

The epidermal lesions produced by the virus are unique among previously described poxviruses of wild rodents, being proliferative rather than ulcerative in nature, and lacking systemic involvement (*7*,*11*). The

population-level implications of this poxvirus on hosts are unclear. The 2 mice in this study represent the spectrum of observed pathology, from severe (mouse 1) to mild (mouse 2). The virus was confirmed at 2 different localities, and field notes indicate similar lesions were observed in other rodent species. During 2013–2017 (*12*,*13*), among ≈1,800 rodents captured during field research in east central Texas, we documented proliferative lesions on the tail or feet of ≥17 individual rodents of 3 species (*B. taylori*, n = 12; *Chaetodipus hispidus*, n = 2; *Sigmodon hispidus*, n = 3). The combined distributional range of these 3 host species includes ≥20 US states and a large portion of Mexico (*14*).

Novel poxviruses identified in wildlife populations might be useful for the identification of threats to human and animal health. The description of these new viruses contributes to the study of viral diversity and pathogenesis. Propagation of the Brazospox virus in cell culture for use in infection studies, coupled with expanded field surveillance, examination of museum specimens, and full genome analysis could yield additional clues to the origin, pathogenesis, and potential host range of this novel poxvirus.

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# Foot-and-Mouth Disease in the Middle East Caused by an A/ASIA/G-VII Virus Lineage, 2015–2016

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Phylogenetic analyses of foot-and-mouth disease type A viruses in the Middle East during 2015–2016 identified viruses belonging to the A/ASIA/G-VII lineage, which originated in the Indian subcontinent. Changes in a critical antigenic site within capsid viral protein 1 suggest possible evolutionary pressure caused by an intensive vaccination program.

Foot-and-mouth disease (FMD) can decrease productivity in the cloven-hooved livestock industry. As this disease spreads rapidly over large distances, it is regarded as one of the most economically devastating diseases of livestock. FMD is caused by FMD virus (FMDV; family *Picornaviridae*, genus *Aphthovirus*), which has 7 immunologically distinct serotypes, O, A, C, Asia 1, SAT 1, SAT 2, and SAT 3. Worldwide, ecologic niches of FMDV circulation have been defined as 7 virus pools. Pools 1–3 are present in Asia, where only serotypes O, A, and Asia 1 are present.

Serotype A viruses are considered to be the most variable (genetically and antigenically) of Eurasian serotypes. Three topotypes (ASIA, AFRICA, and EURO-SA [Europe–South America]) and multiple diverse lineages and sublineages have been identified (*1*). The ASIA topotype is widespread and is found in most countries in Asia; there have been sporadic incursions into North Africa. Although the G-VII lineage (also known as genotype 18) (*2*,*3*) usually circulates in countries containing virus pool 2 (commonly in Bangladesh and India, rarely in Bhutan and Nepal, but until now not in Sri Lanka), this lineage has also been reported in Saudi Arabia in 1995, Albania and the former Yugoslav Republic of Macedonia in 1996, and Myanmar in 2010.

In India, viruses of the A/ASIA/G-VII lineage were isolated in 1983 (*4*) and until 2001 were co-circulating with the A/ASIA/G-VI lineage (*2*). After 2001, only the G-VII lineage has been reported (*5*). Overall incidence of FMD outbreaks caused by serotype A in India during 2011–2016 was low (3.1% of the total reported outbreaks). During the same time, 86.8% of outbreaks were caused by serotype O, and 10.1% by serotype Asia 1 (*6*).

Despite the low number of outbreaks investigated, emergence of a capsid viral protein (VP) 3 deletion variant, $VP^{59}$, was reported in the A/ASIA/G-VII lineage during 2002 (*4*). Currently, a group of viruses described as clade C, a subgroup within the $VP3^{59}$-deletion variant, is speculated to be the dominant group of the A/ASIA/G-VII lineage prevalent in India (*2*). We report foot-and-mouth disease in the Middle East during 2015–2016 caused by an A/ASIA/G-VII virus lineage.

## The Study

During outbreak investigations of FMD in Saudi Arabia in 2015, a virus of the A/ASIA/G-VII lineage ($VP3^{59}$-deletion variant), was identified in cattle (*7*). The outbreak spread quickly to several strictly monitored dairy farms that had high rates of vaccination, as well as to nomadic herds. Concurrently, related viruses were found in Armenia, Iran, and Turkey in 2015 and continued to circulate in Saudi Arabia, Turkey, and Iran in 2016. Sequences of VP1-coding regions from samples submitted to the Food and Agriculture Organization of the United Nations World Reference Laboratory for FMD (Pirbright, UK) were determined by using described methods (*8*). VP1 sequences from outbreaks in Armenia, Bangladesh, and Turkey were determined at the Federal Centre for Animal Health (Vladimir, Russia); the University of Dhaka (Dhaka, Bangladesh); and the Foot-and-Mouth Disease Institute (Ankara, Turkey), respectively (Table).

We performed maximum-likelihood analyses to compare VP1 coding sequences with other contemporary sequences of the A/ASIA/G-VII lineage and grouped them within the $VP3^{59}$-deleted C clade (*2*) (Figure 1,

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panel A). We estimated time-resolved phylogenetic trees for 101 serotype A FMDV G-VII VP1 sequences by using BEAST version 1.8.4 (*9*) and incorporated the general time-reversible model with gamma-distributed rate variation among sites and 0.5 prior proportion of invariant sites, the Bayesian Skyline tree before accounting for demographic uncertainty, and a log-normal uncorrelated relaxed clock across branches (*10*). We ran Markov Chain Monte Carlo analysis for 200 million steps and sampled trees every 20,000 steps after a

**Table.** Characteristics of 57 strains **of** foot-and-mouth disease viruses used in analysis of foot-and-mouth disease caused by an A/ASIA/G-VII virus lineage, Middle East, 2015–2016

| Virus designation | Country | Location | Species | Date collected | GenBank accession no. |
|---|---|---|---|---|---|
| A/ARM/1/2015* | Armenia | Armavir, Arazap | Cattle | 2015 Dec 25 | KY982279 |
| A/ARM/2/2015* | Armenia | Armavir, Arazap | Cattle | 2015 Dec 25 | KY982280 |
| A/ARM/3/2015* | Armenia | Armavir, Arazap | Cattle | 2015 Dec 25 | KY982281 |
| BAN/CH/Sa-304/2016 | Bangladesh | Chittagong | Cattle | 2016 Sep 27 | KY077630 |
| A/IRN/8/2015 | Iran | Qom | Cattle | 2015 Aug 30 | KY982282 |
| A/IRN/12/2015 | Iran | Qom | Cattle | 2015 Sep 9 | KY982283 |
| A/IRN/13/2015 | Iran | Qom | Cattle | 2015 Sep 28 | KY982284 |
| A/IRN/14/2015 | Iran | Qom | Cattle | 2015 Sep 28 | KY982285 |
| A/IRN/17/2015 | Iran | Qom | Cattle | 2015 Oct 9 | KY982286 |
| A/IRN/18/2015 | Iran | Tehran | Cattle | 2015 Oct 10 | KY982287 |
| A/IRN/21/2015 | Iran | Qom | Cattle | 2015 Oct 24 | KY982288 |
| A/IRN/22/2015 | Iran | Tehran | Cattle | 2015 Oct 24 | KY982289 |
| A/IRN/25/2015 | Iran | East Azerbaijan | Cattle | 2015 Oct 28 | KY982290 |
| A/IRN/27/2015 | Iran | Kermanshah | Cattle | 2015 Nov 8 | KY982291 |
| A/IRN/1/2016 | Iran | Qom | Cattle | 2016 Jan 4 | KY982292 |
| A/IRN/8/2016 | Iran | Tehran | Cattle | 2016 Feb 4 | KY982293 |
| A/IRN/11/2016 | Iran | Qazvin | Cattle | 2016 Feb 27 | KY982294 |
| A/IRN/12/2016 | Iran | Ardebil | Cattle | 2016 Feb 29 | KY982295 |
| A/IRN/20/2016 | Iran | Yazd | Cattle | 2016 Feb 4 | KY982296 |
| A/IRN/23/2016 | Iran | Alborz | Cattle | 2016 Apr 7 | KY982297 |
| A/SAU/1/2015 | Saudi Arabia | Farm A, Durma | Cattle | 2015 Sep 2 | KU127247 |
| A/SAU/2/2015 | Saudi Arabia | Farm A, Durma | Cattle | 2015 Sep 2 | KY982298 |
| A/SAU/3/2015 | Saudi Arabia | Farm B, Al Kharj | Cattle | 2015 Oct 9 | KY982299 |
| A/SAU/4/2015 | Saudi Arabia | Farm B, Al Kharj | Cattle | 2015 Oct 19 | KY982300 |
| A/SAU/5/2015 | Saudi Arabia | Farm C, Al Kharj | Cattle | 2015 Oct 16 | KY982301 |
| A/SAU/6/2015 | Saudi Arabia | Farm A, Durma | Cattle | 2015 Oct 21 | KY982302 |
| A/SAU/7/2015 | Saudi Arabia | Farm D, Al Kharj | Cattle | 2015 Oct 23 | KY982303 |
| A/SAU/8/2015 | Saudi Arabia | Farm D, Al Kharj | Cattle | 2015 Dec 30 | KY982304 |
| A/SAU/9/2015 | Saudi Arabia | Al Kharj | Cattle | 2015 Oct 5 | KY982305 |
| A/SAU/14/2015 | Saudi Arabia | Al Kharj | Sheep | 2015 Oct 26 | KY982306 |
| A/SAU/15/2015 | Saudi Arabia | Al Kharj | Sheep | 2015 Oct 26 | KY982307 |
| A/SAU/16/2015 | Saudi Arabia | Al Kharj | Sheep | 2015 Oct 26 | KY982308 |
| A/SAU/17/2015 | Saudi Arabia | Al Kharj | Sheep | 2015 Oct 26 | KY982309 |
| A/SAU/21/2015 | Saudi Arabia | Al Kharj | Cattle | 2015 Dec 22 | KY982310 |
| A/SAU/15/2016 | Saudi Arabia | Farm D, Al Kharj | Cattle | 2016 Mar 27 | KY982311 |
| A/SAU/19/2016 | Saudi Arabia | Farm C, Al Kharj | Cattle | 2016 Oct 14 | KY982312 |
| A/SAU/20/2016 | Saudi Arabia | Farm C, Al Kharj | Cattle | 2016 Oct 14 | KY982313 |
| A/SAU/21/2016 | Saudi Arabia | Mekkah | Cattle | 2016 Oct 19 | KY982314 |
| A/SAU/22/2016 | Saudi Arabia | Mekkah | Cattle | 2016 Oct 19 | KY982315 |
| A/SAU/24/2016 | Saudi Arabia | Mekkah | Cattle | 2016 Oct 19 | KY982316 |
| A/SAU/37/2016 | Saudi Arabia | Al Kharj | Cattle | 2016 Dec 29 | KY982317 |
| A/SAU/40/2016 | Saudi Arabia | Al Kharj | Cattle | 2016 Dec 29 | KY982318 |
| A/SAU/41/2016 | Saudi Arabia | Al Kharj | Cattle | 2016 Dec 29 | KY982319 |
| A/SAU/42/2016 | Saudi Arabia | Al Kharj | Cattle | 2016 Dec 29 | KY982320 |
| A/TUR/175/2015.712* | Turkey | Van | Cattle | 2015 Sep 29 | KY982321 |
| A/TUR/198/2015.808* | Turkey | Van | Cattle | 2015 Oct 15 | KY982322 |
| A/TUR/203/2015.827* | Turkey | Van | Cattle | 2015 Oct 22 | KY982323 |
| A/TUR/219/2015.865* | Turkey | Düzce | Cattle | 2015 Nov 10 | KY982324 |
| A/TUR/305/2015.923* | Turkey | Yozgat | Cattle | 2015 Nov 24 | KY982325 |
| A/TUR/331/2015.923* | Turkey | Kütahya | Cattle | 2015 Nov 27 | KY982326 |
| A/TUR/48/2016.019* | Turkey | Iğdir | Sheep | 2016 Jan 8 | KY982327 |
| A/TUR/1008/2016.500* | Turkey | Muş | Cattle | 2016 Jun 29 | KY982328 |
| A/TUR/1193/2016.731* | Turkey | Kastamonu | Cattle | 2016 Sep 21 | KY982329 |
| A/TUR/1210/2016.750* | Turkey | Kars | Cattle | 2016 Sep 26 | KY982330 |
| A/TUR/1218/2016.769* | Turkey | Tokat | Cattle | 2016 Oct 3 | KY982331 |
| A/TUR/1225/2016.769* | Turkey | Gümüşhane | Cattle | 2016 Oct 6 | KY982332 |
| A/TUR/1227/2016.750* | Turkey | Ardahan | Cattle | 2016 Sep 30 | KY982333 |

*Non–World Reference Laboratory for Foot-and-Mouth Disease (Pirbright, UK) reference number.

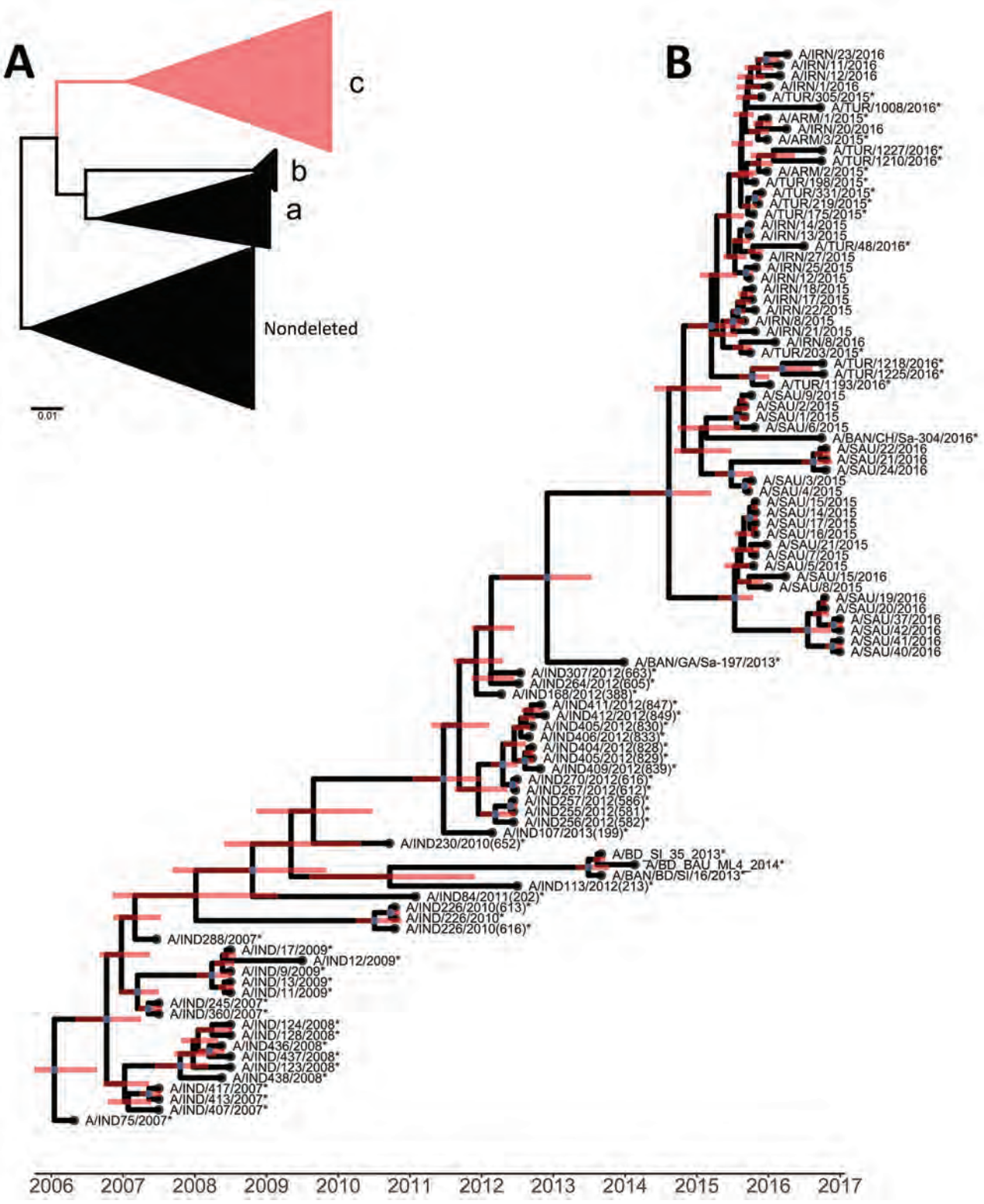


**Figure 1.** Phylogenetic analyses of viral protein 1–coding sequences of foot-and-mouth disease viruses classified within the A/ASIA/G-VII lineage (isolated during 2006–2017) and reference viruses. A) Maximum-likelihood analysis showing 4 clades (sublineages). Scale bar indicates nucleotide substitutions per site. B) Bayesian phylogenetic analysis of viruses grouping within clade C. Red lines indicate 95% high posterior density of the most recent common ancestor. *Non–World Reference Laboratory for Foot-and-Mouth Disease (Pirbright, UK) reference number. ARM, Armenia; BAN, Bangladesh; IND, India; IRN, Iran; SAU, Saudi Arabia; TUR, Turkey.

burn-in of 20 million steps. We assessed convergence and good mixing of the Markov Chain Monte Carlo C chain by using Tracer version 1.6 (http://beast.community/tracer).

VP1 coding region–based Bayesian analyses identified ≥2 independent introductions of G-VII virus into the study region, ≥1 to Saudi Arabia and 1 to Iran (Figure 1, panel B).

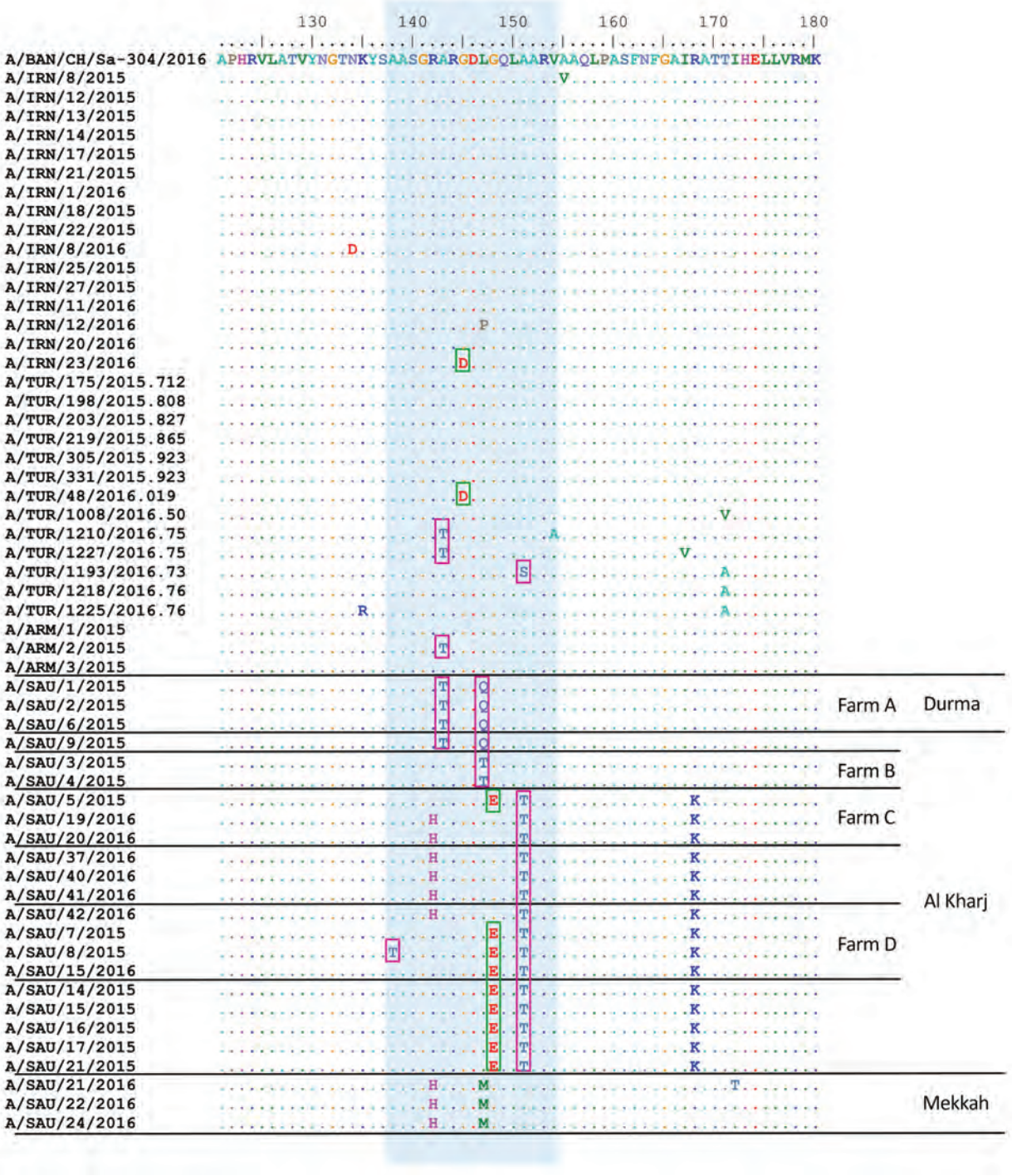


**Figure 2.** Comparison of predicted amino acid sequences of foot-and-mouth disease viruses showing changes in major antigenic sites. Predicted amino acid sequences for samples collected during outbreaks of foot-and-mouth disease during 2015–2016 were compared with A/BAN/CH/Sa-304/2016 virus sequence. Blue shading indicates conservative changes within antigenic site 1, pink boxes indicate hydrophobic to hydrophilic substitutions, and green boxes indicate hydrophobic to acidic substitutions. Dots indicate sequence identity. Amino acid residues are colored according to their physicochemical properties. Four large dairy farms (containing >1,000 lactating cows), which were multiply sampled, are indicated. ARM, Armenia; BAN, Bangladesh; IRN, Iran; SAU, Saudi Arabia; TUR, Turkey.

However, lack of sequences for recent viruses circulating in the Indian subcontinent makes it difficult to resolve more precisely the number of introductions. Closely related viruses might be circulating in a wider geographic area, thus being a source of the outbreaks. However, this speculation is not supported by available epidemiologic information.

Outbreaks in Armenia, Iran, and Turkey were closely related and most likely originated from the same source. The most recent common ancestor (MRCA) was dated to March 2015 (95% high posterior density [HPD] October 2014–July 2015). The MRCA of the C clade was dated to January 2006 (95% HPD June 2005–April 2006), which is consistent with the first isolate obtained in India during 2007 (*2*). The MRCA of the phylogenetic cluster grouping Middle East (Saudi Arabia, Iran, and Turkey) isolates was dated to July 2015 (95% HPD April–October 2015). Potential movement of G-VII FMDV lineages from the Indian subcontinent might be dated to October 2014 (95% HPD January 2014–February 2015). Evolution of the G-VII C clade lineage was estimated as having a mutation rate of $1.1 \times 10^{-2}$ nt/site/y (95% HPD $8.0 \times 10^{-3}$ to $1.4 \times 10^{-2}$ nt/site/y).

Amino acid substitutions on the FMDV surface, particularly in the G-H loop of VP1 (antigenic site 1), have been implicated in antigenic variation of the virus in vitro and in vivo (*11*). Predicted amino acid sequences obtained from samples collected during outbreaks in 2015–2016 were compared with the A/BAN/CH/Sa-304/2016 sequence (most closely related virus from the Indian subcontinent), and 8 nonconservative substitutions were identified at 6 positions (138, 143, 145, 147, 148, and 151) within the VP1 antigenic site 1 (*12*,*13*) (Figure 2; Table).

Changes from hydrophobic (alanine and leucine) to hydrophilic (threonine, glutamine, and serine) amino acid residues were most common, found at 4 positions; changes from hydrophobic (glycine) to acidic (aspartic acid and glutamic acid) amino acids were found at 2 positions. Antigenic variation at site 1 of type C viruses is often based on alternate switching between alanine and threonine residues without accumulation of amino acid substitutions (*14*). In addition, 2 independent changes that did not alter the amino acid characteristics were identified at positions 142 and 147. We also showed that changes at antigenic site 1 were conserved mainly within but differed between farms, supporting independent selection pressures. Although the same vaccine was used, the intensive and frequent vaccination regimen routinely used on the affected farms in Saudi Arabia might have led to an independent antigenic evolution on an individual farm level from chance substitutions. Nevertheless, occasional substitutions within antigenic site 1 were also observed in Armenia, Turkey, and Iran (Figure 2).

## Conclusions

As reported for the O/ME-SA/Ind-2001d virus lineage (*15*), A/ASIA/G-VII is the second FMDV lineage believed to have originated in the Indian subcontinent since 2013 and resulted in extensive outbreaks outside its usual area of distribution. Similar to the Ind-2001d outbreaks, current outbreaks caused by the G-VII lineage appear to be linked to multiple introductions of the virus from the Indian subcontinent; the virus then spread among susceptible ruminant populations in Saudi Arabia, Iran, Turkey, and Armenia.

It is a concern that in vitro vaccine matching data (by virus neutralization) provide poor confidence that commercially available vaccines would offer effective protection against the G-VII lineage (A. Ludi, pers. comm., May 2016). To improve control programs, it is crucial to identify expected routes of FMDV escape (e.g., international trade in animals and animal products) outside historically defined geographic distribution, and to establish transmission pathways within affected areas. To reconstruct likely transmission pathways at greater resolution, genome sequencing of viruses described in this report is currently in progress.

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### About the Author

Dr. Bachanek-Bankowska is a senior research scientist at the Food and Agriculture Organization of the United Nations World Reference Laboratory for FMD at The Pirbright Institute, United Kingdom. Her primary research interests are molecular epidemiology and development of molecular diagnostic methods for FMDV.

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# Novel *Salmonella enterica* Serovar Typhimurium Genotype Levels as Herald of Seasonal Salmonellosis Epidemics

**Cristina Sotomayor, Qinning Wang, Alicia Arnott, Peter Howard, Kirsty Hope, Ruiting Lan, Vitali Sintchenko**

We examined the population dynamics of *Salmonella enterica* serovar Typhimurium during seasonal salmonellosis epidemics in New South Wales, Australia, during 2009–2016. Of 15,626 isolates, 5%–20% consisted of novel genotypes. Seasons with salmonellosis epidemics were associated with a reduction in novel genotypes in the preceding winter and spring.

Nontyphoidal *Salmonella* spp. cause an estimated 93.8 million salmonellosis infections and 155,000 deaths globally each year (*1*). However, the population dynamics of human salmonellosis remain poorly understood, which can undermine the effective use of public health resources (*2*). *Salmonella enterica* serovar Typhimurium is a highly diverse serovar and the dominant cause of salmonellosis worldwide (*3*,*4*), experiencing continuous evolution, persistence, and adaptation within different ecologic niches. Whereas the complexities of the clonal structure of *Salmonella* Typhimurium populations have been recognized (*4*–*7*), the effect of temporal change in subtype diversity on disease incidence is not well understood.

Multilocus variable-number tandem-repeat (VNTR) analysis (MLVA) has been used as a high-resolution *Salmonella* typing method amenable to harmonization (*8*,*9*). In our study, we sought to determine if *Salmonella* Typhimurium subtype diversity can be used to predict incidence of human salmonellosis. We examined *Salmonella* Typhimurium isolates recovered during 2009–2016 in the comparatively low prevalence setting of New South Wales (NSW), the most populous state of Australia (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/6/17-1096-Techapp1.pdf).

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## The Study

We used MLVA to genotype all *Salmonella* Typhimurium isolates referred to the NSW Enteric Reference Laboratory at the Centre for Infectious Diseases and Microbiology, NSW Health Pathology (Sydney, NSW, Australia), during August 2009–March 2016. We conducted multiplex PCR to amplify VNTRs (STTR9, STTR5, STTR6, STTR10pl and STTR3) and subsequent analyses as described previously (*9*,*10*). We reported MLVA results as a string of 5 numbers representing relevant repeats (*11*). We used *Salmonella* Typhimurium reference strain LT2 (GenBank accession on. NC_003197) as a control throughout and consistently generated the expected MLVA type 4-13-13-10-0211. We defined a cluster as ≥2 isolates with the same MLVA type collected within 12 weeks (*10*) and used a $\chi^2$ test to determine the significance of differences observed; we considered a p value of <0.05 significant. We determined population diversity by calculating the Simpson index of diversity (*12*), and population richness using the McIntosh dominance index (*13*). This study was approved by our local Human Research Ethics Committee (LNR/17/WMED/25).

After excluding duplicate isolates from the same episode of the disease (99% of all cases recorded in NSW), we examined a total of 15,626 human *Salmonella* Typhimurium isolates. We defined seasons as spring (September–November), summer (December–February), autumn (March–May), and winter (June–August). We observed a substantial fluctuation in the number of infections during the study period (Figure 1). The relative contributions of common definitive phage types (DT) and MLVA types to local *Salmonella* Typhimurium activity varied over 8 seasonal peaks. DT135 dominated in 2008 but was subsequently replaced by DT170 as the most common type in 2009 and 2010; an increase in the activity of DT9 occurred in 2014 and 2015. In total, we observed 667 different MLVA types. Six related STM DT170 MLVA types (2-7-6-12-0212; 2-7-7-12-0212; 2-7-6-13-0212; 2-7-6-11-0212; 2-7-6-14-0212 and 2-7-7-11-0212) represented 30% of all isolates. Increases in *Salmonella* Typhimurium cases during summer and autumn months (December–May in the

Southern Hemisphere), with 2 cycles of 2009–2011 and 2012–2014, were mirrored by an increase in the number of unique MLVA types detected. The proportions isolates included in clusters also demonstrated expected seasonal fluctuations corresponding to increases in incidence (Figure 1, panel A), although we found no significant change in the number of clusters or their average size during the study period ($p<0.05$).

The average number of *Salmonella* Typhimurium cases for the 8 summer–autumn seasons investigated during the study period was 1,301. We categorized all summer–autumn seasons as either high (epidemic) or low on the basis of whether case numbers were above or below the average. We confirmed the designations by comparing the seasonal averages calculated for this study with yearly rates of salmonellosis notifications. These 2 approaches congruently assigned summer–autumn seasons of 2010, 2011, 2014, and 2015 as high or epidemic, whereas summer–autumn seasons of 2009, 2012, 2013, and 2016 were classified as low; the difference between case numbers in high and low seasons was significant ($p<0.02$) (Figure 1, panels B, C). The average annual number of foodborne community outbreaks recorded by the national public health network during high seasons was 63 and during low seasons was 48 (http://www.ozfoodnet.gov.au).

At the beginning of the study, we considered all MLVA types novel. The ratio of novel, previously unreported MLVA types to all types stabilized within 5 months; after the fifth month, 10%–40% of all MLVA types detected at any given time consisted of novel MLVA types (Figure 2). We detected no changes in the age distribution of human

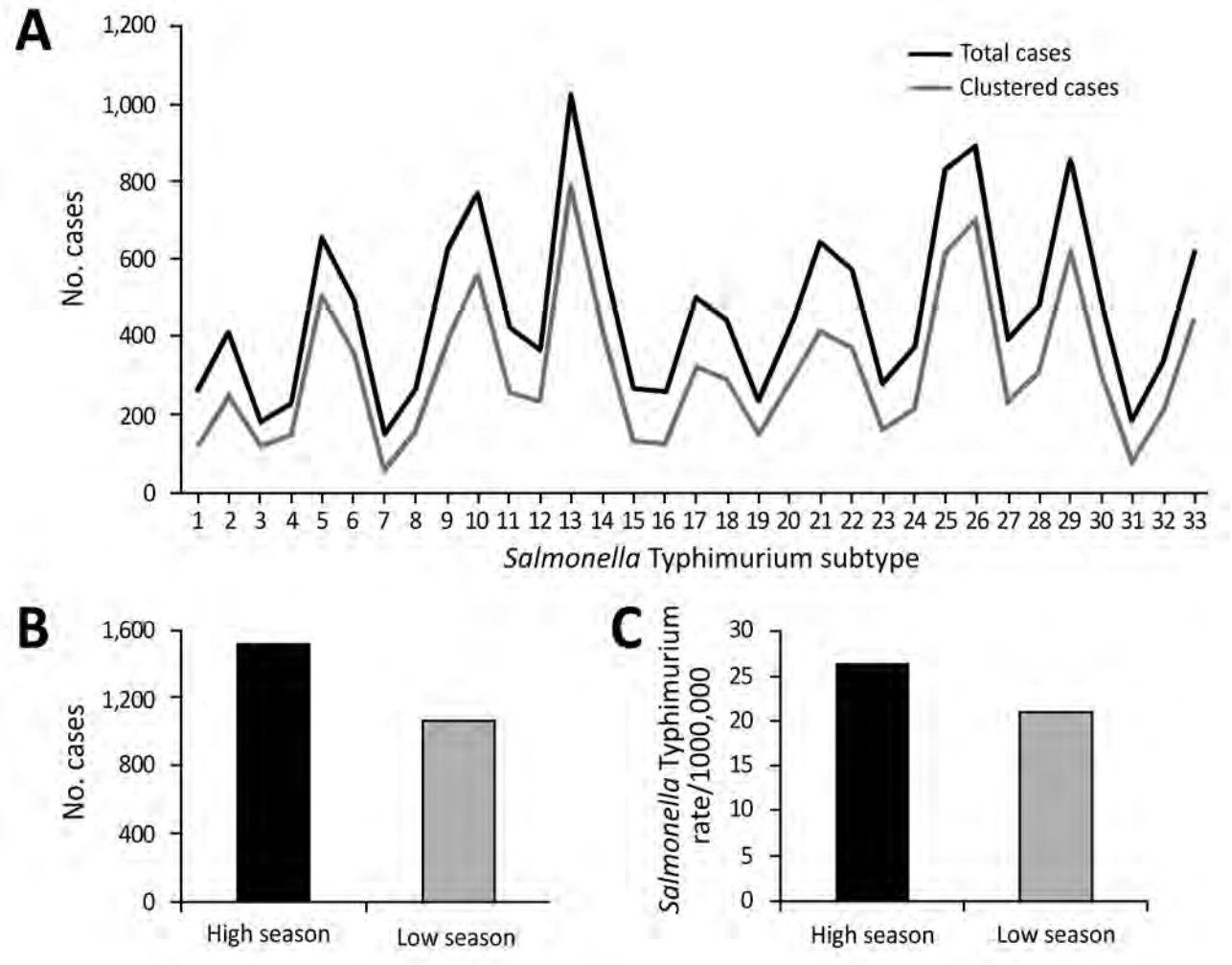


**Figure 1.** Trends of *Salmonella enterica* serovar Typhimurium notifications and multilocus variable-number tandem-repeat analysis (MLVA) patterns, New South Wales, Australia, 2009–2016. A) Quarterly counts of total cases and cases clustered by MLVA. B) Mean sum of *Salmonella* Typhimurium notifications for summer and autumn quarters for high and low seasons observed ($p = 0.01$). C) Differences in mean yearly rates of salmonellosis cases reported to the New South Wales Health Department between years corresponding to high and low seasons ($p = 0.02$). Data source: National Notifiable Diseases Surveillance System (http://www9.health.gov.au/cda/source/).

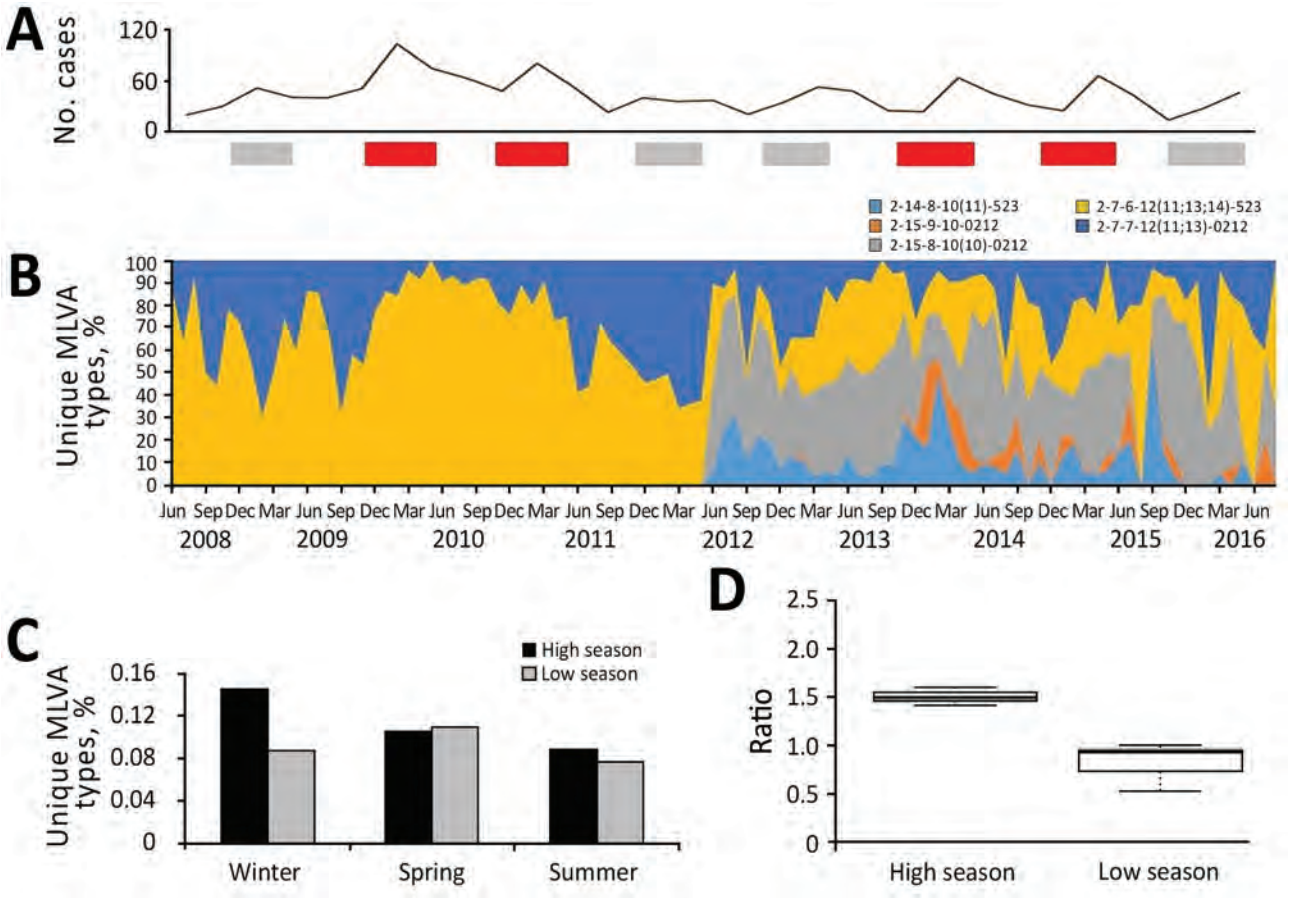


**Figure 2**. Population dynamics of *Salmonella enterica* serovar Typhimurium MLVA types, New South Wales, Australia, 2009–2016. A) Total number of novel or unique MLVA types. Red bars indicate high season and gray bars low season. B) Temporal dynamics of the most common MLVA types expressed as proportions by type. C) Quarterly counts of novel MLVA types during winter, spring, and summer for high and low seasons (p = 0.05). D) Box plots of the mean ratio of novel MLVA type counts during high and low seasons (p = 0.006). Box top and bottom indicate third and first quartiles, respectively; horizontal lines within boxes indicate medians; whiskers indicate CIs; dotted vertical lines indicate the spread of values in the subgroup. We built box plots with BoxPlotR (http://shiny.chemgrig.org). MLVA, multilocus variable-number tandem-repeat analysis.

populations affected by dominant types; 41.1% of all infections occurred in those <14 years of age.

The diversity of the *Salmonella* Typhimurium population remained relatively constant over time; the McIntosh dominance index of diversity fluctuated between 0.6 and 0.9 during both high and low seasons (p = 0.478). However, we observed a rapid decrease in the proportions of unique MLVA types from winter to spring (i.e., $U_w/U_{sp}$ ratio<1) before epidemic *Salmonella* Typhimurium activity. In contrast, the proportion of unique MLVA types increased from winter to spring preceding low seasons ($U_w/U_{sp}$ ratio>1) (Figure 2). This ratio also correlated with incidence of *Salmonella* Typhimurium in NSW over the study period (r = 0.922). Of note, the percentage of unique MLVA types recovered from patients <14 years of age during winter of high/epidemic seasons was also significantly lower than during the winter months of low seasons of salmonellosis (15.1% vs. 28.2%; p<0.001).

Our observations potentially reflect the decrease in *Salmonella* Typhimurium diversity resulting from the reduction of genome variation under selection pressure, which is associated with the emergence of successful unique clones capable of causing epidemics in immunologically naive hosts. However, longer-term monitoring of subtype diversity and disease incidence is warranted to confirm these trends. Although an analogous selection-driven reduction in genetic diversity has been observed in other pathogens, such as influenza virus (*14*), it has not previously been observed in *Salmonella*. Our findings also suggest that the timely recognition of novel *Salmonella* Typhimurium subtypes may be of significance for surveillance and that the conventional diversity indices alone may not be sufficient to detect subtle changes in circulating subtypes. As the estimated ratio of accumulation of MLVA repeats in different loci to single nucleotide polymorphisms is 1:6.9 (*15*), changes in the composition of *Salmonella* Typhimurium

subtypes might offer insight into the relevance of population diversity for fluctuations in the incidence of salmonellosis.

## Conclusions

Substantial increases in seasonal epidemics of *Salmonella* Typhimurium can be associated with a reduction in newly identified MLVA types in the preceding winter and spring, reflecting the emergence of successful *Salmonella* Typhimurium clones under selection pressure. The proportion of novel MLVA types in winter and spring may serve as an early warning sign in public health surveillance. These observations add further insights into the epidemiology of *Salmonella* Typhimurium infections in a low-incidence setting. Although they may not be readily applicable to high-incidence *Salmonella* Typhimurium settings with frequent co-infections and different diagnostic or public health practices, the epidemiology of *Salmonella* Typhimurium and public health responses in Australia are similar to those in other industrialized countries, supporting the generalizability of our findings. Prospective monitoring of *Salmonella* Typhimurium population diversity and identifying new MLVA types as reservoirs from which future epidemics might emerge can improve the assessment of risks of seasonal increase in *Salmonella* Typhimurium incidence.

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# Urban Wild Boars and Risk for Zoonotic *Streptococcus suis*, Spain

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Urban wild boars (*Sus scrofa*) from Barcelona, Spain, harbor great diversity of *Streptococcus suis* strains, including strains with the *cps*2 gene and with the same molecular profile as local human cases. The increasing trend of potential effective contacts for *S. suis* transmission is of public health concern.

Populations of the European wild boar (*Sus scrofa*) have been increasing (*1*). The wild boar's high adaptability to human-dominated landscapes and its increase in human tolerance have also prompted its presence in urban areas, leading to conflicts with local humans (*2*,*3*). Health risk assessment should therefore be performed in this new scenario, in which wild boars and their interactions with humans are becoming common in heavily populated areas (*3*).

*Streptococcus suis* is an emerging zoonotic pathogen (*4*). Among the bacterium's 35 described serotypes, serotypes 2, 5, and 14 are those most related to human disease (*4*,*5*). Several risk factors have been associated with *S. suis* infection in humans, such as consumption of undercooked pig meat or work in pig-related occupations (*5*). The wild boar has also been identified as a source of human infection in relation to hunting activities (*6*). However, the increasing presence of synanthropic wild boars may also pose a novel public health risk for nonhunters. The objective of this study was to investigate the presence of *S. suis* strains with zoonotic potential in wild boars from the metropolitan area of Barcelona, Spain, and assess the risk of transmission to humans.

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## The Study

The metropolitan area of Barcelona is heavily populated (>3.2 million) and includes Collserola Natural Park, 8,000 hectares of Mediterranean forest (Figure 1). The wild boar population in this forest is ≈1,200, and habituation to humans has become common (*2*). A total of 108 apparently healthy wild boars were sampled in the Barcelona area for the presence of *S. suis* during April–November 2015; the animals had been hunted in regular campaigns, captured by box traps and euthanized for population control, or captured with the aid of anesthetic darts and euthanized because of public risk. All procedures were performed under the regulation of the competent public administrations and in compliance with current guidelines for ethical use of animals in research, following European (2010/63/EU) and Spanish (R.D. 53/2013) legislation.

At postmortem examination, we collected tonsil and nasal swab specimens from each wild boar and cultured them on chocolate agar. For each sample, a maximum of 4 presumptive *S. suis* colonies were subcultured and subsequently identified with a *S. suis*–specific gene *recN* PCR (*7*). We molecularly serotyped *Streptococcus suis* isolates (*8*) and further serotyped positive isolates for *cps*2 gene by coagglutination (*9*). We also included 2 local *S. suis* serotype 2 strains of human origin for molecular characterization (Table 1). The source of the infection was not identified in 1 of these 2 patients (*10*), but the second patient acquired the infection through an accidental cut on his hand with a tusk while manipulating a wild boar hunted 50 km from Barcelona. In the days immediately following his injury, this patient developed pain in his hand; 5 days after the injury, he sought care at an emergency department. He had fever (body temperature 39.5°C), leukocytosis ($35 \times 10^9$ cells/L; reference range $3.9–10 \times 10^9$ cells/L), limited arm mobility, headache, and lethargic mental status. At this stage, the patient received diagnoses of arthritis and meningitis.

We isolated *S. suis* from 91 (84.3%; 95% CI 76.2%–89.9%) wild boars, identifying 332 isolates from tonsil (n = 141) and nasal (n = 191) swab specimens. We found

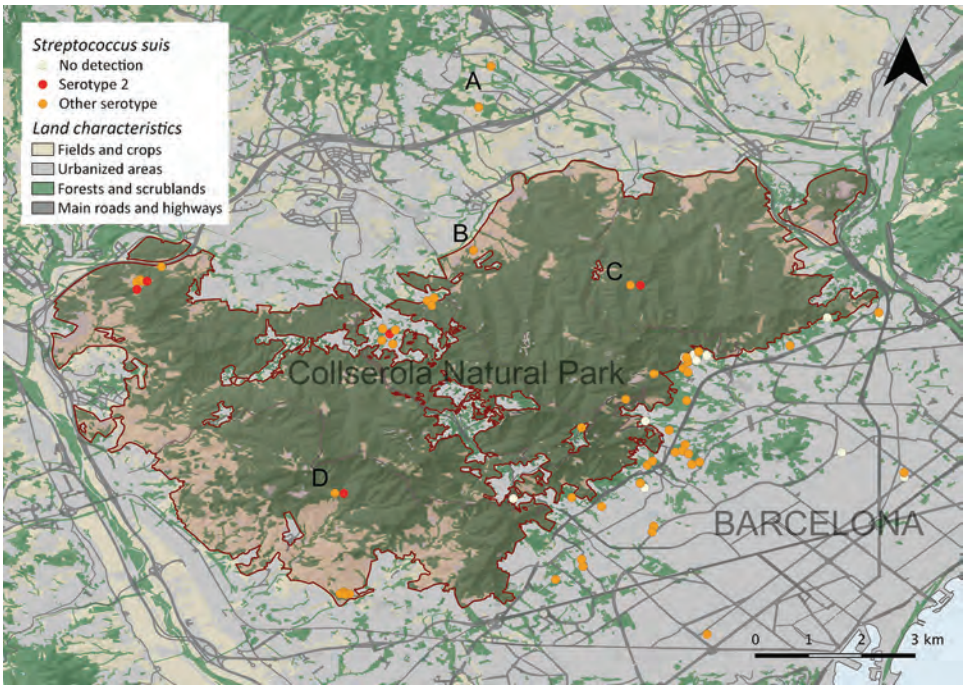


**Figure 1**. Part of the metropolitan area of Barcelona, Spain, showing land characteristics, Collserola Natural Park, the location of the wild boars sampled, and results of *Streptococcus suis* serotype 2 strains, identified by both isolation and molecular detection. Letters indicate locations where several wild boars were sampled, obtained by box traps (A, n = 21) or regular hunting campaigns (B, n = 9; C, n = 14; D, n = 5).

17 different serotypes and nontypeable strains in the wild boar population (Table 2). Three of the colonies were serotype 2, isolated from 2 wild boars, and were confirmed along with the human strains as serotype 2 by coagglutination test. We further characterized serotype 2 strains (both wild boar and human origin) using multilocus sequence typing (*11*) and by assessing the presence of the genes muramidase-released protein (*mrp*), extracellular factor (*ef*), and hemolysin suilysin (*sly*), associated with invasiveness and zoonotic potential (*12*). These serotype 2 strains belonged to clonal complex 1 and had the same molecular characterization (sequence type [ST] 1; *cps*2/*mrp*+/*ef*+/*sly*+), with the exception of 1 human isolate that was ST3 (a single-locus variant of ST1) (Table 1). Results from enterobacterial repetitive intergenic consensus (ERIC) PCR (*13*) showed a unique fingerprint profile for all serotype 2 strains and 32 different fingerprints for 36 other isolates (originating from 10 wild boars). In addition, 4 of 94 wild boars (4.3%; 95% CI 1.7%–10.4%) tested positive for detection of the *cps2* gene directly in nasal swab specimens.

To evaluate the occurrence and trend of potential effective contacts between urban wild boars and humans, we examined 174 veterinary field interventions related to wild boar incidents in Barcelona during 2013–2016. We found that both the number of interventions and the percentage of interventions motivated by violent physical contacts or aggressive interactions with humans (charges, bites, or injuries) showed an increasing trend (Figure 2).

## Discussion

The isolation of *S. suis* in apparently healthy wild boars from the metropolitan area of Barcelona highlights its reservoir role and identifies this species as a potential source for human infections in urban areas. The high number of wild boars carrying *S. suis* confirms results obtained in a previous study, in which *S. suis* and *S. suis*–like strains were detected together through the use of a PCR for the *gdh* gene (*14*). Our results show a high diversity of *S. suis* serotypes, with and without pathogenic relevance for humans (*4*). A high percentage of isolates was nontypeable, as previously found in both domestic pig and wild boar carriers (*4*). These results

**Table 1**. Sequence type and virulence associated gene profile of the *Streptococcus suis* serotype 2 strains isolated from wild boars and humans in metropolitan area of Barcelona, Spain, 2012–2015*

| Source | Year | Clinical infection | MLST | Virulence genes | | | References |
|---|---|---|---|---|---|---|---|
| | | | | *mrp* | *ef* | *sly* | |
| Human male, 57 y of age | 2012 | Meningitis, arthritis, bacteremia | ST3 | + | + | + | (*10*) |
| Human male, 48 y of age | 2014 | Meningitis, arthritis | ST1 | + | + | + | This study |
| Wild boar | 2015 | No | ST1 | + | + | + | This study |
| Wild boar | 2015 | No | ST1 | + | + | + | This study |
| Wild boar | 2015 | No | ST1 | + | + | + | This study |

*MLST, multilocus sequence typing; ST, sequence type.

**Table 2**. Frequency of *Streptococcus suis* serotypes identified by multiplex PCR in isolates from 108 wild boars from the metropolitan area of Barcelona, Spain, 2012–2015*

| | Isolates | | | | Wild boar | |
|---|---|---|---|---|---|---|
| Serotype | No. nasal samples positive | No. tonsillar samples positive | Total no. samples positive | Prevalence, % (95% CI) | No. animals positive* | Prevalence % (95% CI) |
| 2 | 2 | 1 | 3 | 0.9 (0.3–2.6) | 2 | 1.8 (0.5–6.5) |
| 4 | 3 | 7 | 10 | 3.0 (1.6–5.4) | 10 | 9.2 (5.1–16.2) |
| 6 | 5 | 0 | 5 | 1.5 (0.6–3.5) | 2 | 1.8 (0.5–6.5) |
| 7 | 0 | 2 | 2 | 0.6 (0.2–2.2) | 2 | 1.8 (0.5–6.5) |
| 8 | 3 | 1 | 4 | 1.2 (0.5–3.1) | 3 | 2.8 (0.9–7.8) |
| 9 | 7 | 18 | 25 | 7.5 (5.1–10.9) | 13 | 12.0 (7.2–19.5) |
| 10 | 4 | 1 | 5 | 1.5 (0.6–3.5) | 3 | 2.8 (0.9–7.8) |
| 12 | 0 | 1 | 1 | 0.3 (0.0–1.7) | 1 | 0.9 (0.0–5.1) |
| 15 | 0 | 4 | 4 | 1.2 (0.5–3.1) | 3 | 2.8 (0.9–7.8) |
| 16 | 27 | 7 | 34 | 10.2 (7.4–14.0) | 17 | 15.7 (10.1–23.8) |
| 17 | 1 | 0 | 1 | 0.3 (0.0–1.7) | 1 | 0.9 (0.0–5.1) |
| 21 | 30 | 0 | 30 | 9.0 (6.4–12.6) | 12 | 11.1 (6.5–18.4) |
| 23 | 3 | 0 | 3 | 0.9 (0.3–2.6) | 3 | 2.8 (0.9–7.8) |
| 27 | 2 | 2 | 4 | 1.2 (0.5–3.1) | 4 | 3.7 (1.4–9.1) |
| 28 | 1 | 2 | 3 | 0.9 (0.3–2.6) | 3 | 2.8 (0.9–7.8) |
| 31 | 20 | 13 | 33 | 9.9 (7.1–13.6) | 23 | 21.3 (14.6–29.9) |
| 33 | 3 | 0 | 3 | 0.9 (0.3–2.6) | 2 | 1.8 (0.5–6.5) |
| Nontypeable | 80 | 82 | 162 | 48.8 (43.5–54.1) | 70 | 64.8 (55.4–73.2) |
| Total | 191 | 141 | 332 | 100 | 108† | 100 |

*Number of wild boars that were positive for that serotype, either from nasal or tonsillar isolates.
†Total count does not coincide with the column sum because each wild boar can harbor ≥1 serotype and *S. suis* was not isolated from 17 wild boars.

further suggest that *S. suis* and serotype 2 strains are widely distributed in European wild boar populations (*14*).

Most human disease cases reported worldwide are associated with invasive serotype 2 strains that typically cause meningitis and, ultimately, neurologic sequelae or death (*4*,*5*). ST1, belonging to clonal complex 1, is one of the most frequent sequence types found in human and pig disease cases from Europe and other regions; it often involves the encoding virulence genes *mrp*, *ef*, and *sly* found in *cps2*-positive wild boar strains (*15*). Despite the high diversity of genomic fingerprints found in wild boar isolates, a unique fingerprint included all serotype 2 strains, suggesting no consistent differences between human and wild boar isolates. However, wild boars harboring *cps2*-positive strains were apparently healthy carriers. Other serotypes found in apparently healthy wild boars, such as 5, 9, 14, 16, and 21, have also been occasionally associated with severe human disease (*4*,*5*).

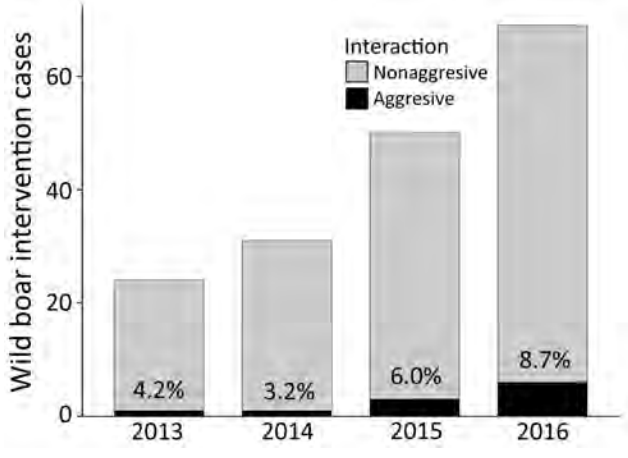


**Figure 2**. Occurrence and temporal trend of veterinary interventions related to wild boar removal in Barcelona, Spain, 2013–2016, and percentage of interventions with aggressive interactions. Aggressive interaction involves both violent physical contacts (charging or pushing for food) and aggressions to humans (bites).

At least 7 human cases of wild boar bites were treated at the emergency department of a main local hospital (2012–2015, Communication Unit of Vall d'Hebron, Barcelona), yet small wounds not attended in health centers can be enough to acquire systemic infections (*4*). Human *S. suis* infections are associated mainly with pig farming and the food industry, being considered an occupational disease or foodborne disease in developing countries (*5*). The increasing trend of potential effective contacts for *S. suis* transmission between urban wild boars and humans in Barcelona suggests that *S. suis* can be acquired by humans who belong to apparently low-risk collectives.

## Conclusions

Wild boars from the metropolitan area of Barcelona harbor *S. suis* strains with the same enterobacterial repetitive intergenic consensus PCR fingerprints and virulence gene profiles as invasive strains of local human origin; thus, they have zoonotic and invasive potential. The increasing interactions of wild boars with humans in urban areas pose risks for *S. suis* or other directly or indirectly transmitted zoonoses, making the presence of urban wild boars a public health concern.

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# Human Endophthalmitis Caused By Pseudorabies Virus Infection, China, 2017

**Jing-Wen Ai,[1] Shan-Shan Weng,[1] Qi Cheng, Peng Cui, Yong-Jun Li, Hong-Long Wu, Yi-Min Zhu, Bin Xu, Wen-Hong Zhang**

We report human endophthalmitis caused by pseudorabies virus infection after exposure to sewage on a hog farm in China. High-throughput sequencing and real-time PCR of vitreous humor showed pseudorabies virus sequences. This case showed that pseudorabies virus might infect humans after direct contact with contaminants.

Pseudorabies virus (PRV) primarily infects swine and has several secondary hosts, including cattle, dogs, and cats. PRV, also called Aujeszky disease virus or Suid herpesvirus 1, is a member of the *Alphaherpesvirinae* subfamily within the family *Herpesviridae*. PRV infection has not been confirmed in humans (*1*), but previous reports have suggested the possible presence of PRV infection in 3 immunocompetent humans in whom fever, sweating, and neurologic complaints developed; virus neutralization and immune precipitation tests were positive for PRV antibody (*2*,*3*). We report a human case of human infectious endophthalmitis caused by PRV in a woman from Jiangxi Province, China, in July 2017.

## The Study

On June 14, 2017, sewage from a hog farm spilled onto a 46-year-old woman from Jiangxi Province, China, who worked as a swineherder; her daily work was to feed swine and clean hoggery sewage. The next day, she had a headache and fever of 39.5°C. Three days later, she became visually impaired and was admitted to a local hospital, where she was treated empirically with meropenem, vancomycin, and acyclovir. On June 29, after no significant improvement, she was transferred to Huashan Hospital of Fudan University (Shanghai, China), to treat unresponsive fever, headaches, and visual impairment.

On examination, she had palpebral conjunctival congestion, and visual acuity to light perception of both eyes had worsened. Slit-lamp examination showed keratic precipitates and Tyndall effect flare. Funduscopic examination revealed vitreous opacity (Figure 1, panel A) and a pale white lesion on the posterior pole of the right eye (Figure 1, panel B), which suggested acute retinal necrosis and occlusive vasculitis. Results of routine laboratory testing were normal, including serology tests for HIV and hepatitis B and C, T-SPOT.*TB* test (Oxford Immunotec Ltd., Oxford, UK), blood culture, cryptococcal latex agglutination test, autoantibodies, and cerebrospinal fluid test. Test results for plasma Epstein-Barr virus and cytomegalovirus (CMV) IgG were positive; IgM was negative for both pathogens.

The on-staff ophthalmologist diagnosed endophthalmitis in the patient, considering viral infection as most likely. The patient was transferred to the ophthalmology department for vitrectomy surgery on the right eye on June 30. During the operation, ≈2 mL of vitreous humor was taken for culture and next-generation sequencing (NGS) (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/6/17-1612-Techapp1.pdf)

On July 2, NGS results showed 4,832 unique sequence reads of PRV in vitreous humor, covering 84% of the nucleotide sequences (Table; Figure 2, panel A); NGS results for cerebrospinal fluid (CSF) were negative for PRV. Other detected sequences were within laboratory reference ranges. On the basis of NGS results, the physicians, who suspected that the patient may have acquired PRV infection, immediately initiated valacyclovir therapy. Sanger sequencing (online Technical Appendix Figure 1) and PCR analysis (online Technical Appendix Figures 2, 3) confirmed identification of PRV in vitreous humor (*4*–*6*). Phylogenetic analysis disclosed a close connection between the isolated strain and 3 emergent and highly pathogenic PRV variants in China (Figure 2, panel B; online Technical Appendix) (*7*,*8*).

To further validate our results, we constructed a plasmid and a standard curve for real-time PCR and obtained quantitative results of viral DNA load as $2.7 \times 10^6$ copies/mL (online Technical Appendix Figure 3). To rule out contamination, we performed PCR of the PRV on a control group of 7 persons; all were negative (online Technical Appendix Figure 2). Other possible viral causes of infective endophthalmitis, such as varicella-zoster virus, herpes simplex virus, and cytomegalovirus, were excluded through

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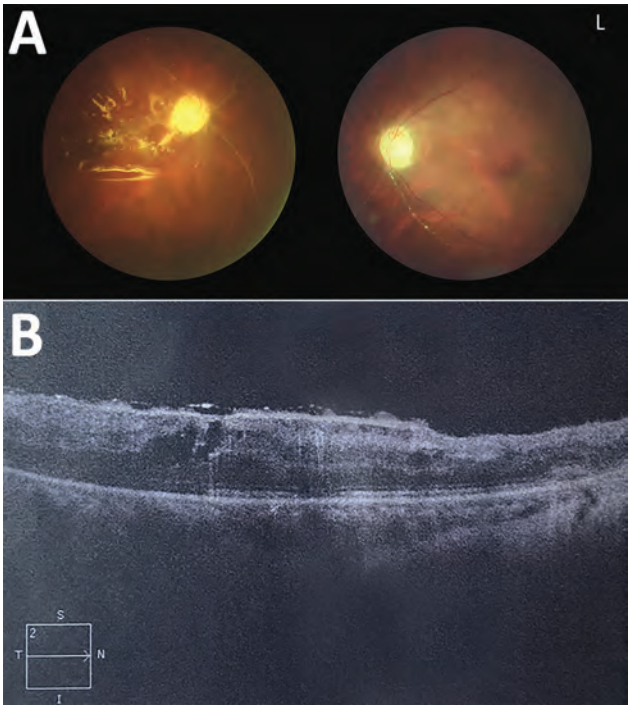


**Figure 1.** Ocular examination conducted 2 months after eye surgery in patient with human endophthalmitis caused by pseudorabies virus, China, 2017. A) Fundus photography of both eyes showing retinal necrosis and occlusive vasculitis and postoperative change after pars plana vitrectomy with silicone oil injection in the right eye. B) Optical coherence tomography of the patient's right eye showed postoperative change after pars plana vitrectomy. 2, scan depth (2 mm); I, inferior, L, left eye; N, nasal side; S, superior; T, temporal side..

PCR. One week after surgery, culture result for vitreous humor was negative; the patient's fever and headache had resolved, and visual acuity had improved slightly. The patient was discharged on July 11; during the last clinical check-up (December 6, 2017), the visual acuity of her left eye had improved to 0.2, and the right eye remained at slight light perception.

During the follow-up period, we obtained the patient's plasma and CSF samples and ordered PRV antibody testing. PRV antibody was detected in all plasma samples at 4 and 5 months after disease onset and in CSF samples at 2 weeks to 2 months after disease onset, indicating the patient's previous contact with PRV (online Technical Appendix Figure 4). Epstein-Barr virus, CMV, and PRV serologic tests were also conducted on control samples and ruled out the possibility of cross-reaction (online Technical Appendix Table 2) (*9*,*10*). Although the patient experienced headache and fever during disease progression and CSF PRV antibody test was positive, routine tests, NGS, and PCR of the CSF all failed to disclose abnormality. Therefore, we do not have enough evidence to suggest possible PRV central nervous system infection. Considering epidemiologic history, clinical symptoms, and serologic and molecular testing results, we diagnosed PRV endophthalmitis.

## Conclusions

The first 2 suspected cases of human PRV infection were reported in 1914, but detection of antibodies or cultivation of the virus had failed. In 1987, Mravak reported 3 suspected cases of human PRV infection with positive serum antibodies (*2*). All 3 patients were immunocompetent and their clinical manifestation occurred 1–3 weeks after possible animal contact. Initial symptoms included fever, sweating, and weakness; later, central nervous system symptoms developed. Some symptoms persisted for months, and serologic PRV antibodies were positive 5–15 months after the onset of clinical symptoms. In our study, the patient had similar symptoms, plus visual impairment and a unique infection route of PRV through direct exposure to contaminants.

Since 2011, prevalence of PRV infection among swineherds in China has risen several times (*11*). Among swineherds in Jiangxi Province, studies have shown varying positive rates for PRV DNA: from 5.5% to 26.5% during 2014, (*12*), and of positive PRV antibodies, from 84.4% to 89.9% during September 2013–September 2015 (*13*). PRV vaccine is still provided for swineherds on a voluntary basis rather than as a requirement in China, and swine were not vaccinated in the hoggery in which this patient worked at that time. On the day of disease onset,

**Table.** Pathogens detected by using next-generation sequencing in vitreous humor from a patient with human endophthalmitis caused by pseudorabies virus, China, 2017

| Pathogen | Coverage, % | Depth, bp | Unique reads |
|---|---|---|---|
| Virus | | | |
| *Suid herpesvirus 1* | 84 | 6.8 | 4,832 |
| *Bovine herpesvirus 5* | 8.9 | 1 | 1 |
| Bacteria | | | |
| *Thermoanaerobacter wiegelii* | 0.52 | 5.3 | 9 |
| *Corynebacterium urealyticum* | 0.56 | 1 | 0 |
| *Haloquadratum walsbyi* | 0.47 | 2.8 | 0 |
| *Brachyspira pilosicoli* | 0.41 | 1 | 1 |
| *Candidatus* Nitrososphaera | 0.46 | 1 | 0 |
| Fungus | | | |
| *Cryptococcus gattii* | 0.46 | 1 | 1 |

the patient's eyes were directly contaminated with sewage containing pig excrement when cleaning pig sties. Although no previous study had reported a confirmed case of PRV-caused infectious endophthalmitis, the atypical infectious route in this case made hoggery the most probable infectious source in this study.

NGS is marked by its rapid diagnostic ability to precisely identify certain pathogens in peripheral blood, respiratory, and CSF samples. In this case, NGS testing in facilitating the diagnosis of infective endophthalmitis is supported. The fact that real-time PCR and Sanger sequencing results were consistent with the NGS results further validated the credibility of this technique. Furthermore, a serologic test was conducted during the follow-up period; results showed that, even 5 months after symptom onset, the antibodies to PRV in plasma were

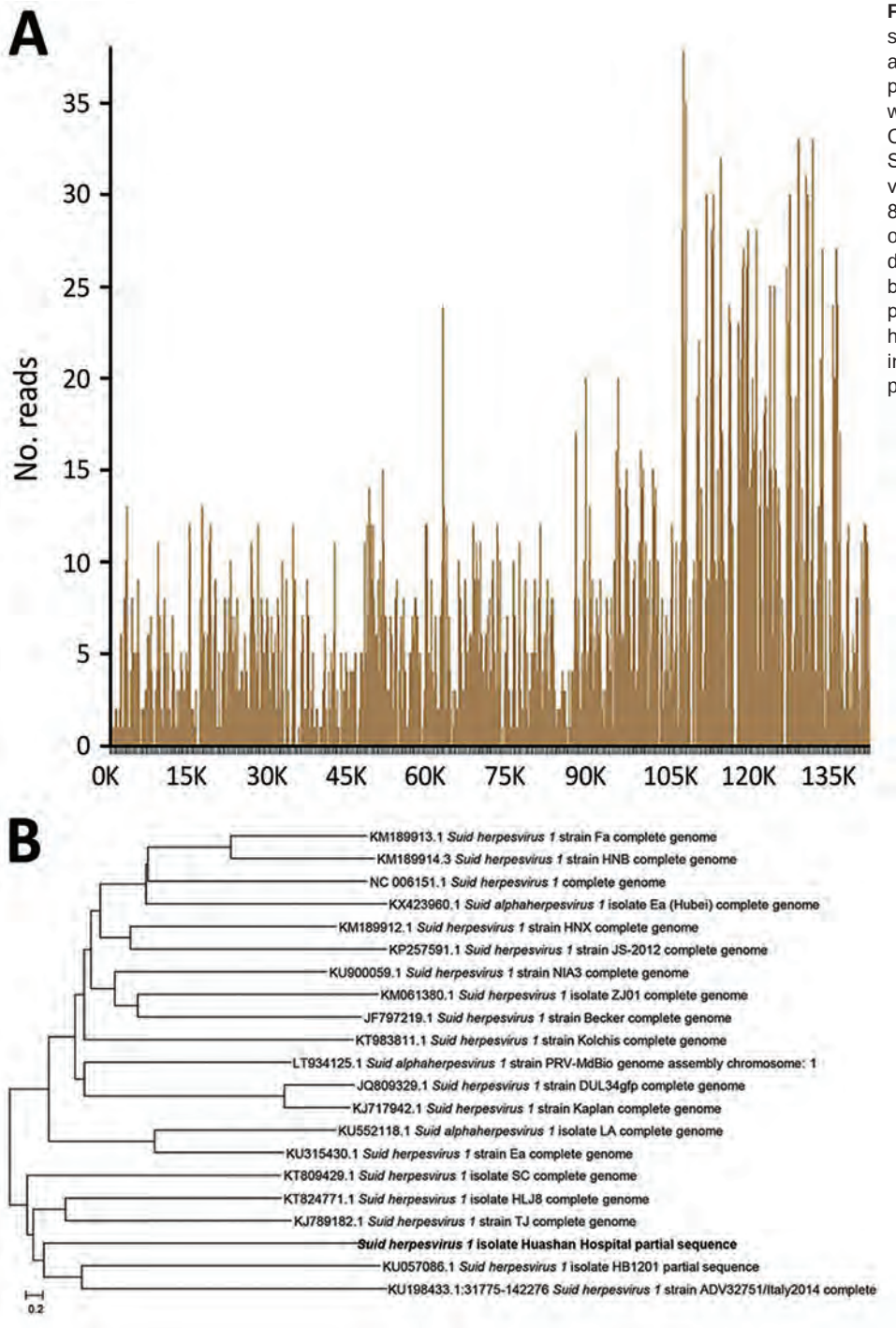


**Figure 2.** Results of gene sequencing, serologic testing, and phylogenetic analysis of pseudorabies virus from patient with human endophthalmitis, China, 2017. A) Sequencing of Suid herpesvirus 1 (pseudorabies virus) yielded a total coverage of 84%. B) Evolutionary relationships of taxa. Phylogenetic analysis disclosed a close connection between the isolate from the patient (boldface) and 4 other Suid herpesvirus 1 strains. Scale bar indicates amino acid substitutions per site.

still active, and the antibodies in CSF persisted during the entire follow-up period (online Technical Appendix Figure 4). This result is similar to that reported in 1987, indicating that PRV antibodies may persist long after the initial infections.

In summary, this case of PRV-caused human infectious endophthalmitis indicates that PRV could affect humans through direct contact with pig contaminants. NGS of the vitreous humor provided a strong technical support for rapid diagnosis of PRV infection in this patient. However, the pathogenesis of PRV infection remains to be explored. This case stresses the importance of mandatory PRV vaccine among swineherds and the necessity for workers in the breeding industry to increase awareness of self-protection when handling animal containments.

### Acknowledgments

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# Pulmonary Infections with Nontuberculous Mycobacteria, Catalonia, Spain, 1994–2014

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In Spain, systematic reporting of pulmonary infections with nontuberculous mycobacteria is not mandatory. Therefore, to determine trends, we retrospectively identified cases for January 1994–December 2014 in Catalonia. Over the 21 years, prevalence increased and was associated with being male. *Mycobacterium avium* complex and *M. abscessus* prevalence increased; *M. kansasii* prevalence decreased.

Nontuberculous mycobacteria (NTM) in low-prevalence settings have been regarded as opportunistic pathogens associated with HIV infection and chronic pulmonary disease. An overall increase in prevalence of pulmonary NTM infections in different geographic areas has been reported (*1*–*7*). The burden of pulmonary NTM infections in Spain is largely unknown because systematic reporting is not mandatory. We report secular trends in the prevalence of pulmonary infections caused by NTM over a 21-year period in a healthcare region of Catalonia, Spain.

## The Study

We performed a descriptive population-based study in 13 municipalities in the Barcelona-South Health Region of Catalonia for the period January 1994–December 2014. The study population comprised inhabitants ≥18 years of age in this area of the health region (population at the period midpoint 600,892). During 1994–2009, specialized healthcare for this population was provided by 3 hospitals and from 2010 on by 4 hospitals. The referral hospital for the area is Bellvitge University Hospital (BUH). Throughout the study period, the BUH mycobacterial laboratory processed all samples from the participating hospitals for identification only or for culture and identification. Samples for mycobacteria isolation were processed according to standard methods (*8*). From April 1994 through March 2009, liquid cultures were processed by use of the BACTEC TB 460 radiometric method (Becton Dickinson, Sparks, MD, USA); from April 2009 on, the BACTEC MGIT 960 system (Becton Dickinson) was used. From 1994 through 2002, *Mycobacterium abscessus* was part of the *M. chelonae-abscessus* group; from 2003 on, it was identified as a separate species.

Using files of NTM isolates from the BUH laboratory, we retrospectively identified cases and included only those for which patients were ≥18 years of age, resided in the study area, and had NTM isolated from ≥1 respiratory specimen. We excluded patients who resided outside the study area and patients from whom *M. gordonae* was isolated. We defined patients as having pulmonary disease if ≥2 cultures were positive or if antimicrobial chemotherapy considered active against the species isolated had been started. The BUH ethics committee approved the study.

We calculated overall and annual prevalence rates with 95% CIs as the number of patients with ≥1 isolate (isolation prevalence) and the number of patients with pulmonary disease (disease prevalence), divided by the population, according to the official census of Catalonia (https://www.idescat.cat/territori/?geomun&langen). We used Poisson regression models to estimate rate ratios of isolates and pulmonary disease caused by NTM per year. We constructed regression models for age groups 18–49, 50–65, and >65 years; to determine the relative prevalence of respiratory specimens, we adjusted all estimates for sex and year of isolation. Results of the regression models are expressed as relative risk with 95% CIs. We performed analyses by using SPSS version 18.0 for Windows (https://www.ibm.com/analytics/data-science/predictive-analytics/spss-statistical-software) and Epidat (https://www.sergas.es/Saude-publica/EPIDAT?idioma=es) statistical packages.

During the 21-year period, we identified 680 patients (mean age 60.5 ± 16.6 years; 77.8% men) from whom NTM had been isolated from respiratory specimens. The overall period prevalence of patients from whom NTM was isolated

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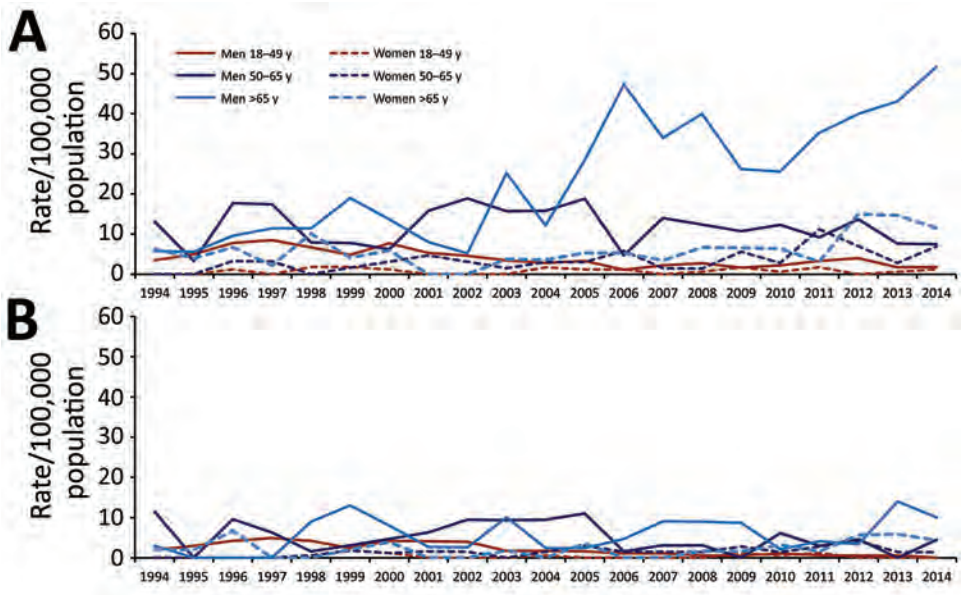


**Figure 1.** Annual prevalence rates per 100,000 population for nontuberculous mycobacteria isolation (A) and pulmonary disease (B), by patient sex and age group, Barcelona-South Health Region, Catalonia, Spain, 1994–2014.

was 113.2 (95% CI 105.0–122.0)/100,000 population; for pulmonary disease, prevalence was 42.8 (95% CI 37.5–48.0)/100,000 population. Prevalence rates were higher for men than for women, for isolation (180.5 and 49.1/100,000 population, respectively), and for disease (68.2 and 18.5/100,000 population, respectively) (Figure 1; online Technical Appendix Tables 1, 2, https://wwwnc.cdc.gov/EID/article/24/6/17-2095-Techapp1.pdf). The regression models showed a 5% annual decrease in prevalence rates of isolation among those 18–49 years of age, an 11% increase among those >65 years of age, and no significant changes among those 50–65 years of age. Male sex remained an independent factor associated with higher rates of isolation of NTM in the 3 age groups (Table 1; Figure 1, panel A). Similarly, prevalence rates of pulmonary disease showed an annual decrease of 9% among those 18–49 years of age, an annual increase of 7% among those >65 years of age, and remained without significant changes among those 50–65 years of age. Male sex remained associated with higher rates of pulmonary disease (Table 1; Figure 1, panel B).

Trends in prevalence rates differed among species of mycobacteria (Table 2; Figure 2). Although prevalence rates rose significantly for *M. avium* complex (MAC) (by 10% for isolation and 13% for pulmonary disease), rates of *M. kansasii* fell (by 9% for isolation and 11% for pulmonary disease). Since 2003, *M. abscessus* isolation increased annually by 22% and pulmonary disease increased by 24%. As for rapidly growing mycobacteria other than *M. abscessus*, isolation but not pulmonary disease increased significantly over the study period (Table 2).

## Conclusions

We found a significant increase in the prevalence of isolation of NTM from respiratory specimens in the study

**Table 1.** Poisson regression analysis of the association between prevalence rates of nontuberculous mycobacteria isolation and pulmonary disease, Barcelona-South Health Region, Catalonia, Spain, 1994–2014*

| Patient age group, sex, and year | Isolation | | | Pulmonary disease | | |
|---|---|---|---|---|---|---|
| | No. patients | aRR (95% CI) | p value | No. patients | aRR (95% CI) | p value |
| 18–49 y | 176 | | | 89 | | |
| Sex | | | | | | |
| F | 29 | 1.0 | <0.0001 | 12 | 1.0 | <0.0001 |
| M | 147 | 5.10 (3.20–8.12) | | 77 | 6.46 (3.04–13.89) | |
| Year of isolation | 176 | 0.95 (0.93–0.97) | <0.0001 | 89 | 0.92 (0.88–0.95) | <0.0001 |
| 50–65 y | 209 | | | 89 | | |
| Sex | | | | | | |
| F | 49 | 1.0 | <0.0001 | 20 | 1.0 | <0.0001 |
| M | 160 | 3.18 (2.20–4.58) | | 69 | 3.38 (2.01–5.24) | |
| Year of isolation | 209 | 1.02 (0.99–1.04) | 0.2 | 89 | 0.99 (0.95–1.04) | 0.7 |
| >65 y | 295 | | | 79 | | |
| Sex | | | | | | |
| F | 73 | 1.0 | <0.0001 | 25 | 1.0 | 0.001 |
| M | 222 | 3.09 (2.39–3.93) | | 54 | 2.15 (1.35–3.41) | |
| Year of isolation | 295 | 1.11 (1.09–1.13) | <0.0001 | 79 | 1.0 (1.02–1.12) | 0.004 |

*aRR, adjusted relative risk.

**Table 2.** Bivariate Poisson regression analysis of changes in prevalence rates of nontuberculous mycobacteria isolation and pulmonary disease by species. Barcelona-South Health Region, Catalonia, Spain, 1994–2014*

| | Isolation | | | Pulmonary disease | | |
|---|---|---|---|---|---|---|
| Species | No. patients | RR (95% CI) | p value | No. patients | RR (95% CI) | p value |
| *Mycobacterium kansasii* | 194 | 0.92 (0.89–0.94) | <0.0001 | 154 | 0.91 (0.87–0.93) | <0.0001 |
| *M. avium* complex | 139 | 1.10 (1.07–1.15) | <0.0001 | 67 | 1.13 (1.08–1.18) | <0.0001 |
| *M. xenopi* | 96 | 1.03 (0.99–1.08) | 0.16 | 16 | 1.06 (0.98–1.13) | 0.10 |
| *M. abscessus*† | 17 | 1.22 (1.11–1.33) | <0.0001 | 11 | 1.24 (1.08.1.42) | 0.002 |
| Other‡ | 234 | 1.10 (1.08–1.12) | <0.0001 | 9 | 1.11 (0.99–1.24) | 0.06 |

*RR, relative risk
†2003–2014 only.
‡Rapidly growing mycobacteria other than *M. abscessus,* and other species of mycobacteria.

area. However, the trends varied according to mycobacteria species. The most common NTM causing lung disease during the first half of the study period, *M. kansasii*, declined progressively from the early 2000s on. In contrast, MAC became the most frequently isolated NTM, and rates increased for *M. abscessus*, eventually equaling those of *M. kansasii*. Rapidly growing mycobacteria other than *M. abscessus* were also increasingly isolated in the most recent years, but most were deemed colonizers (*9*).

We do not believe that the increased prevalence of most NTM can be explained by the 2009 implementation of the new culture system MGIT 960 because its sensitivity is equivalent to that of the BACTEC TB 460 system used previously in combination with solid media (*10*,*11*). Furthermore, changes in MAC and *M. kansasii,* which are easily recovered in culture, cannot be attributed to the implementation of the newer culture media. The changes recorded in temporal trends can be explained, at least in part, by the dynamics of the HIV epidemic in Spain. The reduction of susceptible HIV-infected patients through improvements in antiretroviral therapy in the second half of the 1990s led to a dramatic fall in the prevalence of NTM infections among these patients. Because *M. kansasii* was the most frequently isolated NTM among HIV-infected patients in the Barcelona-South Health Region of Catalonia, its prevalence decreased, paralleling the decrease in susceptible persons with HIV infection (*12*). This reduction in *M. kansasii* infections is also illustrated by the reduction among persons 18–49 years of age because HIV-infected patients in Spain at that time were mostly young users of illicit intravenous drugs. Even so, the decrease among HIV patients does not explain the changes in the NTM ecology in our area, in which MAC and *M. abscessus* seem to be filling the vacancy left by *M. kansasii*. Although the prevalence is still low, the current distribution of mycobacteria species in this area is closer to that of other countries (*1*–*7*,*13*).

Our study has 2 main limitations. First, although we aimed to capture all NTM patients in the area, we may have missed some patients who sought medical care elsewhere. Second, we defined pulmonary disease patients as having had ≥2 positive cultures or having received treatment for the NTM species isolated. Although the first criterion may have overestimated prevalence, the second may have been biased toward inclusion of patients with more severe illness and may thus have underestimated prevalence.

Our data add evidence for the increasing prevalence of NTM pulmonary infections in Catalonia. We also observed concurrent species-specific differences in prevalence trends in this area.

## About the Author

Dr. Santin is an infectious diseases senior consultant at Bellvitge University Hospital, a professor at Barcelona University, and a researcher at Bellvitge Biomedical Research Institute in Barcelona, Spain. His research interests include tuberculosis and nontuberculous mycobacteria.

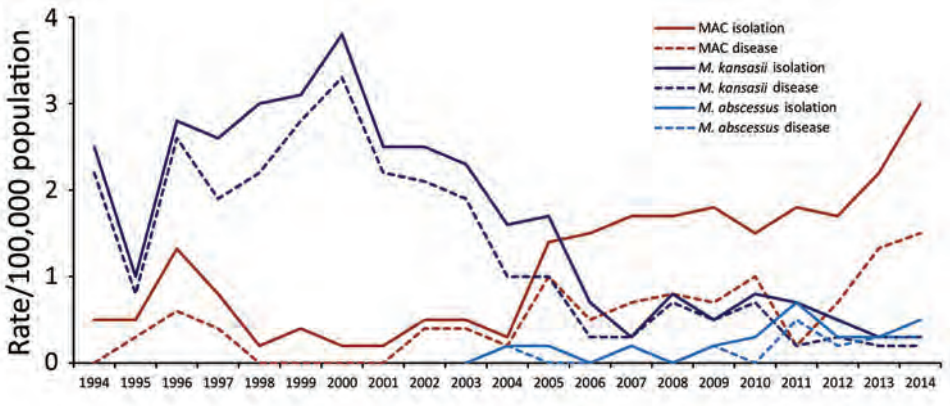


**Figure 2**. Annual prevalence rates per 100,000 population of 3 species of nontuberculous mycobacteria (*Mycobacterium kansasii*, MAC, and *M. abscessus*), Barcelona-South Health Region, Catalonia, Spain, 1994–2014. MAC, *Mycobacterium avium* complex.

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# Westward Spread of Highly Pathogenic Avian Influenza A(H7N9) Virus among Humans, China

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We report infection of humans with highly pathogenic avian influenza A(H7N9) virus in Shaanxi, China, in May 2017. We obtained complete genomes for samples from 5 patients and from live poultry markets or farms in 4 cities. Results indicate that H7N9 is spreading westward from southern and eastern China.

Avian influenza A(H7N9) virus caused 5 waves of human infection in China from its emergence in 2013 (*1*) through May 17, 2017. During that period, 1,564 laboratory-confirmed human cases and 612 deaths were reported; about half occurred during the fifth wave (*2*). The fifth wave not only infected many more persons but also spread north; previous H7N9 outbreaks had been documented only in eastern and southern China. Moreover, viruses recently isolated from human case-patients in Guangdong Province (A/Guangdong/17SF003/2016 and A/Guangdong/17SF006/2017) in southern China have been confirmed as highly pathogenic avian influenza (HPAI) A(H7N9) viruses, on the basis of their molecular (*3*) and biologic (*4*) characteristics.

We report cases of human infection with H7N9 virus, including in 1 person who was infected with a highly pathogenic variant, in Shaanxi Province, western China, during April and May 2017. We obtained complete genome sequences of H7N9 viruses from 5 patients and from 21 environmental samples obtained from live poultry markets (LPMs) and from poultry farms in 4 cities.

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## The Study

A case of HPAI H7N9 virus infection in a human from Shaanxi Province was identified in the city of Yulin on May 26, 2017. On day 1 of illness onset, fever and fatigue developed; after 2 days, the patient received medical attention. On day 4, the patient was admitted to an intensive care unit, and on day 6, HPAI H7N9 infection was laboratory confirmed. After treatment with oseltamivir, the patient recovered. An additional 4 cases of infection with low pathogenicity avian influenza (LPAI) A(H7N9) virus among humans were identified during April 30–May 13, 2017, in Shaanxi Province: 2 in Xianyang, 1 in Baoji, and 1 in the capital city, Xi'an. Recorded symptoms included fever (100%); cough and fatigue (75%); and productive cough, diarrhea, and shortness of breath (50%). Patients received medical attention at an average of 3.75 (range 1–5) days after illness onset and were admitted to a hospital at an average of 6 (range 4–8) days after onset. Samples were laboratory confirmed as H7N9 virus at an average of 9 (range 6–14) days after onset. Of the 5 patients, 4 were treated with oseltamivir; 2 of those died.

Respiratory tract specimens (throat swab or sputum sample) were collected from patients and transferred to the national laboratory at the Chinese Center for Disease Control and Prevention (China CDC) for confirmation. Viral RNA was extracted from each sample by using nucleic acid isolation magnetic beads, according to the manufacturer's instructions (TianLong Science and Technology Co. Ltd. Xi'an, China). Avian influenza virus H7 and N9 gene segments were detected in the total RNA by using an H7N9 dual-channel Taqman probe reverse transcription PCR kit (BioPerfectus Co. Ltd. Taizhou, China).

During May 2017, we collected 496 environmental samples (poultry feces and poultry cage swabs from LPMs and poultry farms) from 4 cities (Xi'an, Baoji, Xianyang, and Yuli) in Shaanxi Province (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/6/17-1135-Techapp1.pdf). A total of 14 samples from LPMs were positive for H7N9 virus.

We extracted total RNA from the patient respiratory specimens and from H7N9-positive environmental samples and sequenced avian influenza virus genomes by using next-generation sequencing on an Illumina MiSeq (Illumina Inc., Shanghai, China). Sequence comparisons

[1]These authors contributed equally to this article.

showed that the viruses we isolated from 1 patient in Yulin and from the 15 environmental samples had the same polybasic (PB) amino acid sequence (PEVPKRKRTAR/GL) at the hemagglutinin (HA) cleavage site as that in previously confirmed HPAI H7N9 viruses (A/Guangdong/17SF003, A/Guangdong/17SF006, and A/Taiwan/1/2017) (*3*–*5*), indicating that the viruses we isolated are also HPAI viruses. In addition, the HPAI viruses we isolated appear to have inherited the HA amino acid mutation G186V from LPAI viruses and reverted to 226Q from 226L, as observed in previously confirmed HPAI H7N9 viruses (*4*–*6*). Because G186V and Q226L indicate an increased level of binding to human-type influenza receptors (*7*,*8*), the ability of the HPAI viruses we isolated here to infect humans is likely similar to that of previously reported HPAI H7N9 viruses. Furthermore, the HA genes of all Shaanxi HPAI H7N9 viruses we isolated were >99% identical to those of previously isolated HPAI H7N9 viruses.

We analyzed each of 8 gene segments of H7N9 viruses by using phylogenetic methods, together with available sequences from the GISAID EpiFlu database (http://platform.gisaid.org/; accession numbers in online Technical Appendix Table 3). The HA and neuraminidase (NA) phylogenies indicate that the viruses isolated in Shaanxi belong to clade W2-C (*9*). In the HA tree (Figure 1; online Technical Appendix Figure 1), the 16 HPAI H7N9 viruses we isolated clustered with previously detected HPAI H7N9 viruses from Guangdong Province. The HA gene of the Shaanxi HPAI H7N9 viruses has a common ancestor with the Guangdong HPAI H7N9 viruses, indicating that HPAI H7N9 viruses have spread from Guangdong to other provinces (online Technical Appendix Figure 2, panel B). Further, the 7 LPAI H7N9 viruses we identified in Shaanxi Province clustered with LPAI H7N9 viruses isolated from various districts in eastern (Anhui, Fujian, Zhejiang, Jiangsu), central (Chongqing, Henan, Hunan, Hubei), and

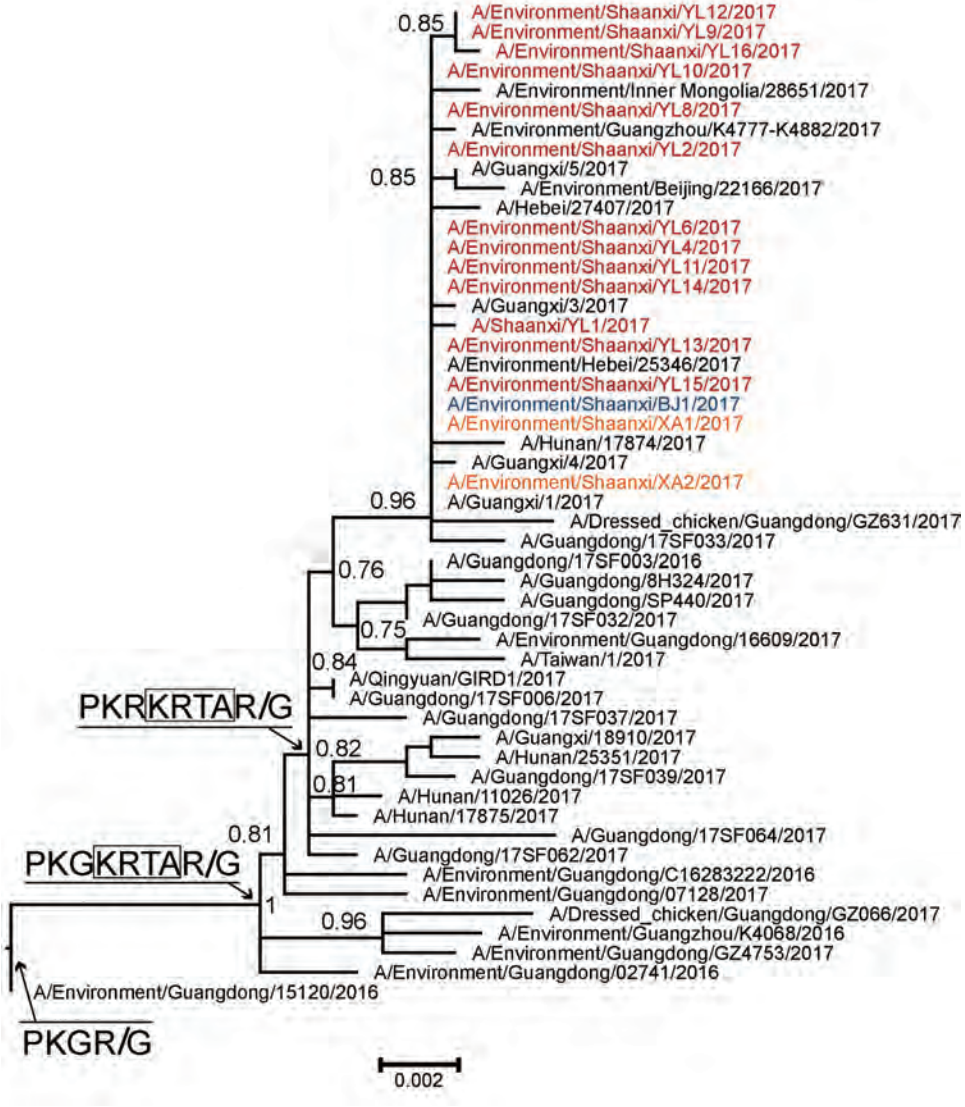


**Figure 1.** Detail of highly pathogenic avian influenza A(H7N9) viruses isolated from human and environmental sources, Shaanxi Province, China, 2016–2017, showing Shimodaira-Hasegawa-like local bootstrap support values. Amino acid changes within the hemagglutinin cleavage site are indicated on basal branches. The low pathogenicity strain A/Environment/Guangdong/15120/2016 is used as an outgroup. Colors indicate sampling locations of the H7N9 viruses obtained in this study: red, Yulin; blue, Baoji; orange, Xi'an. An expanded version of this figure showing comparisons to reference viruses is available in online Technical Appendix Figure 1 (https://wwwnc.cdc.gov/EID/article/24/6/17-1135-Techapp1.pdf). Scale bar indicates amino acid substituions per site.

southern (Guangxi, Guangdong, Hong Kong) China, indicating that LPAI H7N9 viruses have been spreading from eastern and southern China into western China (Figure 2). In the NA phylogeny, the phylogenetic positions of the NA genes of all viruses we sequenced were similar to those observed in the HA phylogeny (online Technical Appendix Figure 2). Phylogenies of the 6 internal genes (online Technical Appendix Figure 3) show that the viruses from Shaanxi Province belong to clades 1 and 2 (*9*) of the ZJ-HJ/07 lineage of H9N2 viruses (*10*) (online Technical Appendix Table 2).

Phylogenetic analyses of HA and NA gene segments reveal that the viruses obtained from human cases in Yulin and Baoji were genetically similar to those from environmental samples collected from local farms or LPMs in Yulin and Baoji (online Technical Appendix Figure 1, panel A; online Technical Appendix Figure 2), suggesting that the human H7N9 infections are related to viruses circulating in local farms or LPMs. This hypothesis is also supported by most internal gene segments, except for the NS segments of viruses from Yulin (no internal gene segments were obtained for isolate A/Environment/Shaanxi/BJ2/2017 sampled in Baoji). Most of the gene segments of the viruses from human cases in Baoji and Xianyang were similar to those of A/Environment/Shaanxi/XA4/2017, the virus isolated from LPMs in Xi'an (with the exception of PB1 from the human case in Baoji and PB2, PB1, and matrix of the viruses from human cases in Xianyang). The NS and polymerase acidic segments of the LPAI virus from a human case in Xi'an (A/Shaanxi/XA1/2017) were genetically similar to the HPAI viruses we isolated; other gene segments were close to LPAI viruses from Anhui Province in southern China.


**Figure 2.** Geographic distribution of the avian influenza A(H7N9) viruses isolated in Shaanxi Province, China, 2016–2017 (solid circles), and of HPAI H7N9 viruses detected in other provinces of China (open circles).

## Conclusions

We report emerging HPAI H7N9 variants in Shaanxi Province, western China. Our phylogenetic analyses support that the LPAI H7N9 viruses in Shaanxi Province originated from eastern and southern China, and the Shaanxi HPAI H7N9 isolates probably originated in Guangdong Province and were transmitted either directly or indirectly through other provinces.

Our results provide evidence that H7N9 viruses infecting humans in Shaanxi Province derived, directly or indirectly, from strains circulating in local farms and LPMs. Previous studies suggest that poultry trade between LPMs may play a key role in spreading H7N9 viruses (*11*). Similarly, we hypothesize that live poultry or poultry product transport may facilitate the spread of HPAI H7N9 viruses out of Guangdong Province and within Shaanxi Province.

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# Importation of Human Seoul Virus Infection to Germany from Indonesia

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Seoul hantavirus–associated hemorrhagic fever with renal syndrome cases are rare outside Asia and have not yet been found in Germany. We report clinical and molecular evidence for a Seoul virus infection in a patient in Germany. The infection was most likely acquired during a stay in Sulawesi, Indonesia.

Hantaviruses are globally emerging zoonotic pathogens that cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (*1*). One representative of this negative-sense, single-stranded RNA virus family is rat-associated Seoul virus (SEOV). In comparison to infections by prototypical Hantaan virus (HTNV), infection with SEOV is believed to lead to somewhat milder disease with shorter clinical phases (*2*). The most characteristic manifestations of SEOV infection are prominent abdominal symptoms, including hepatomegaly, hepatic dysfunction, and mild renal failure (*2*). A clear distinction from infections by related hantaviruses, such as HTNV, by serodiagnostic means is difficult and complicates interpretation of current clinical studies on (putatively) SEOV-infected patients.

SEOV infections have been found in rats and humans mainly in Asia but also worldwide (*3*). In Europe, molecular analysis has shown circulation of SEOV in wild brown rats (*Rattus norvegicus*) and in pet rats in the United Kingdom, France, Belgium, the Netherlands, and Sweden; however, human SEOV infections have been diagnosed by using only serologic analysis (*4*). Unequivocal molecular proof of human SEOV infections in Europe has been shown only for 4 patients in France (*5*) and 1 patient in the United Kingdom (https://www.gov.uk/government/publications/hantavirus-infection-in-people-sero-surveillance-study-in-england).

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In Germany, SEOV-specific antibodies or SEOV RNA have not been detected in rats or humans. We report on a case of molecularly proven hantavirus disease caused by SEOV infection in a patient in Germany who probably acquired the infection in Indonesia.

## The Patient

On April 25, 2017, a 70-year-old man from Germany visited the emergency department at Asklepios Klinik Harburg (Hamburg, Germany) and reported severe diarrhea, thoracic/back pain, and bronchopulmonary symptoms. He also reported a fever that emerged at the end of a multiweek vacation on the island of Sulawesi in Indonesia a few days before his return to Germany on April 12. On May 2, he was hospitalized because of acute kidney injury.

We obtained laboratory findings for blood samples collected during the inpatient period of 11 days (Table). An initially low platelet count (66/nL) at admission returned to a reference value at day 8 of hospitalization. Serum creatinine levels were increased; maximum values were observed at days 2 and 3 of hospitalization. The glomerular filtration rate was decreased. Leukocyte counts and levels of C-reactive protein and lactate dehydrogenase were slightly increased. Increased levels of liver enzymes (aspartate aminotransferase, alanine aminotransferase, and γ-glutamyltransferase) indicated hepatic involvement, a characteristic of SEOV infections (*2*). Diuresis returned to reference values during hospitalization, and no polyuria was observed. The patient was discharged from the hospital with a serum creatinine level of 1.8 mg/dL and in largely normalized general condition.

We performed initial laboratory diagnostics (serologic analysis for hantavirus) by using the Hantavirus Profile 1 Immunoblot (Euroimmun, Lübeck, Germany) in the hospital laboratory and the *recom*Line HantaPlus IgG and IgM assays (Mikrogen, Martinsried, Germany) in our laboratory. The Profile 1 blot does not contain SEOV antigen, but serum from the patient showed reactivity for Dobrava-Belgrade virus (DOBV) IgG and HTNV IgM. The *recom*-Line IgG and IgM blots showed reactivity for DOBV, HTNV, and SEOV; the weakest result was for SEOV nucleocapsid protein.

For molecular typing, we tested the first blood sample collected during hospitalization by using reverse transcription for hantavirus RNA. We obtained partial

[1]These authors contributed equally to this article.

**Table.** Biochemical parameters of a case-patient during 11 days of hospitalization who was infected with Seoul virus imported to Germany from Indonesia*

| Parameter | Reference range | Day 1 | Day 2 | Day 3 | Day 9 | Day 11 |
|---|---|---|---|---|---|---|
| Platelet count | 160–370/nL | 66 | 85 | 112 | 394 | 490 |
| Creatinine | 0.7–1.2 mg/dL | 3.7 | 5.5 | 5 | 2.3 | 1.8 |
| GFR CKD-EPI | >60 mL/min | 16 | 10 | 11 | 28 | 37 |
| Leukocyte count | 3.5–9.8 cells/nL | 12.8 | 15.7 | 16.8 | 12.1 | 11.7 |
| CRP | <5.0 mg/L | 54.4 | ND | 26.5 | 10.4 | ND |
| LDH | <250 U/L | 735 | 561 | ND | 285 | ND |
| GGT | <60 U/L | 137 | 115 | 121 | 159 | ND |
| ALT | <50 U/L | 75 | 119 | 234 | 120 | ND |
| AST | <50 U/L | ND | 173 | ND | 46 | ND |
| Urea | 18–55 mg/dL | ND | 240 | ND | 73 | 48 |
| Hemoglobin | 13.5–17.5 g/dL | 17.6 | 16 | 15.1 | 12.9 | 11.4 |

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; CKD-EPI, chronic kidney disease epidemiology collaboration; CRP, C-reactive protein; GFR, glomerular filtration rate using the CKD-EPI formula; GGT, γ-glutamyltransferase; LDH, lactate dehydrogenase; ND, not determined.

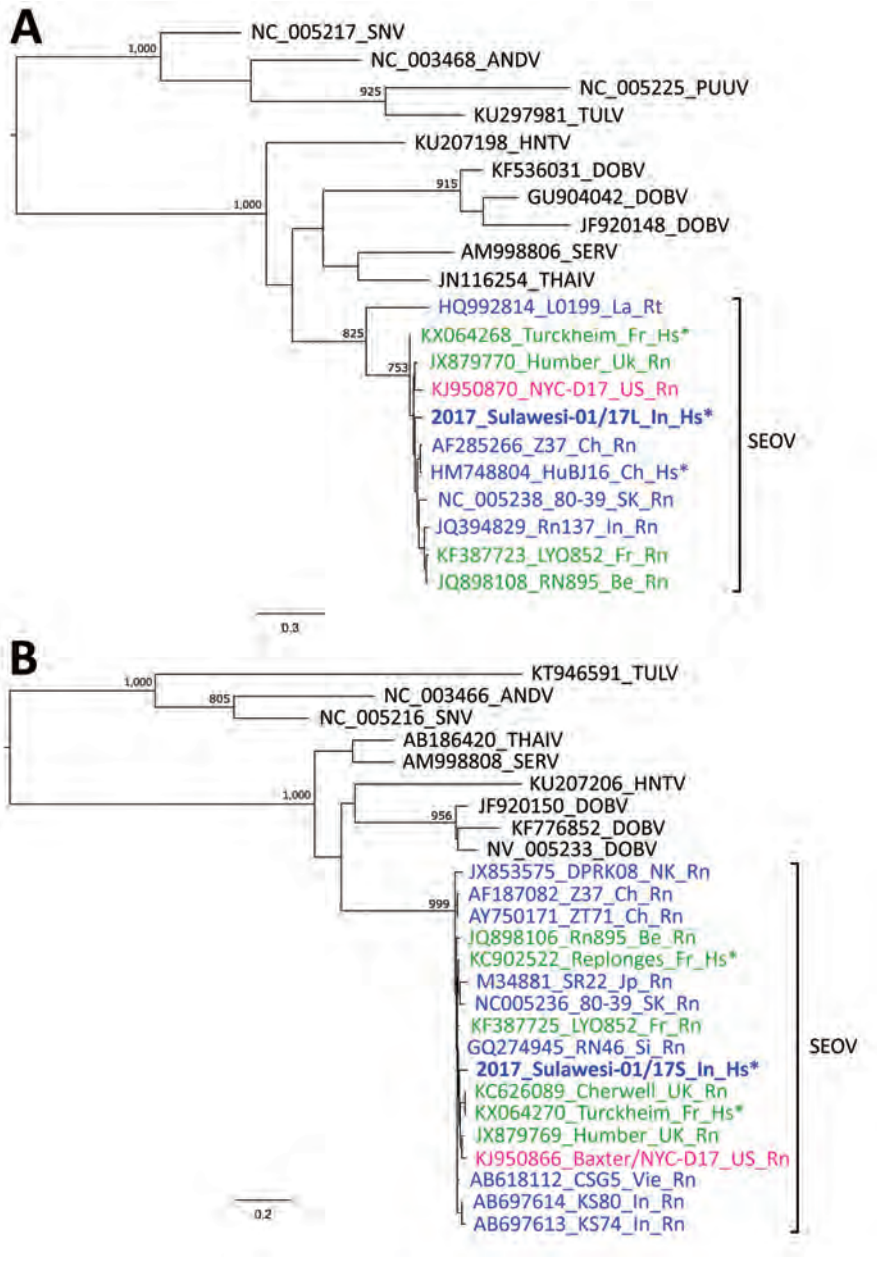


**Figure.** Maximum-likelihood phylogenetic trees of partial RNA segments of orthohantaviruses. A) Large RNA segments based on a 347-nt alignment and the general time reversible plus gamma distribution model of nucleotide substitution. B) Small RNA segments based on a 318-nt alignment and the Hasegawa–Kishino–Yano 85 plus gamma distribution model. Trees were constructed by using PhyML3.0 (*8*) and the best-fitting model according to smart model selection in this software and 1,000 bootstrap replicates. Values along branches are bootstrap values >75% for major clades. GenBank accession number, strain, country of origin, and host are shown for each virus isolate. Bold indicates SEOV isolated from the patient in this study. Blue indicates SEOV strains from Asia, green indicates SEOV strains from Europe, and red indicates SEOV strains from the Americas. Scale bars indicate nucleotide substitutions per site. *Sequences from viruses of human origin. ANDV, Andes virus; Be, Belgium; Ch, China; DOBV, Dobrava-Belgrade virus; Fr, France; HNTV, Hantaan virus; Hs, *Homo sapiens*; In, Indonesia; Jp, Japan; La, Laos; NK, North Korea; PUUV, Puumala virus; Rn, *Rattus norvegicus*; Rt, *R. tanezumi*; SEOV, Seoul virus; SERV, Serang virus; Si, Singapore; SK, South Korea; SNV, Sin nombre virus; THAIV, Thailand virus; TULV, Tula virus; UK, United Kingdom; US, United States; Vie, Vietnam.

sequences of genes coding for hantavirus polymerase (large [L] RNA segment) by using HAN-L primers (*6*). We also obtained nucleocapsid protein (small [S] RNA segment) sequences by using primers S1 and S2 (*7*) and primers S598 (5′-ATG AAG GCA GAA GAG ATT ACA CC[TA] GG-3′) and S6HC (5′-CCA GCA AAC ACC CAT ATT GAT GAT-3′) as nested primers. Sequences of the strain Sulawesi-01/17 were deposited in GenBank under accession nos. MG386252 for the L segment and MG386253 for the S segment. We showed by phylogenetic analysis that L and S sequences obtained from the patient segregated to the main cluster of SEOV strains, which clearly demonstrated that SEOV was the causal agent of infection (Figure).

## Conclusions

We report an infection with SEOV in a patient in Germany. If one considers the clinical course, this case of HFRS appeared moderate, and the outcome for this case-patient was favorable and showed a full recovery. As expected for SEOV-associated hantavirus disease (*2*), severe gastrointestinal symptoms and liver involvement were observed, but kidney dysfunction was mild and no hemodialysis was needed. However, the clinical course appeared unusually protracted (≈6 weeks) between the febrile phase and discharge from hospital. Typing of the causative hantavirus by using 2 commercial immunoblots was misleading, but sequence data obtained from the L and S segments unequivocally confirmed that the patient was infected with SEOV.

Molecular phylogenetic analysis of genetically characterized SEOV strains resulted in creation of 4 phylogroups (*9*). Most strains, including all strains from locations other than mountainous areas of China, belong to major phylogroup A and have probably spread from Asia as a result of distribution of rats during trade activities by humans (*9*). Although SEOV strains are found in ports and countries with overseas traffic, no SEOV infections of rats or humans have been reported in Sulawesi in Indonesia. SEOV RNA in rats, but no proof of human infections, was reported in Jakarta, on the island of Java in Indonesia (*10*,*11*).

Because of the wide spread of SEOV caused by extensive movement of its natural host (rats), analysis of the nucleotide sequence of the SEOV strain from our patient does not enable identification of the place of infection. This strain is related to SEOV strains from Asia, Europe, and the United States (Figure). This finding contrasts with those for other hantaviruses, for which there are clear spatial association between virus strains and carrier hosts, such as vole-associated Puumala virus (*6*,*12*).

Although molecular characterization of the SEOV strain isolated from the patient enabled diagnosis of SEOV infection, these results did not identify the original place of infection. The clinical course, including onset of disease, makes it highly probable that the patient acquired the infection during his stay in Indonesia. Because he did not report any further travel activities during this stay, we conclude that he had acquired the infection in Sulawesi in Indonesia.

In summary, we report a human SEOV infection imported to Germany from Indonesia. Our results demonstrate that extended molecular diagnostics are required for reliable hantavirus typing, especially for patients with travel histories.

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# Detection of Low Pathogenicity Influenza A(H7N3) Virus during Duck Mortality Event, Cambodia, 2017

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In January 2017, an estimated 3,700 (93%) of 4,000 Khaki Campbell ducks (*Anas platyrhynchos domesticus*) died in Kampong Thom Province, Cambodia. We detected low pathogenicity avian influenza A(H7N3) virus and anatid herpesvirus 1 (duck plague) in the affected flock; however, the exact cause of the mortality event remains unclear.

Avian influenza viruses (AIVs) are negative-sense, single-stranded RNA viruses normally found in wild aquatic birds, the natural reservoir (*1*). Typically, AIVs do not cause severe disease in domestic poultry; however, 2 AIV subtypes, H5 and H7 influenza A viruses, are capable of mutating to form highly pathogenic avian influenza (HPAI) variants that can cause high rates of disease and death in poultry flocks (*2*). In addition, establishment of AIVs in domestic poultry increases the probability of zoonotic transmission to humans. Recent attention has focused on H7 AIVs (particularly subtype H7N9 in China) that have become established in domestic poultry and repeatedly transmitted to humans since 2013 (*3*). Influenza A(H7N3) and A(H7N7) viruses have also been the causative agent in historical poultry and human infections in Europe and the Americas (*2*). Overall, the pathogenic and zoonotic potential of H7 strains makes them a substantial economic and public health concern.

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Cambodia is a lower-middle-income country in Southeast Asia with a large socioeconomic dependence on agriculture. In 2015, a total of 57% of all households in Cambodia had agricultural holdings, and 87% of these households raised poultry (*4*). Poultry are generally reared in backyards or on small-scale farms with minimal or no biosecurity. Therefore, poultry diseases such as HPAI can have devastating economic consequences. In 2013 alone, ≈25 million chickens and 3.3 million ducks were either traded or disposed of (slaughtered for sale or died) in Cambodia. Of these, 22% of chickens and 18% of ducks were reported to have died from illness (*5*). Although control measures for HPAI in Cambodia include culling of poultry that is infected, suspected to be infected, or in contact with infected/suspected poultry, reporting is minimal, and no compensation mechanism exists. Since 2004, a total of 58 reported AIV outbreaks (mostly HPAI) have occurred in poultry and wild birds (as of April 2018), and 56 human influenza A(H5N1) cases (37 fatalities [case-fatality rate 66%]) have been reported in Cambodia (*6*).

## The Study

In early January 2017, a free-range production flock of 4-month-old Khaki Campbell ducks located in Kampong Thom Province in central Cambodia were found with loss of appetite, depression, weakness, white-bluish diarrhea, and swollen heads and eyes (Figure 1). Within days, ≈93% (an estimated 3,700 of 4,000) had succumbed to disease. The remainder of the flock was slaughtered. As part of a routine investigation into the causative agent, we obtained oropharyngeal and cloacal swab specimens and organs from 4 of the affected ducks. The National Animal Health and Production Research Institute of Cambodia performed initial screening for the presence of AIV by real-time quantitative reverse transcription PCR (qRT-PCR). The Institut Pasteur du Cambodge (IPC) verified the results and conducted further analysis.

IPC confirmed by qRT-PCR that 2 of 4 ducks were positive for influenza A virus. IPC successfully isolated viruses from both samples in embryonated chicken eggs (*7*) and designated them A/duck/Cambodia/b0116502/2017 and A/duck/Cambodia/b0120501/2017. Next, the World Health Organization's Collaborating Centre for Reference and Research on Influenza (Melbourne, VIC, Australia)


**Figure 1.** Location of duck mortality event and detection of influenza A(H7N3) virus in Kampong Thom Province (gold shading), Cambodia, January 2017. Open circle indicates exact location of the mortality event.

performed whole-genome sequencing on isolate RNA using the Ion Torrent next-generation sequencing (NGS) platform (Life Technologies, Carlsbad, CA, USA) and analyzed NGS data by using CLC Genomic Workbench 10 (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench). Sanger sequencing with segment-specific primers filled in any sequencing gaps (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/24/6/17-2099-Techapp1.pdf) using Big Dye Terminator Reaction Mix (Applied Biosystems, Foster City, CA, USA) on an ABI 3500xL Genetic Analyzer (Applied Biosystems). IPC, in conjunction with the World Health Organization's Collaborating Centre for Reference and Research on Influenza, used Geneious 9.1.8 (Biomatters Ltd., Auckland, New Zealand) to collate NGS and Sanger sequencing data, align strains, and analyze molecular markers. IPC submitted all sequences to GenBank (accession nos. MG591682–MG591697; online Technical Appendix Table 2). We used the maximum-likelihood method based on the general time-reversible model to infer phylogenic relationships and tree construction for each gene in MEGA version 7 (*8*) with 500 bootstrap replicates for robustness.

Sequencing revealed that both isolates belonged to the H7N3 subtype. Identification of H7 is not novel in Cambodia. Prior studies in 2013 and 2015 in live bird markets have identified low pathogenicity AIV (LPAIV) subtype H7 circulating in chickens and ducks (*6*,*7*). Phylogenetic analyses indicated that all of the gene segments from both H7N3 isolates from Cambodia showed the highest degree sequence similarity to each other and fell into the Eurasian lineage of H7 viruses circulating in Asia, predominantly during 2012–2015. Neither strain shared genes with H7N9 viruses associated with human cases in China (Table 1; Figure 2; online Technical Appendix Figure 1–6). On a molecular level, we determined both isolates to be LPAIVs, showing a monobasic cleavage site, avian receptor specificity, and genetic indications of susceptibility to neuraminidase and matrix protein ion channel inhibitors (Table 2).

Because molecular data indicated that the H7N3 strains in this event were LPAIVs, these viruses were

**Table 1.** Sequence similarity between influenza A virus subtype H7N3 isolates and other influenza viruses, Cambodia*

| Gene segment | Strain with highest sequence identity | Position, nt | Identity, % | GenBank accession no. |
|---|---|---|---|---|
| PB2 | A/duck/Hunan/S11682/2015(H7N9) | 1–2280 | 97 | MF630450 |
| PB1 | A/duck/Hunan/S11682/2015(H7N9) | 1–2274 | 97 | MF630451 |
| PA | A/duck/Jiangxi/15867/2013(H10N3) | 1–2151 | 97 | KP285492 |
| HA | A/wild bird/Jiangxi/34458/2013(H7N7) | 1–1683 | 97 | KP417103 |
| NP | A/duck/Nha Trang/84/2014(H6N6) | 1–1497 | 98 | LC050632 |
| NA | A/duck/Vietnam/OIE-2329/2009(H11N3) | 4–1413 | 94 | AB545596 |
| MP | A/duck/Hunan/S4443/2011(H11N9) | 1–982 | 99 | CY146719 |
| NS | A/duck/Vietnam/HU1–16/2014(H11N7) | 1–838 | 98/99 | LC070024 |

*HA, hemagglutinin; MP, matrix protein; NA, neuraminidase; NP, nucleoprotein; NS nonstructural; PA, polymerase; PB1, polymerase basic protein 1; PB2, polymerase basic protein 2.

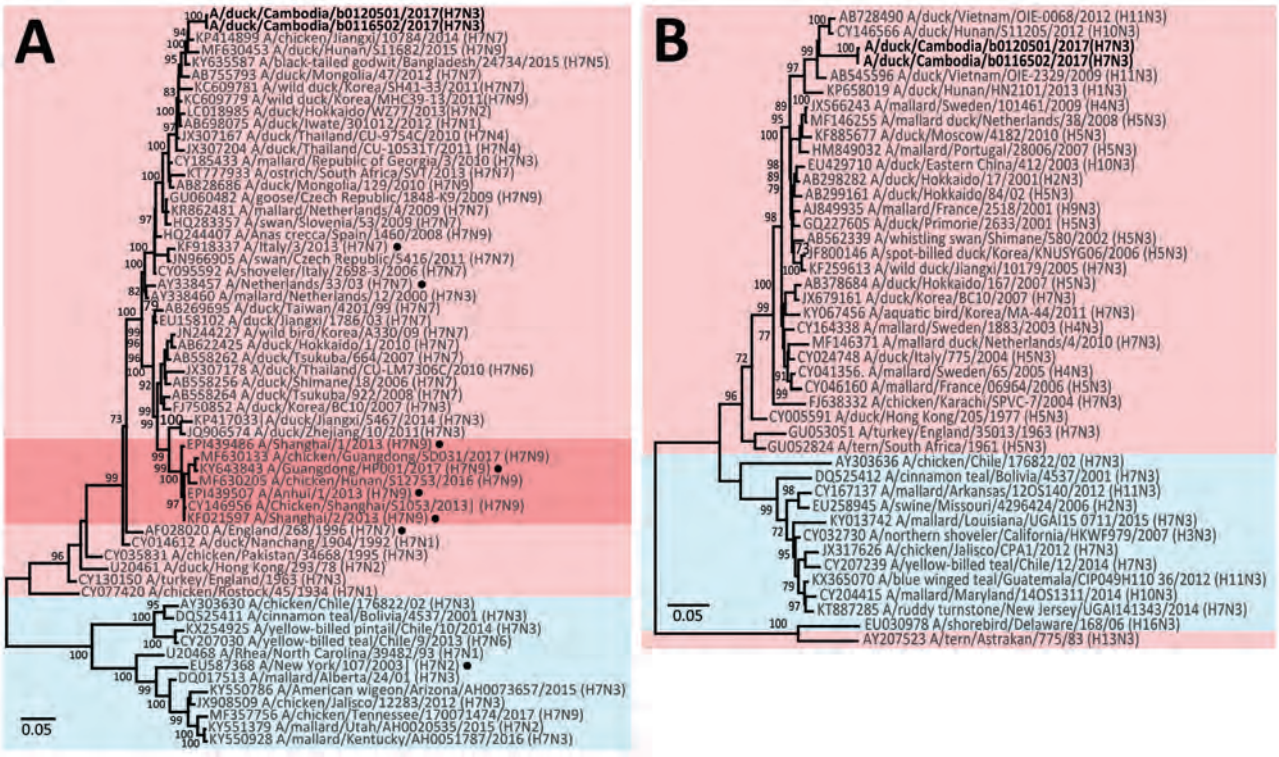


**Figure 2.** Phylogenetic analysis of the H7 hemagglutinin (HA) gene (A) and N3 neuraminidase (NA) gene (B) of influenza viruses isolated from ducks in Kampong Thom Province, Cambodia (boldface), and reference isolates. Trees were generated using the maximum-likelihood method based on the general time-reversible model. Bootstrap values (n = 500) >70 are indicated. Light pink shading indicates strains from Eurasia, blue shading indicates strains from North and South America, and dark pink shading indicates H7N9 strains from China. Black dots indicate strains from human infections. GenBank accession numbers are provided for reference isolates. Scale bars indicate substitutions per site.

probably not the sole causative agent for such high mortality during this outbreak. However, infection with LPAIV H7N3 might have contributed to lethality by increasing susceptibility to secondary infections. In an effort to identify other possible pathogens, we screened swab samples and internal organs of the same ducks from the outbreak

**Table 2.** Genetic risk characteristics of influenza A virus subtype H7N3 isolates, Cambodia*

| Gene segment and risk factor | Amino acid change | Isolates in Cambodia | Conclusions† | Reference |
|---|---|---|---|---|
| PB2 | | | | |
| Mammalian host range marker and increased viral pathogenicity | E627K | E | Avian specificity | (*9*) |
| | D701N | D | | |
| HA | | | | |
| Multibasic cleavage site causing increased pathogenicity | Multibasic | PEPPKGR/GLF | Monobasic | (*10*) |
| Increased mammalian receptor specificity | Q226L‡ | Q | Avian specificity | (*11*) |
| | G228S‡ | G | | |
| NA | | | | |
| Resistance to NA inhibitor antivirals | H275Y§ | H | Sensitive to oseltamivir | (*12*) |
| | E119K¶ | E | | |
| | R292K | R | | |
| MP | | | | |
| Resistance to M2 inhibitor antivirals | L26F | Q | Sensitive to M2 inhibitors | (*13*) |
| | V27A | R | | |
| | A30T | D | | |
| | S31N | V | | |
| | G34E | G | | |

*HA, hemagglutinin; MP, matrix protein; MP2, matrix protein 2; NA, neuraminidase; PB2, polymerase basic protein 2.
†Receptor binding specificity and antiviral sensitivity is predicted based on sequence information and has not been experimentally confirmed.
‡H3 numbering.
§N1 numbering.
¶N2 numbering.

for the presence of anatid herpesvirus 1 (AnHV-1), commonly known as duck plague (*14*). Two of the 3 carcasses screened were positive for AnHV-1 in the liver; however, direct comparisons to swab samples cannot be made because no information was available on the correlation between swab samples and duck carcasses. AnHV-1 is known to cause high mortality in duck flocks globally, including in Cambodia, and co-infection with H7N3 virus and AnHV-1 might have contributed to the outbreak (*15*). From swab samples, we confirmed co-infection with AnHV-1 in the 2 H7N3 virus–positive ducks and 1 of the 2 H7N3 virus–negative ducks; however, our outbreak investigation revealed that ducks were retrospectively vaccinated against AnHV-1 with live attenuated vaccine once the flock began to show signs of illness. Consequently, because AnHV-1 vaccine can be detected by qRT-PCR up to 6 days postvaccination (*14*) and no specific date was available for vaccination before sample collection, no direct conclusions can be made about the associated contribution of AnHV-1 and H7N3 to the mass mortality during this outbreak. The extent and effect of such a co-infection need to be investigated further.

## Conclusions

Given the endemicity of AIVs in Southeast Asia, especially in Cambodia, understanding the prevalence and effect of AIV in the region is vital. Although the H7 viruses identified during this outbreak were determined to be low pathogenicity and their role as causative agents of duck mortality remains unclear, continued active and passive surveillance, as well as molecular characterization and risk assessment, is crucial to identify, control, and prevent AIVs in this region. Further work is also necessary to understand the interplay of AIVs with other diseases of poultry to determine the etiology of bird mortality events in the region.

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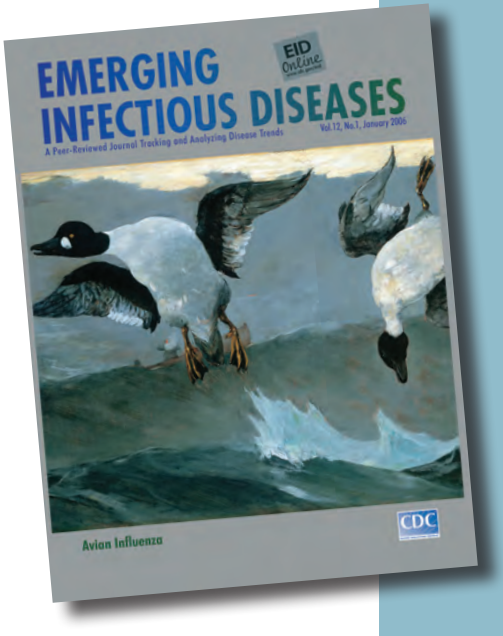
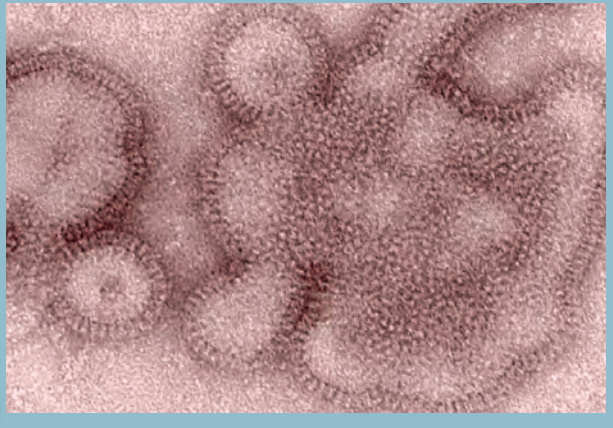
# etymologia revisited

## Influenza

Acute viral infection of the respiratory tract. From Latin influentia, "to flow into"; in medieval times, intangible fluid given off by stars was believed to affect humans. The Italian influenza referred to any disease outbreak thought to be influenced by stars. In 1743, what Italians called an influenza di catarro ("epidemic of catarrh") spread across Europe, and the disease came to be known in English as simply "influenza."



Sources: Dorland's illustrated medical dictionary. 30th ed. Philadelphia: Saunders; 2003; and Quinion M. World wide words. 1998 Jan 3 [cited 2005 Dec 5]. http://www.worldwidewords.org/topicalwords/tw-inf1.htm

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***https://wwwnc.cdc.gov/eid/article/12/1/et-1201_article***

# *Rickettsia parkeri* in *Dermacentor parumapertus* Ticks, Mexico

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During a study to identify zoonotic pathogens in northwestern Mexico, we detected the presence of a rickettsial agent in *Dermacentor parumapertus* ticks from black-tailed jackrabbits (*Lepus californicus*). Comparison of 4 gene sequences (*gltA, htrA, ompA,* and *ompB*) of this agent showed 99%–100% identity with sequences of *Rickettsia parkeri*.

*Rickettsia parkeri* is an emerging pathogen that causes a spotted fever group rickettsiosis, transmitted to humans primarily by several species of ticks of the genus *Amblyomma*, including *A. maculatum*, *A. triste*, *A. tigrinum*, and *A. ovale*. However, *R. parkeri* has also been detected in other hard ticks, including *A. americanum*, *A. aureolatum*, *A. dubitatum*, *A. longirostre*, *A. nodosum*, *A. parkeri*, *Ixodes scapularis*, *Rhipicephalus sanguineus*, *Dermacentor parumapertus*, and *D. variabilis*, in 8 countries in North (United States), Central (Belize), and South (Colombia, Peru, Brazil, Bolivia, Uruguay, Argentina) America (*1*,*2*) (Figure 1, panel A).

Recently, a distinct strain of *Rickettsia parkeri*, designated *R. parkeri* Black Gap, was isolated and characterized from the tick *Dermacentor parumapertus* collected from black-tailed jackrabbits in Texas, USA (*3*). This isolate is phylogenetically related to *Rickettsia* sp. strain Atlantic rainforest, a well-known pathogenic lineage of *R. parkeri* associated with a mild rickettsiosis of humans in Brazil (*4*). In addition, the presence of *R. parkeri* in *A. triste* and *A. maculatum* ticks has been confirmed in several locations in Arizona (*5*,*6*). Although *R. parkeri*–infected ticks have now been identified in several US states that border Mexico, no studies have demonstrated the presence of *R. parkeri* in ticks in Mexico.

## The Study

We conducted a study to identify zoonotic pathogens in northwestern Mexico; the study area comprised a region within the Janos Biosphere Reserve in Chihuahua state (30°51′50″N, 108°30′09″W) and in the San Pedro River Basin in Sonora state (31°30′90″N, 110°10′70″W) (Figure 1, panel B). The area is in a transition zone between the Sonoran Desert, the Sierra Madre Occidental, and the Chihuahuan Desert and comprises a mosaic of grasslands, mesquite scrublands, and oak forests. During September 2013–September 2014, we sampled lagomorphs in 6 trapping locations (Chihuahuan locations: Casa de Janos, El Cuervo, Monte Verde, Pancho Villa, and Rancho El Uno; Sonoran location: Palmitas). Lagomorphs were live-trapped in box traps and leg-hold traps during a separate study to evaluate *Bartonella* genotypes in wild carnivores (*7*) under permission no. FAUT-0250 of the Secretaría de Medio Ambiente y Recursos Naturales. We identified captured animals as to species, sex, and age (juvenile or adult) using a standard field guide (*8*). We physically restrained the lagomorphs and visually examined them for ticks, removed the ticks manually, and deposited them in cryovials containing 96% ethanol. We then released the hosts in situ. We performed morphological identification of ticks with specialized taxonomic keys (*9*).

We collected 29 ticks: 23 *D. parumapertus* adults from 21 black-tailed jackrabbits (*Lepus californicus*), 2 *D. parumapertus* adults from 3 white-sided jackrabbits (*Lepus callotis*), and 2 *D. parumapertus* adults and 2 *Amblyomma* sp. nymphs from 4 desert cottontails (*Sylvilagus audubonii*). We deposited 1 female and 2 male *D. parumapertus* ticks in the Colección del Laboratorio de Acarología, Facultad de Ciencias, Universidad Nacional Autónoma de México (UNAM) in Mexico City.

For the remaining specimens, we performed DNA extraction individually using the Chelex100 Chelating Resin (Bio-Rad, Hercules, CA, USA) protocol (*10*). For the initial screening, we amplified a conserved fragment of 805 bp of the *gltA* gene, which is present in all *Rickettsia* species

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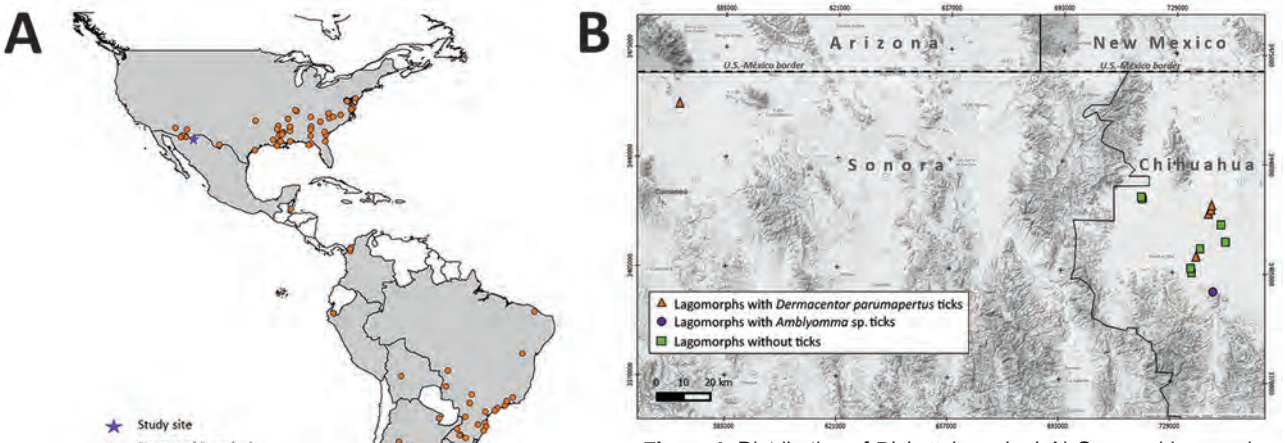


**Figure 1**. Distribution of *Rickettsia parkeri*. A) Geographic records of *R. parkeri* in the Americas based on reports from the literature. We used a layer of Google Maps (www.google.com/maps/) for the construction of the figure. B) Area of study of *Rickettsia* in Mexico. Squares and triangles represent the sites where lagomorphs carrying ticks were captured.

(*11*). We characterized all positive samples by the amplification of 3 additional gene fragments (*ompA, ompB,* and *htrA*) using primers and conditions described elsewhere (*11*,*12*). The reaction mixture consisted of 12.5 μL of GoTaq Green Master Mix 2X (Promega, Madison, WI, USA), the corresponding pair of primers (100 ng each), 6.5 μL nuclease-free water, and 100 ng of DNA in a final volume of 25 μL. In all reactions, we included a negative (reaction mix without DNA) and a positive (reaction mix with *Rickettsia lusitaniae* DNA detected in *Ornithodoros yumatensis* from a previous study in southern Mexico [*11*]) control. We sent amplicons of the expected size to Laboratorio de Biología Molecular y de la Salud, UNAM, for purification and sequencing. We compared the sequences obtained with those deposited in GenBank using BLAST (*13*). We deposited sequences recovered in this study in GenBank (accession nos. MG578509–MG578512). We performed global alignments for each gene using the ClustalW algorithm in MEGA 6.0 (https://www.megasoftware.net) and then concatenated them in BioEdit (https://www.mbio/ncsu.edu/BioEdit/bioedit.html). We selected the nucleotide substitution model based on the lowest AICc (Akaike information criterion, corrected). We then generated a maximum likelihood phylogenetic tree with 10,000 bootstrap replications in MEGA 6.0, using the close neighbor interchange method. Gaps were excluded from the analysis.

Of the 24 *D. parumapertus* ticks tested, 1 female and 3 males (16.6% total) were positive for the amplification of the gene *gltA*. Neither of the *Amblyomma* nymphal ticks was positive for *Rickettsia* DNA. In addition, we were able to amplify the other 3 genes in the 4 positive samples analyzed. The sequences obtained from the 4 ticks were 100% identical to each other for each corresponding gene. Comparison of 4 gene sequences exhibited 99%–100% identity with the corresponding sequences of *R. parkeri* (Table). The final supermatrix consisted of 2,308 bp (731 bp for *gtlA*, 429 bp for *ompA*, 744 bp for *ompB*, and 404 bp for *htrA* genes), with 670 variable sites, 494 singletons, and 176 parsimony informative sites. Additionally, our phylogenetic analysis corroborates the identity of the *Rickettsia* detected, as our sequences and those of references of the different strains of *R. parkeri* form a monophyletic clade with a support value of 98% (Figure 2).

## Conclusions

We identified DNA of *R. parkeri* in *D. parumapertus* ticks from Chihuahua and Sonora, nearly identical to the Black Gap strain of *R. parkeri* reported previously from *D. parumapertus* ticks from Texas. This tick species is widely distributed across much of northern Mexico, including the states of Baja California, Baja California Sur, Chiapas,

**Table.** Results of analysis of *Rickettsia* sequences recovered from ticks in Mexico to *Rickettsia parkeri* sequences from GenBank*

| Gene | Comparison strain (accession no.) | Sequence identity |
|---|---|---|
| *gltA* | *R. parkeri* Black Gap (KY124257.1) | 731/731, 100% |
| *ompA* | *R. parkeri* RAmova (MF034495.1) | 429/429, 100% |
| | *R. parkeri* Atlantic rainforest (KX137902.1) | 429/429, 100% |
| *ompB* | *R. parkeri* Black Gap (KY113111.1) | 735/744, 98.7% |
| *htrA* | *R. parkeri* (U17008.1) | 404/404, 100% |

*Analysis conducted using BLAST (*13*).

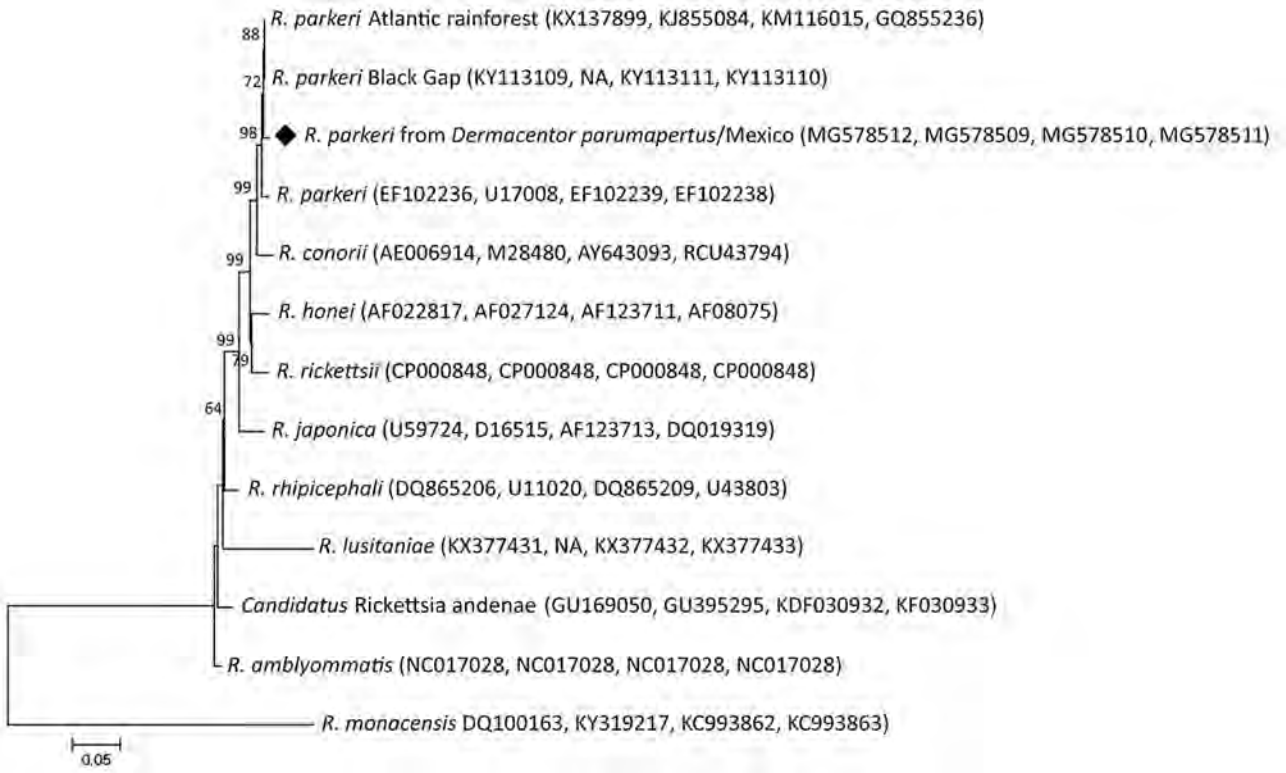


**Figure 2**. Maximum-likelihood phylogenetic tree generated by using the general time-reversible model using discrete gamma distribution for a total of 2,308 bp of the *gltA*, *htrA*, *ompB*, and *ompA* genes concatenated from a few members of the genus *Rickettsia*. Diamond indicates isolate obtained from ticks in Mexico. Bootstrap values >50% are indicated at the nodes (–ln = –6514.38). Numbers in parentheses are GenBank accession numbers. Scale bar indicates nucleotide substitutions per site. NA, not available.

Chihuahua, Coahuila, Durango, Hidalgo, Mexico City, San Luis Potosi, and Sonora (*8*,*14*), which suggests that this *Rickettsia* species could be present in other localities in Mexico. *D. parumapertus* ticks have a marked preference for lagomorphs; nonetheless, various other human-biting *Dermacentor* and *Amblyomma* tick species also parasitize lagomorphs, and there is a possibility that these species could acquire *R. parkeri* by co-feeding and subsequently transmit this agent to humans in these regions. The prevalence of *R. parkeri* in ticks from Mexico was consistent with other studies from Texas and Arizona, in which the prevalence ranged from 14% to 24% (*3*,*5*,*6*). We conducted our study in a region of Mexico where spotted fever is endemic; the incidence of spotted fever group rickettsiosis in Chihuahua in 2016 was 0.11 human cases/100,000 inhabitants and that in Sonora was 1.21 human cases/100,000 inhabitants (*15*). All these cases have been attributed to *R. rickettsii*, the cause of Rocky Mountain spotted fever. Although no confirmed cases of disease in humans have been attributed to *R. parkeri* Black Gap infections, previous experimental studies have shown that this isolate could be pathogenic in a guinea pig model (*3*). In addition, its phylogenetic relatedness with *R. parkeri* strain Atlantic rainforest, a well-recognized human pathogen (*4*), will have great meaning for healthcare workers in Mexico. However, further studies should be done to identify the potential of *R. parkeri* Black Gap and the strain of *R. parkeri* we identified as human pathogens. Our findings highlight the importance of studying rickettsial agents in wildlife to identify pathogens of potential public health concern.


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# Novel Focus of Sin Nombre Virus in *Peromyscus eremicus* Mice, Death Valley National Park, California, USA

**Joseph E. Burns,[1] Marco E. Metzger,[1] Sharon Messenger,[1] Curtis L. Fritz, Inger-Marie E. Vilcins, Barryett Enge, Lawrence R. Bronson, Vicki L. Kramer, Renjie Hu**

The deer mouse (*Peromyscus maniculatus*) is the primary reservoir for Sin Nombre virus (SNV) in the western United States. Rodent surveillance for hantavirus in Death Valley National Park, California, USA, revealed cactus mice (*P. eremicus*) as a possible focal reservoir for SNV in this location. We identified SNV antibodies in 40% of cactus mice sampled.

Hantaviruses constitute a worldwide group of predominantly rodentborne zoonotic pathogens, some of which have emerged as distinctive human health hazards. In North America, Sin Nombre virus (SNV) is the most widespread hantavirus and is of primary public health importance because of the high case-fatality rate (>35%) associated with hantavirus pulmonary syndrome (HPS) (*1*). The principal reservoir of SNV is the deer mouse, *Peromyscus maniculatus* (*2*), a habitat generalist. Evidence of virus infection can be detected in populations of these mice throughout their range (*3*). Several other hantavirus strains have been identified in other species of mice in the family Cricetidae, but pathogenicity of these strains to humans remains unresolved (*4*,*5*).

Field and laboratory studies in North America have confirmed a close association between rodent species and specific hantavirus strains; limited sustained interspecies infection have been documented (*6*). In California, detection of elevated serum antibody titers to hantavirus in rodents other than deer mice have been assumed to represent El Moro Canyon virus in western harvest mice (*Reithrodontomys megalotis*); Isla Vista virus in California voles (*Microtus californicus*); Limestone Canyon virus in brush mice (*P. boylii*); or "spillover" of SNV infection from *P. maniculatus* to other species (*1*). It is not uncommon to detect serum antibodies to SNV in a small percentage (<5%) of sampled species that share habitat with *P. maniculatus* mice, including the closely related *P. boylii*, *P. fraterculus*, *P. eremicus*, and *P. truei* mice, as well as wood rats, *Neotoma* spp. (*3*). In California, SNV-seropositive rodents typically have not been found to exceed the average *P. maniculatus* infection prevalence of ≈14%, although infection prevalence estimates have slightly exceeded 14% among certain populations of *R. megalotis* mice (*3*,*7*). These presumptive spillover infections are believed to be incidental and not likely to result in sustained transmission in the secondarily infected species (*1*,*3*).

We conducted a survey in Death Valley National Park, California, USA, to document the presence and estimate the infection prevalence of hantavirus in rodents living in and around buildings within select developed areas of the park. The ultimate objective was to assess potential occupational risk to staff and incidental risk to visitors in a highly visited geographic area of the state that was previously unstudied (*8*,*9*).

## The Study

Death Valley National Park is the largest national park in the contiguous 48 United States and is well known for having some of the hottest desert valleys in North America. Nearly 1 million persons visit the park each year; most visitation occurs during late autumn through mid-spring. Scotty's Castle, located in the northeastern region of the park (37.031°N, 117.340°W, elevation 950 m), is a popular tourist attraction that has ≈100,000 visitors annually (US National Park Service, pers. comm., 2015). The attraction consists of an extensive 2-story historic villa and associated outbuildings, used as offices, residences, a visitor's center, and storage, designed in Mission and Spanish Colonial Revival architecture. Below the villa lies a complex of service tunnels that are also included in tours. A year-round natural spring provides surface water flow for ≈0.3 km, creating an oasis in the desert.

We sampled rodents on the Scotty's Castle grounds in March 2010, April 2011, and October 2011. During each sampling event, we placed 100 aluminum Sherman live-traps (HB Sherman Traps Inc., Tallahassee, FL, USA) throughout the compound, including the riparian zone, inside and around occupied and unoccupied buildings, and in the service tunnels below the villa. We baited traps with dry oats and peanuts, set late in the afternoon, and retrieved captured rodents the following morning. We

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[1]These authors contributed equally to this article.

**Table**. Sin Nombre virus test results among *Peromyscus* mouse species, by test type, for sampling conducted in March 2010, April and October 2011, Death Valley National Park, California, USA

| | No. positive/no. tested (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | March 2010 | | | April 2011* | October 2011 | | |
| Species | Seropositive | RNA positive | Both | Seropositive | Seropositive | RNA positive | Both |
| *P. eremicus* | 2/32 (6.2) | 0/32 (0) | 11/32 (34.4) | 13/40 (32.5) | 5/28 (17.9) | 6/28 (21.4) | 9/28 (32.1) |
| *P. maniculatus* | 0/1 (0) | 0/1 (0) | 1/1 (100) | 0/1 (0) | 1/3 (33.3) | 1/3 (33.3) | 0/3 (0) |
| *P. crinitus* | 0/0 | 0/0 | 0/0 | 0/2 (0%) | 0/2 (0%) | 0/2 (0%) | 0/2 (0%) |

*PCR testing was not performed on April 2011 samples.

anesthetized the rodents, then collected reproductive and morphometric data, and identified the rodents to species. We collected a minimum of 11 μL of blood from the retrobulbar sinus of each mouse for ELISA testing for SNV IgG (*10*); all *Peromyscus* spp. mice were humanely euthanized for SNV molecular testing. Blood samples and carcasses were analyzed at the California Department of Public Health Viral and Rickettsial Disease Laboratory (Richmond, CA, USA).

A total of 109 mice were captured during the 3 sampling events (300 trap nights): 100 (91.7%) *P. eremicus,* 5 (4.6%) *P. maniculatus,* and 4 (3.7%) *P. crinitus*. For *P. eremicus* mice, antibodies reactive to SNV antigen, PCR positive tissue, or both were detected in 13 (40.6%) of

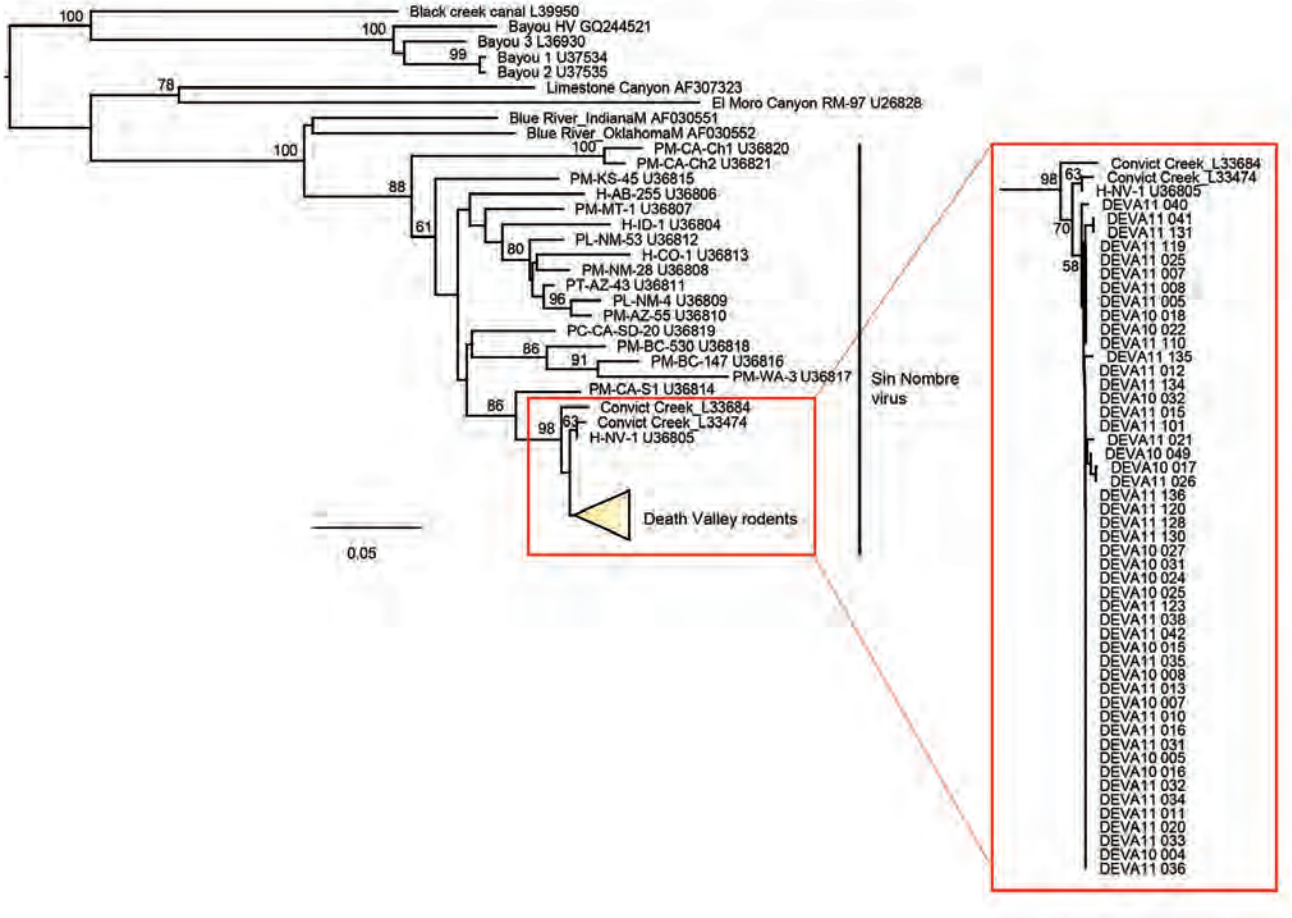


**Figure.** Phylogenetic tree of Gn glycoprotein sequences comparing hantaviruses sampled from 48 *Peromyscus eremicus* and 1 *P. maniculatus* (DEVA 10 022) mice collected in Death Valley National Park, California, USA (detail in inset box; GenBank accession nos. MG992890–MG992938). Representative reference sequences of hantaviruses in the United States were downloaded from GenBank (accession numbers included in taxon labels). The tree was reconstructed by analysis of 370 bases of the glycoprotein precursor (GPC) gene by using the neighbor-joining method, employing the HKY model, to estimate genetic distances. We estimated support for relationships by using a nonparametric bootstrap analysis (1,000 replicates). Nodes with bootstrap percentages >50% are indicated. Similar tree topologies were generated from maximum-likelihood (RAxML) and Bayesian (Mr. Bayes) phylogenetic analyses (not shown), implemented by using Geneious version 10.0 (Biomatters; Newark, NJ, USA). Scale bar represents genetic distance (nucleotide substitutions per site). DEVA, Death Valley National Park.

32 collected in March 2010, 13 (32.5%) of 40 collected in April 2011, and 20 (71.4%) of 28 collected in October 2011. For *P. maniculatus* mice, antibodies reactive to SNV antigen, PCR positive tissue, or both were detected in 1 (100%) of 1 collected in March 2010, 0 (0%) of 1 collected in April 2011, and 2 (66.6%) of 3 collected in October 2011 (Table). None of the 4 *P. crinitus* mice tested positive for SNV antibodies or viral RNA. We compared viral RNA sequence (Gn) results for 48 *P. eremicus* and 1 *P. maniculatus* mice collected in each of the 3 collection periods to related hantaviruses and found a close consensus (>98%) to Convict Creek viruses 74 and 107 ([GenBank accession nos. L33474 and L33684) (*11*) (Figure).

## Discussion

The *P. maniculatus* deer mouse is recognized as the primary reservoir for SNV in the western United States. Published estimates for SNV seroprevalence are consistently higher in *P. maniculatus* mice than for any other *Peromyscus* species. Rodent hantavirus surveillance in California during 2001–2010 (*7*) detected the serum antibody to SNV among 14% (1,058/7,621) of deer mice statewide; concurrently, SNV seroprevalence for *P. eremicus* mice was significantly lower at 3.7% (102/2,723) and did not exceed this highest site-specific estimate at any individual surveillance site.

We identified serum antibodies to SNV in 40% of *P. eremicus* mice sampled. The consistently high seroprevalence over 3 sample periods suggests that SNV is efficiently transmitted and maintained within this population. The sequence characterization of viral RNA from seropositive *P. eremicus* mice further substantiates that the virus closely resembles type strains of pathogenic SNV associated with HPS.

The factors necessary to sustain a virus–reservoir relationship are both intrinsic and extrinsic. Hantaviruses are believed to have coevolved with their respective rodent hosts (*1*). *P. eremicus* mice are most closely phylogenetically related to *P. maniculatus* and *P. leucopus* mice, reservoirs for hantaviruses SNV and Monogahela virus, respectively, which are recognized to cause HPS (*12*). The close genetic similarity among these species may best enable *P. eremicus* mice among *Peromyscus* spp. mice in California to serve as a viable alternative host for SNV or to harbor a coevolved hantavirus of similar SNV lineage.

Hantaviruses are transmitted between rodent hosts through direct contact. Thus, a minimum population density is required to sustain transmission within an isolated group. The optimal habitat provided by the oasis setting of our study enables the typically solitary cactus mouse (*13*) to achieve a greater population density not generally found in most of the sylvan desert habitats where these mice are native (0.21–3.3/hectare) (*14*). Further studies in similar high density/optimal peridomestic habitats, which are associated with higher SNV infection prevalence (*1*), are needed to establish whether this high level of infection is reflective of the unique environment or an alternate phenotypic expression or strain, considering the enhanced transmission and maintenance of SNV in cactus mice detected at this location.

These findings underscore the importance of rodent exclusion and management in and around rural and semirural buildings where risk for contact between rodents and humans is high, even in the absence of *P. maniculatus* mice (*9,15*). Park leadership and staff were notified of our study results and given training on hantavirus awareness and prevention, and hantavirus pamphlets were made available for visitors to the park.

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Animal handling techniques were performed in accordance with guidelines of the American Society of Mammalogists for the use of wild mammals in research (https://doi.org/10.1644/10-MAMM-F-355.1).

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# Listeriosis Outbreaks Associated with Soft Cheeses, United States, 1998–2014[1]

**Kelly A. Jackson, L. Hannah Gould, Jennifer C. Hunter, Zuzana Kucerova, Brendan Jackson**

Since 2006, the number of reported US listeriosis outbreaks associated with cheese made under unsanitary conditions has increased. Two-thirds were linked to Latin-style soft cheese, often affecting pregnant Hispanic women and their newborns. Adherence to pasteurization protocols and sanitation measures to avoid contamination after pasteurization can reduce future outbreaks.

*Listeria monocytogenes* is a widely distributed environmental bacterium that can grow at refrigeration temperatures. Infection can cause severe illness and death. Persons at higher risk for infection include older adults, persons with weakened immune systems, and pregnant women and their newborns.

Listeriosis outbreaks have been associated with refrigerated ready-to-eat foods, including hot dogs, delicatessen meats, soft cheeses, milk, and other dairy products. For soft-ripened cheeses, the risk for listeriosis per serving is estimated to be 50- to 160-fold greater for cheese made from unpasteurized milk than pasteurized milk (*1*). Pasteurization kills *L. monocytogenes*; however, milk labeled as pasteurized and dairy products made from pasteurized milk can become contaminated due to inadequate hygiene practices after pasteurization. The earliest reported listeriosis outbreak in the United States in 1985, associated with Latin-style cheese (in particular, queso fresco and cotija), resulted in 142 illnesses, 28 deaths, and 20 fetal losses (*2*). Although the cheese was labeled as made from pasteurized milk, raw milk was inadvertently introduced into the pasteurized milk.

A US retail survey of several soft cheeses (Latin-style, blue-veined, mold-ripened) from 2000–2001 detected *L. monocytogenes* in 1.3% of cheeses made from unlabeled or unpasteurized milk and 0.5% of cheeses from pasteurized milk (*3*). However, pasteurized-milk cheese is much more commonly consumed than cheese made from unpasteurized milk. In a survey of food exposures conducted in 10 US states during 2006–2007, respondents reported eating types of soft cheeses (15.3% for blue-veined cheese, 6%–11% for other soft cheeses; pasteurization status unknown) more frequently than they reported eating cheeses made from unpasteurized milk in the previous 7 days (1.6%) (*4*). We describe outbreaks linked to soft cheese (both soft-ripened and acid-coagulated–ripened cheeses), demographic characteristics of the persons affected, and possible contributing factors to help inform prevention messaging for persons at higher risk.

## The Study

Health departments in the United States electronically submit reports of foodborne disease outbreaks to the Foodborne Disease Outbreak Surveillance System (FDOSS). FDOSS captures information on etiology; implicated food; number of illnesses, hospitalizations, and deaths; and other features. We queried FDOSS for *L. monocytogenes* outbreaks (≥2 cases) in the United States from 1998, when pulsed-field gel electrophoresis was first used to investigate listeriosis outbreaks, through 2014. We obtained information on fetal losses; deaths; number of cheese types; pasteurization status of milk used to make the cheese; recall issuance; and isolate subtyping from published reports (*5*–*11*), unpublished data, and food recall announcements. We considered infections in pregnant women or infants ≤28 days of age to be pregnancy-associated. We considered outbreaks multistate if exposure to the implicated food occurred in >1 state.

Of 58 listeriosis outbreaks reported during 1998–2014, a total of 17 (30%) were associated with soft cheese (Figure), and resulted in 180 illnesses, 14 fetal losses, and 17 deaths (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/6/17-1051-Techapp1.pdf). Most patients (146, 88%) were hospitalized. Of 116 patients for whom we had information on ethnicity, 38 (33%) were Hispanic. Of 140 cases with available data, 62 (44%) were pregnancy-associated. Median outbreak size was 8 cases (range 2–34 cases). Ten outbreaks were multistate, and 16 were associated with commercial products, of which 14 involved cheeses produced in the United States. The proportion of listeriosis outbreaks linked to soft cheese made from pasteurized milk (12 outbreaks, 33%) was significantly higher during 2007–2014 than during 1998–2006 (1 outbreak, 5%;

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[1]Preliminary results from this study were presented at the International Conference on Emerging Infectious Diseases; March 11–14, 2012; Atlanta, GA, USA.

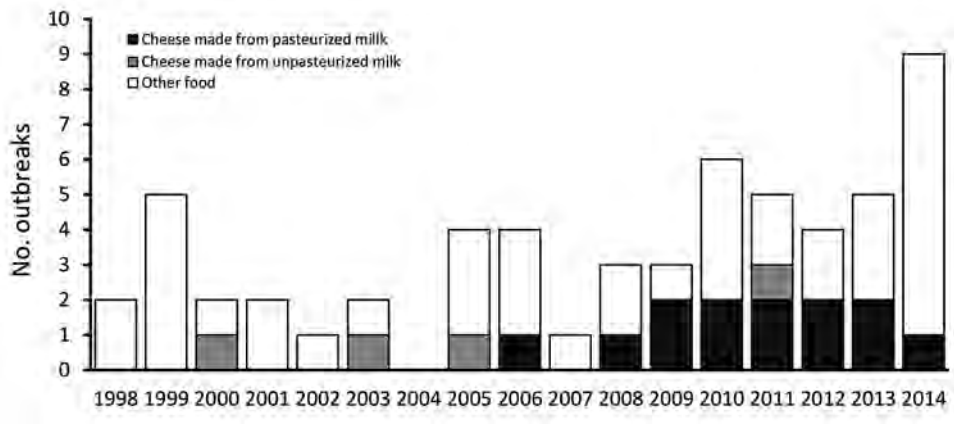


**Figure**. Listeriosis outbreaks associated with soft cheeses and other foods, United States, 1998–2014. The Centers for Disease Control and Prevention began pulsed-field gel electrophoresis subtyping of clinical *Listeria monocytogenes* isolates in 1998 and launched the use of standardized interview questions in 2004; the routine use of whole-genome sequencing was introduced in 2013.

p = 0.009). Clinical isolates from soft-cheese outbreaks predominantly fell in lineage I (14 outbreaks, 82%). We found 2 sequence type (ST) and clonal complex (CC) combinations in multiple outbreaks (ST5/CC5, 5 outbreaks; ST6/CC6, 2 outbreaks), whereas other ST and CC combinations appeared in single outbreaks (e.g., ST663 or ST558).

Latin-style cheeses were implicated in 11/17 (65%) outbreaks, accounting for 98 (54%) cases of listeriosis. The remaining outbreaks involved sheep's-milk cheese, Middle Eastern– or Eastern European–style cheeses, Italian-style cheese, blue-veined cheese, and soft-ripened cheeses (1 outbreak each). Nearly all outbreaks (13/17) resulted in recalls.

FDA inspections of cheese-making facilities associated with outbreaks found sanitation and hygiene deficiencies (e.g., roof leaks over manufacturing equipment, an open sewer vent in a manufacturing room, and food-contact aprons stored in restrooms) (*7*–*9*); pest infestations (e.g., cockroaches, flying insects) (*8*); failure to hold food at proper temperature (*8*); and presence (*8*,*11*,*12*) or persistence of *L. monocytogenes* in environmental niches of processing plants (*9*).

## Conclusions

Consumption of contaminated soft cheese made under unsanitary conditions continues to be a common cause of listeriosis outbreaks in the United States. Multiple types of soft cheeses have been implicated in outbreaks, with most outbreaks linked to Latin-style soft cheese. These outbreaks disproportionately affect Hispanic pregnant women and their neonates, a group with 24 times higher risk for listeriosis than that of the general US population (*13*). The proportion of listeriosis outbreaks caused by consumption of soft cheese made from pasteurized milk has increased in recent years. Reasons for the increase may include the growing US Hispanic population (which increased from 11% in 1998 to 17% in 2014 [*14*]); a 2.5-fold increase in per capita consumption of cheese from 1980 to 2013 (*15*); consumer demand for certain types of cheeses; and an increase in the number of small producers, some of which had sanitary deficiencies. Better outbreak detection due to improved molecular subtyping and epidemiologic methods have resulted in a greater number of solved outbreaks; however, we did not observe a similar increased proportion of outbreaks linked to other foods during the same period. This finding suggests that changes in outbreak detection are unlikely to be the only contributor.

Despite the much higher risk for listeriosis per serving of cheese made from unpasteurized rather than pasteurized milk, during the study period, only about one quarter (4/17) of all outbreaks were linked to consumption of soft cheese made from unpasteurized milk. This result may be due, in part, to public health messages advising consumers at higher risk for listeriosis not to eat these cheeses.

For instances in which information was available, we noted environmental contamination and sanitation deficiencies in all outbreaks associated with cheese made from pasteurized milk. Although some of these deficiencies were unlikely to contaminate cheese directly, they indicate a lack of attention to sanitation and hygiene. This finding highlights the importance of robust sanitation and *L. monocytogenes* monitoring programs for cheese manufacturers.

Consumers, particularly persons at high risk for listeriosis, are advised to avoid unpasteurized milk and dairy products made from unpasteurized milk. Soft cheeses made with pasteurized milk, including commercial cottage cheese, cream cheese, and processed mozzarella, are generally considered safe. However, some soft cheeses made with pasteurized milk, particularly Latin-style soft cheeses, have been produced in facilities with improper processing conditions, resulting in *L. monocytogenes* contamination. Consumers cannot evaluate the conditions under which a cheese was made on the basis of labeling or other attributes of the product. We advise persons at higher risk for listeriosis (the elderly, persons with immunocompromising conditions, and pregnant women) to carefully consider whether to consume Latin-style and other soft cheeses implicated in previous outbreaks.

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# Intense Focus of Alveolar Echinococcosis, South Kyrgyzstan

**Bakhadyr Bebezov, Nurlan Mamashev, Tilek Umetaliev, Iskender Ziadinov, Philip S. Craig, Deborah E. Joekel, Peter Deplazes, Felix Grimm, Paul R. Torgerson**

Human alveolar echinococcosis (AE) is a highly pathogenic zoonotic parasitic disease caused by *Echinococcus multilocularis*. An ultrasound study in southern Kyrgyzstan during 2012 revealed a prevalence of 4.2% probable or confirmed AE and an additional 2.2% possible AE, representing an emerging situation. The risk for probable or confirmed AE was significantly higher in dog owners.

Human alveolar echinococcosis (AE), caused by the larval stage of *Echinococcus multilocularis*, is a lethal parasitic zoonosis if untreated (*1*,*2*). In China, hyperendemic foci of disease have been described (*3*) with prevalences >5%. AE incidence recently has increased in Europe (*4*). In Kyrgyzstan, the disease incidence has increased rapidly since 2000; a total of 148 AE cases were reported in 2013 (*5*).

Hospital records for AE notifications identified a cluster of cases in the Alay Valley in southern Kyrgyzstan. Therefore, in 2012, we conducted an ultrasound study of the population of Sary Mogol (location 39.66°N, 72.88°E) to determine the extent of infection and to investigate the epidemiology of the disease in this district.

## The Study

The study was a census type of design. We obtained informed consent from each study participant or, for children, consent from parents. Participants were interviewed using a questionnaire in Kyrgyz and given an abdominal ultrasound examination. For participants with hepatic lesions suspected to be AE or cystic echinococcosis (CE) or who reported previous treatment for echinococcosis, a venous blood sample was taken for further analysis. The Ministry of Health of the Kyrgyz Republic provided ethics approval for this study.

We detected specific IgG from collected serum in 3 genus-specific ELISAs based on *E. granulosus* hydatid fluid, native protoscolex antigens, and antigen B (*6*). Specific antibodies against *E. multilocularis* were demonstrated using affinity purified Em2G11 antigen (*6*) and the recombinant Em18 antigen (*7*). We further investigated persons who were negative in these ELISAs with a commercial Western blot (*Echinococcus* western blot IgG; LDBio Diagnostics, Lyon, France).

Where possible, we followed participants to treatment. For some patients we obtained samples from resected lesions. DNA was isolated, followed by amplification of part of the *E. multilocularis* mitochondrial 12S rRNA gene (*8*). Confirmation of diagnosis was also achieved by histologic examination of the resected lesions. Possible AE cases were those with ultrasound lesions and no follow-up. Probable cases additionally had positive serologic results, and confirmed cases were positive by histology, PCR, or both.

We analyzed data with all AE cases and with probable or confirmed AE as the dependent variable using a relative risk generalized linear model (GLM). We analyzed differences in lesion sizes between seropositive and seronegative groups by the Wilcoxon test and used Fisher exact test to examine differences in seroprevalence between persons with confirmed AE and persons with only an ultrasound diagnosis.

We examined 1,617 persons (Figure 1) (48% of the population of the district; online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/6/16-1641-Techapp1.pdf). Of these, 106 persons had ultrasound findings consistent with AE (including 1 concomitantly infected with CE). Probable or confirmed cases (online Technical Appendix) with ≥2 diagnostic criteria (Figure 1) were subsequently diagnosed in 68 (4.2%) persons leaving 36 (2.2%) with possible AE. Three (0.2%) additional persons had lesions suggestive of CE. For 9 persons, images were recorded as inconclusive.

The GLM demonstrated an increase in the risk for possible infection with AE in dog owners, male patients, and persons who practiced home slaughter of livestock. Only dog ownership increased the risk for probable AE infection (Table 1).

Of the 106 persons in whom AE was diagnosed, we detected specific antibodies in 40 (42.1%) of the 95 available serum samples by 3 different ELISAs (Table 2). Western blot analysis of negative serum identified specific antibodies on *Echinococcus* genus level in 9 additional patients. Thus, 49 of the 95 persons had serologic evidence of infection. Lesions, measured in 53 patients, ranged from

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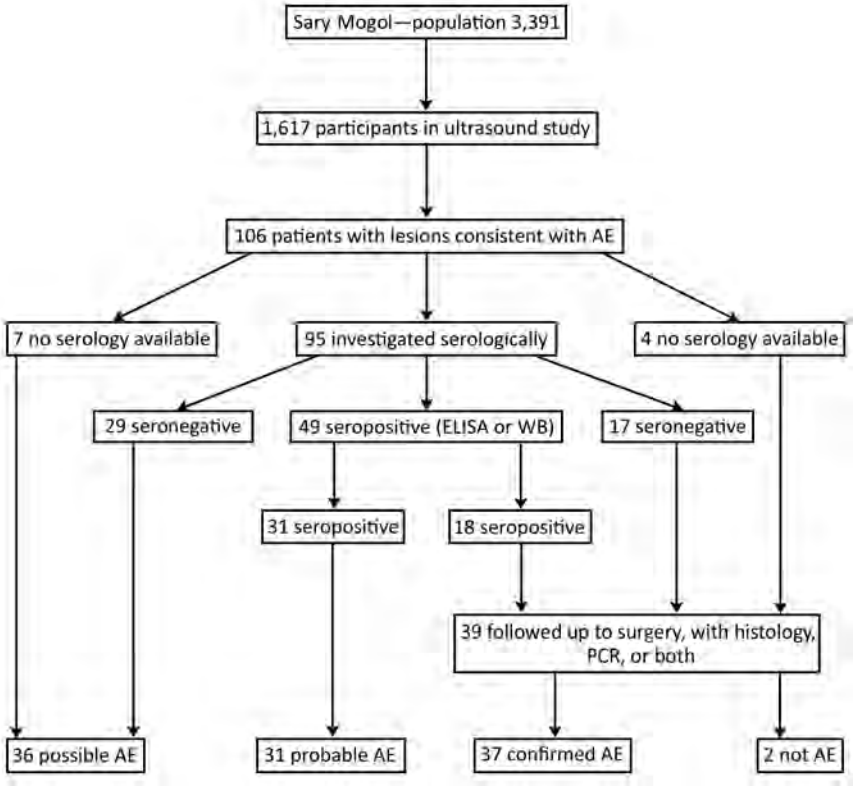


**Figure 1.** Flowchart of patient selection, ultrasound investigation, serologic testing, and case definitions in a study of AE, southern Kyrgyzstan, 2012. AE, alveolar echinococcosis; WB, Western blot.

5 to 197 mm (mean 28 mm). The mean size of lesions in the 22 ELISA- or Western blot–seropositive persons was 46.1 mm, significantly larger than the mean size of 11.0 mm for lesions from the 27 seronegative patients (p = 0.01; Figure 2).

By September 2017, a total of 39 persons were known to have been treated by hepatic surgery. Among them, AE was confirmed in 37 (94.7%) by histology, PCR, or both. From these 37 persons, 35 serum samples were available; 18 (51.4%) showed serologic evidence of infection. This finding did not differ significantly from the proportion of persons without follow-up data who had serologic evidence of infection (31/61) (Figure 1).

The decreasing risk for possible AE with increasing age, contrasting with findings in areas of China where AE is highly endemic (*9*), indicates different dynamics and hence reflect an emerging epidemic of human AE in Kyrgyzstan. Consequently, this observation supports the hypothesis that the epidemic could be linked to the dissolution of the Soviet Union in 1991 (*5*). In our study, the higher risk for possible AE in male than in female patients contrasts with risk in areas of western China where AE is endemic. The reasons for this difference are unclear but might reflect behavioral (e.g., rates of dog contact) or cultural reasons that result in a greater risk for exposure for female persons in China (*9*) and for male persons in Kyrgyzstan. However, both risk factors disappear if only probable or confirmed AE is used as the case definition for AE.

The poorer sensitivity (≈50%–60%) of the serologic tests as compared with the validations in Switzerland (*6*) might result from cases in Switzerland being

**Table 1.** Relative risk from multivariable analysis of persons with an ultrasound diagnosis of AE and persons with a probable or a confirmed diagnosis of AE, southern Kyrgyzstan, 2012*

| Dependent variable, risk factor | Relative risk (95% CI) | p value |
|---|---|---|
| Ultrasound diagnosis of AE | | |
| Patient age† | 0.982 (0.969–0.995) | 0.0074 |
| Male sex | 1.56 (1.07–2.29) | 0.021 |
| Dog ownership | 1.82 (1.24–2.72) | 0.0025 |
| Home slaughter of livestock | 1.60 (1.03–2.56) | 0.043 |
| Dog ownership among persons with probable and confirmed AE‡ | 2.81 (1.64–5.09) | 0.00033 |

*Probable diagnosis: ultrasound and serology; confirmed diagnosis: ultrasound and histology/PCR. AE, alveolar echinococcosis.
†Median age of persons with possible AE was 24 y; median age of AE-negative persons was 28 y.
‡Dog ownership was the only risk factor remaining as significant when only probable and confirmed AE were analyzed as the dependent variables.

**Table 2.** Ultrasound results partially confirmed with PCR/histology for AE or CE in relation to serology, southern Kyrgyzstan, 2012*

| | ELISA | | | | WB† | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Ultrasound results | No. available samples‡ | Neg | AE/CE§ | AE¶ | No. available samples‡ | Neg | AE/CE | CE | AE |
| AE, n = 106 | 95 | 55 | 25 | 15 | 43 | 34 | 6 | 3 | 0 |
| Confirmed AE,# n = 37 | 33 | 18 | 10 | 5 | 13 | 10 | 3 | 0 | 0 |
| Inconclusive, n = 9 | 6 | 5 | 1 | 0 | 4 | 4 | 0 | 0 | 0 |
| CE, n = 3 | 3 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| CE and AE, n = 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Negative but with history of CE/AE, n = 13 | 6 | 1 | 3 | 2 | 1 | 1 | 0 | 0 | 0 |

*AE/CE indicates the test is specific only to genus level. AE, alveolar echinococcosis; CE, cystic echinococcosis; neg, negative; WB, Western blot.
†ELISA-negative serum only.
‡Not every patient for whom echinococcosis was diagnosed by ultrasound had inconclusive results or a history of echinococcosis provided a blood sample. For some of these patients, insufficient serum was available from the blood sample to undertake both ELISA and WB.
§*E. granulosus* hydatid fluid and/or native protoscolex antigens and/or antigen B positive but Em18 and EmG11 negative.
¶Em18 and/or EmG11 positive (*E. granulosus* hydatid fluid and/or native protoscolex antigens and/or antigen B positive or negative).
#Diagnosis confirmed by PCR and/or histology of resected lesion.

at a more advanced stage of clinical disease. The fact that persons with larger lesions were more likely to be seropositive indicates that seroconversion might not occur either during the early stages of the disease or when only abortive lesions are present. Similar patterns of low seroreactivity were observed in the AE endemic focus in south Gansu (China) (*10*) and included persons with possible abortive forms of the disease. In addition, the mean age of ultrasound-positive persons in our study is 9 years younger than those receiving surgical treatment resulting from clinical disease, indicating that our study has detected an early stage of the disease in these persons. In the patients followed up, the seropositivity rate for those with AE confirmed by histopathology did not differ significantly from the rate for those with only an ultrasound diagnosis. Thus, we can conclude that the same proportion of patients without histologic or PCR confirmation (to date) are likely to have AE. Although the diagnostic efficiency of ultrasound should be estimated with caution, these results might indicate a specificity as high as 99.7% (online Technical Appendix). However, including only probable or confirmed cases in the regression analysis increased the association with dog ownership while eliminating other risk factors. This finding might indicate that some of the possible AE cases are not AE. Nevertheless, specificity of ultrasound in this scenario remains at 97.4%.

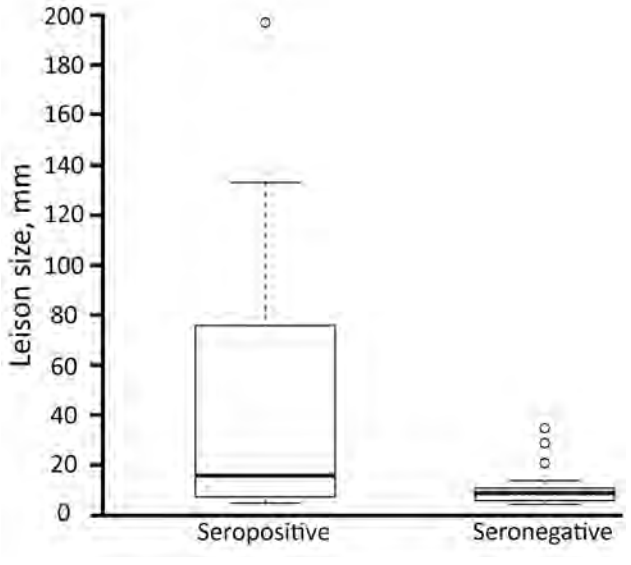


**Figure 2.** Differences in sizes of lesions (n = 49 serum samples available from 57 patients with measured lesions) diagnosed in persons seropositive by ELISA, Western blot, or both (n = 27) or seronegative (n = 22) in a study of alveolar echinococcosis, southern Kyrgyzstan, 2012. Box plots indicate interquartile range (box top and bottom), median (black horizontal line), 1.5 times interquartile range (error bars), and extreme values (circles).

## Conclusions

We documented a highly endemic focus of human AE in which the prevalence of confirmed or probable AE was ≈4.2% in southern Kyrgyzstan. A characteristic of communities with high levels of human AE are concomitant high prevalences of *E. multilocularis* in the dog population, such as western Sichuan Province (*11*). The mole vole (*Ellobius tancrei*) has recently been confirmed as a natural intermediate host of *E. multilocularis* in Sary Mogol and has identical DNA sequence for the *E. multilocularis* haplotype described in feces of local domestic dogs (*12*). In Kyrgyzstan, prevalences in dogs of 20% have been observed (*13*). In our study, dog owners had 1.8 times higher risk for infection than non–dog owners, increasing to 3.3 times for confirmed or probable infection, thus providing evidence that dogs are involved in transmission to humans.

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# Pathogenic *Leptospira* Species in Insectivorous Bats, China, 2015

**Hui-Ju Han, Hong-Ling Wen, Jian-Wei Liu, Xiang-Rong Qin, Min Zhao, Li-Jun Wang, Li-Mei Luo, Chuan-Min Zhou, Ye-Lei Zhu, Rui Qi, Wen-Qian Li, Hao Yu, Xue-Jie Yu**

PCR amplification of the *rrs2* gene indicated that 50% (62/124) of insectivorous bats from eastern China were infected with *Leptospira borgpetersenii*, *L. kirschneri*, and several potentially new *Leptospira* species. Multilocus sequence typing defined 3 novel sequence types in *L. kirschneri*, suggesting that bats are major carriers of *Leptospira*.

Leptospirosis is a zoonotic disease caused by the pathogenic spirochetes of the bacterial genus *Leptospira* (*1*). Although leptospirosis is mainly prevalent in tropical and subtropical countries (*2*), it is considered an emerging or reemerging zoonosis of global public health concern (*1*). In China, leptospirosis is listed as a category B notifiable disease (*3*). Globally, rodents are recognized as important reservoir hosts (*4*); however, a growing number of studies highlight the potential role of bats in the epidemiology of *Leptospira* (*4*). So far, knowledge on *Leptospira* in bats in China is lacking. Therefore, we screened archived bat kidney samples for *Leptospira* species to evaluate the potential role of bats in the ecology of *Leptospira* in China.

## The Study

During July–October 2015, we captured 124 insectivorous bats with nets in Mengyin County, Shandong Province, China; the bats were initially intended for viral metagenomic analysis. Details regarding anesthetization of bats and tissue sampling were described previously (*5*). We collected the kidneys, storing them at –80°C until analysis. We identified bat species by using PCR amplification and DNA sequencing of the cytochrome B (*cytB*) gene as described previously (*6*). The 124 bats were classified into 4 species of the Vespertilionidae family (26 *Eptesicus serotinus* bats from 2 farmers' houses, 30 *Myotis fimbriatus* bats and 10 *M. ricketti* bats from a city sewer, and 58 *Myotis pequinius* bats from a karst cave).

We designated bat kidney samples by using the abbreviation of Shandong plus the sample identification number (e.g., SD-49). We extracted DNA from bat kidney samples by using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. To identify the species of *Leptospira* in bats, we amplified the 16S rRNA gene (*rrs2*) by using nested PCR with primers Lepto 16S-1st-F, Lepto 16S-1st-R, Lepto 16S-2nd-F, and Lepto 16S-2nd-R (*7*). We cloned the *rrs2* PCR products (642 bp) into the pMD 19-T vectors (TaKaRa, Shiga, Japan) and randomly picked 1 colony for Sanger sequencing using M13 universal primers on both DNA strands.

On the basis of *rrs2* amplification results, 62 (50.0%) of 124 bats tested were positive for *Leptospira*. All the *E. serotinus* bats (0/26, 0.0%) were negative for *Leptospira*, whereas the *M. fimbriatus* bats (19/30 [63.3%]), *M. ricketti* bats (6/10 [60.0%]), and *M. pequinius* bats (37/58 [63.7%]) showed high rates of infection.

Using ClustalW with MEGA 7.0 (http://www.megasoftware.net), we aligned the *rrs2* sequences from this study with reference *Leptospira* species downloaded from GenBank. We constructed a neighbor-joining phylogenetic tree (also by using MEGA 7.0) (*8*). On the basis of the *rrs2* phylogeny, all *Leptospira* detected in bats in Mengyin County clustered into the pathogenic group and could be divided into ≥14 clades (clades A–N). Although clade H was most likely associated with *L. borgpetersenii*, the *Leptospira* detected in bats in Mengyin County were divergent from any known *Leptospira* species (Figure 1) and any previously published *Leptospira* sequences from bats (Figure 2); therefore, these organisms might represent potentially new *Leptospira* species. We deposited the 62 *rrs2* sequences of *Leptospira* in the Mengyin County bats into GenBank (accession nos. MF498596–657).

To characterize the *Leptospira* species detected in bats from Mengyin County, we attempted multilocus sequence typing (MLST) on 7 housekeeping genes (*glmU, pntA, sucA, tpiA, pfkB, mreA,* and *caiB*), as previously described (*9*). We assigned alleles for all 7 loci by using a publicly available *Leptospira* MLST website (https://pubmlst.org/leptospira) and defined sequence types (STs) by using the allelic profiles (*glmU-pntA-sucA-tpiA-pfkB-mreA-caiB*).

Because we conducted MLST using kidney DNA rather than DNA from isolates, the results were arduous to

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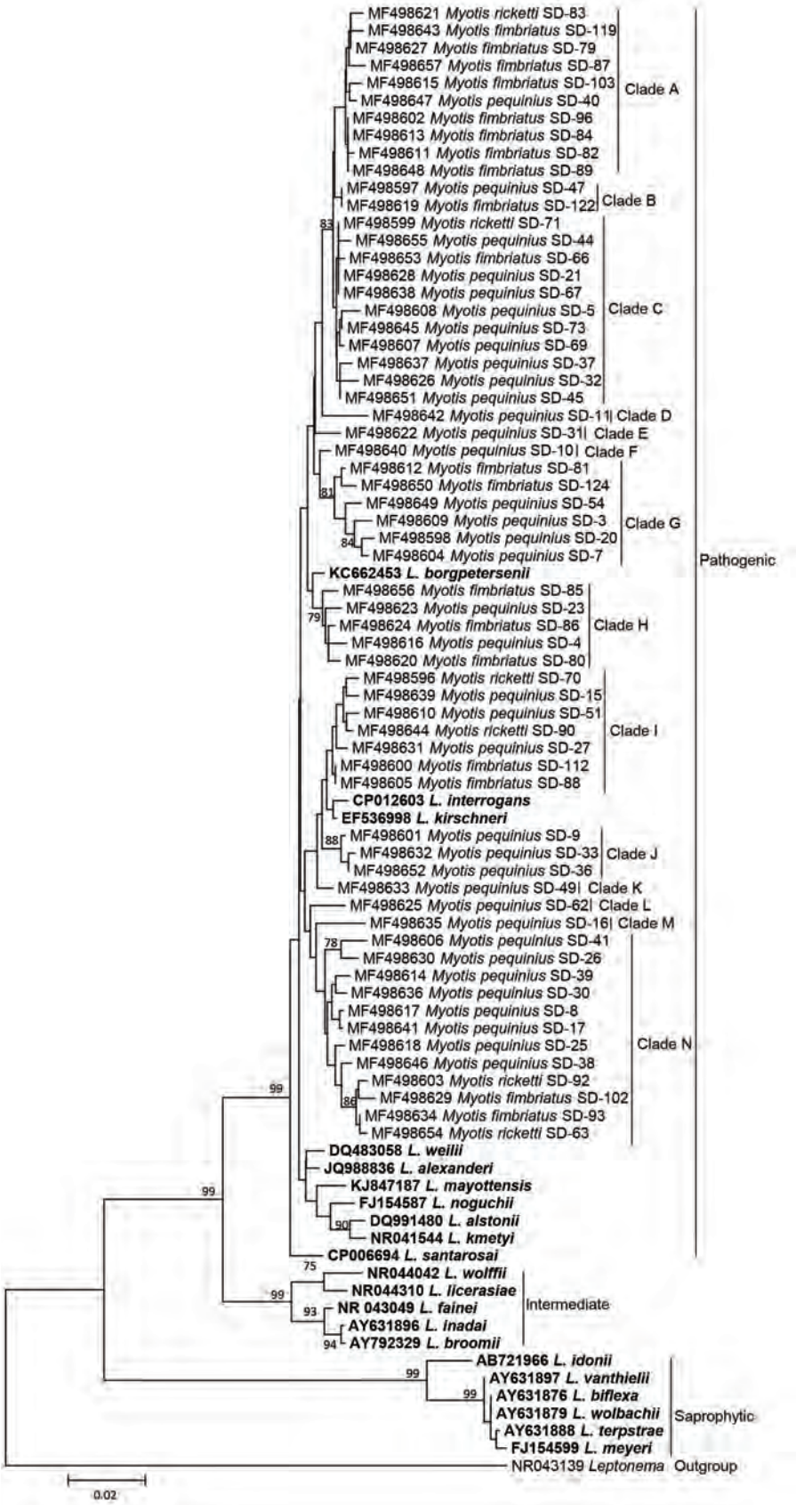


**Figure 1.** Neighbor-joining phylogenetic tree based on *rrs2* gene of *Leptospira* isolates from bats, Mengyin County, Shandong Province, China, and reference *Leptospira* sequences from GenBank (boldface). We constructed the tree with the *rrs2* sequences (642 bp) from this study by using the Kimura 2-parameter model with MEGA 7.0 (http://www.megasoftware.net); we calculated bootstrap values with 1,000 replicates. Sequences of *Leptospira* detected in bats in this study are shown with the GenBank accession number, the Latin name of the bat species in which *Leptospira* was detected, and the corresponding bat number. Only bootstrap values >75% are shown. Scale bar indicates nucleotide substitutions per site.

obtain. Only 35 of the 62 *rrs2*-positive bats were successfully amplified for ≥1 gene. For three samples, SD-49, SD-88, and SD-112, all 7 loci were obtained. We uploaded the allele data to the *Leptospira* MLST database and assigned a novel allele number for each gene (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/6/17-1585-Techapp1.pdf). According to the allelic profiles, we classified the organisms as *L. kirschneri*, assigning all 3 with novel STs (ST244 for SD-49, ST246 for SD-88, and ST245 for SD112).

We obtained 32 incomplete allelic profiles in all. After searching the *Leptospira* MLST database for each gene, we found that the loci of this study could not match with any known alleles and that they represented novel

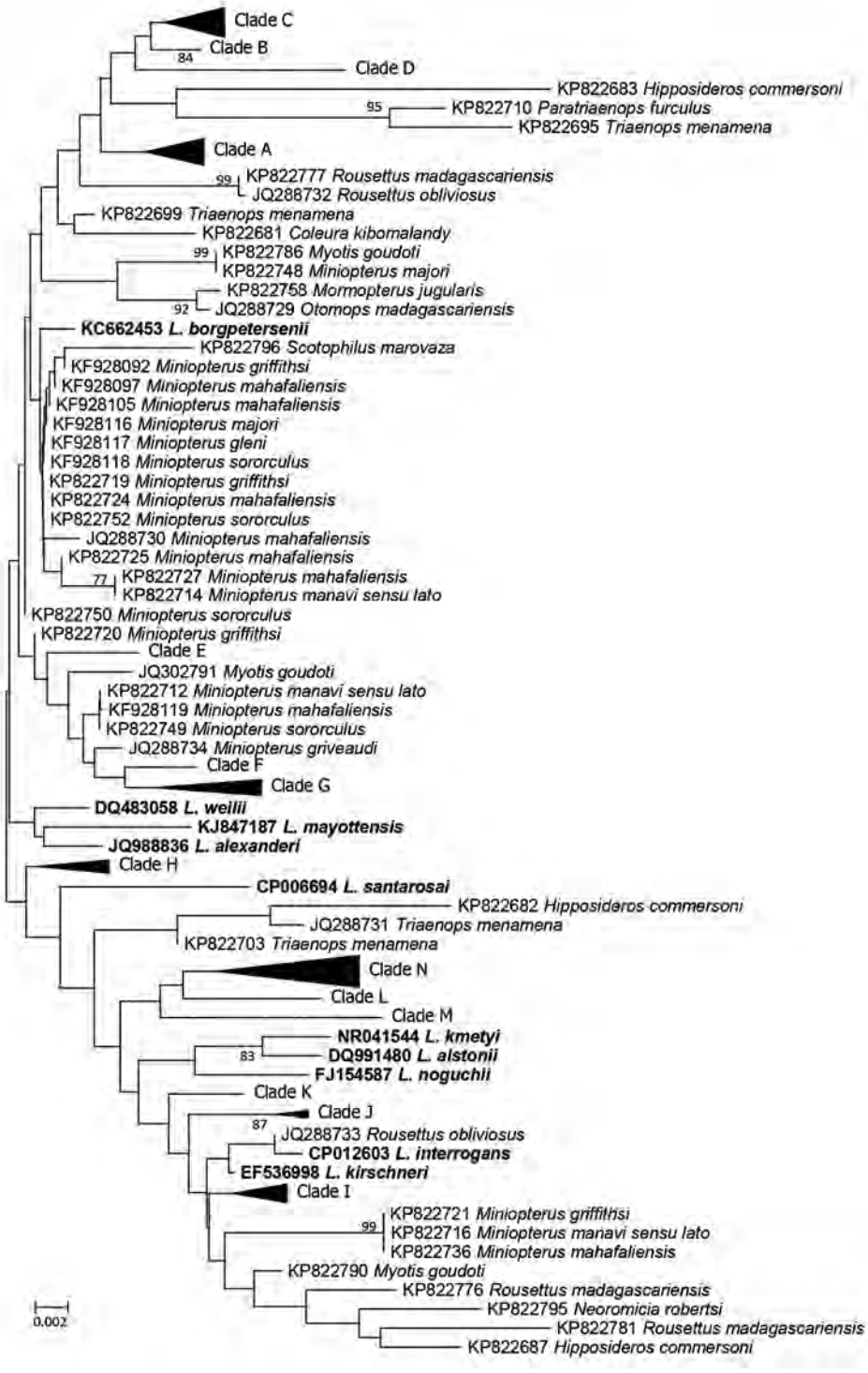


**Figure 2.** Neighbor-joining phylogenetic tree based on *rrs2* gene of pathogenic *Leptospira* isolates from bats, Mengyin County, Shandong Province, China, and reference *Leptospira* sequences from GenBank that had been previously isolated from bats (boldface). We constructed the tree with bat *Leptospira rrs2* sequences (446 bp) from this study and previous studies by using Kimura 2-parameter model with MEGA 7.0 (http://www.megasoftware.net); we calculated bootstrap values with 1,000 replicates. *Leptospira* detected in this study are shown with compressed clades, and bat *Leptospira* sequences from previous studies are shown with the GenBank accession number and the Latin name of the bat species in which *Leptospira* was detected. Only bootstrap values >75% are shown. Scale bar indicates nucleotied substitutions per site.

alleles; however, novel alleles could not be assigned for incomplete allelic profiles. Phylogenies based on each of the 7 genes showed inconsistent topologies for individual bats, indicating co-infection with different *Leptospira* species in the Mengyin County bats (online Technical Appendix Table, online Technical Appendix Figures 1–7). For SD-103, for example, we amplified 5 of the 7 genes. On the basis of *glmU*, *tpiA*, *pfkB*, and *mreA*, SD-103 clustered with *L. borgpetersenii*; however, on the basis of *sucA*, SD-103 fell into the group of the potentially new *Leptospira* sp. 2.

Because we did not conduct culture isolation with the archived bat kidney samples, further isolation with fresh bat kidney or urine samples will be needed to enable a thorough genotyping analysis of *Leptospira* species in the Mengyin County bats. Altogether, our study suggested that bats in Mengyin County carried a wide diversity of *Leptospira*, and the actual genetic diversity is likely even higher.

In our study, all the *E. serotinus* bats were negative for *Leptospira*, whereas *M. fimbriatus*, *M. ricketti*, and *M. pequinius* bats showed a high rate of infection. This finding might be explained by a widely accepted belief that *Leptospira* mainly circulate in humid environments (*10*). *E. serotinus* bats were captured from the eaves of 2 farmers' houses, where the habitats were dry, whereas the *M. fimbriatus*, *M. ricketti*, and *M. pequinius* bats were sampled from the humid city sewer and karst cave.

So far, *Leptospira* has been detected in ≈50 bat species belonging to 8 bat families from tropical and subtropical regions as well as part of Europe (*4*). Although the role of bats as carriers of *Leptospira* associated with human leptospirosis remains uncertain, intrusion into bat habitats and increasing urbanization that results in the cohabitation of bats and humans are likely to increase the opportunity for batborne *Leptospira* spillover (*11*). Moreover, bats might play an important role in the epidemiology of *Leptospira* through transmission between bats and rodents, with rodents being a major source of human infection (*12*).

## Conclusions

*Myotis* spp. bats in Mengyin County in Shandong Province of China showed a high rate of infection with *Leptospira borgpetersenii*, *L. kirschneri*, and several potentially new *Leptospira* species, suggesting that bats are important carriers of *Leptospira* in Mengyin County. To date, knowledge of batborne leptospirosis is lacking, and the monitoring of the potential spillover of batborne *Leptospira* to humans is needed.

The collection of bats for microbiologic studies was approved by the Ethics Committee of Prevention Medicine of Shandong University (approval no. 20150501). Bats were captured with the help of the Mengyin County Center for Disease Control and Prevention. This study was supported by a grant from the National Natural Science Funds of China (grant no. 31570167).

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# *Brucella suis* Infection in Dog Fed Raw Meat, the Netherlands

**Marloes A.M. van Dijk, Marc Y. Engelsma, Vanessa X.N. Visser, Marcel A.H. Spierenburg, Marjolijn E. Holtslag, Peter T.J. Willemsen, Jaap A. Wagenaar, Els M. Broens,[1] Hendrik I.J. Roest[1]**

A *Brucella suis* biovar 1 infection was diagnosed in a dog without typical exposure risks, but the dog had been fed a raw meat–based diet (hare carcasses imported from Argentina). Track and trace investigations revealed that the most likely source of infection was the dog's raw meat diet.

Exposure risks for *Brucella suis* infection typically include contact with wildlife or livestock, breeding, and travel to brucellosis-endemic areas. We report a case of *B. suis* infection in a dog for which the risk was determined to be a raw meat–based diet.

## The Case

In November 2016, a 6-year-old, intact, male American Staffordshire terrier was admitted to a primary care veterinary clinic in the Netherlands, where fever, ascites, and epididymitis/orchitis were detected. Because clinical signs did not improve after a 5-day course of amoxicillin/clavulanic acid (12.5 mg/kg 2×/d), the dog was neutered. During surgery, purulent exudate from the epididymis was noted; this exudate and abdominal fluid were collected and submitted to a routine veterinary diagnostic laboratory. Both samples yielded bacterial growth that was identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Bremen, Germany) as *Brucella* spp. The Dutch National Reference Laboratory identified the isolate by MALDI-TOF mass spectrometry (with an in-house extended database) as *Brucella suis* biovar 1, and the EU reference laboratory confirmed this phenotypically (*1*). One isolate was sequenced and molecularly characterized in silico by multilocus variable-number tandem-repeat analysis (MLVA) as Ms Bruce 06/08/11/12/42/43/45/55/18/19/21/04/07/09/16/30: 2/3/6/10/4/1/5/2/4/38/9.5/5/4/8/5/3 and by multilocus sequence typing (MLST) as sequence type (ST) 14 (*2*–*4*).

After diagnosis confirmation, serum and urine samples were collected from the dog. Serologic testing for *B. suis* yielded a positive result by microscopic agglutination test (MAT; >120 IU/mL) and rose bengal test (*4*,*5*). Serologic test results for *B. canis* (serum agglutination test <50 IU/mL) (*1*) and bacteriologic culture of a urine sample were negative. Despite treatment with doxycycline (10 mg/kg 1×/d for 14 days starting 3 days after neuter), the dog did not recuperate and because of the poor prognosis was euthanized. Postmortem examination of the dog was performed, and samples from kidney, spleen, prostate, liver, and abdominal lymph nodes were tested by PCR (*4*). Only the prostate yielded a positive result for *Brucella* spp.

Because brucellosis is notifiable in the Netherlands, the Incidence Crisis Centre of the Netherlands Food and Consumer Product Safety Authority was notified. The Centre started investigations to track potential transmission and trace the source of infection. The owners of the index dog were asked to list all dogs that had had frequent contact with their dog during the previous 2–3 months. From the 5 contact dogs identified, blood samples were collected (twice, 4 weeks apart) for serologic testing (MAT and rose bengal) and urine samples were collected for bacteriologic culture. Blood from 1 contact dog yielded a weakly positive result for *B. suis* antibodies (MAT 30 IE/mL; rose bengal negative) at both collection times. An acute infection in this dog was considered unlikely because no seroconversion was detected. All other dogs yielded negative serologic results. All urine samples were bacteriologically negative.

The owners of the index dog reported no relevant exposure risks except that the dog was fed a raw meat–based diet (usually commercial mixed raw feed and in June–July 2016 unprocessed heads of hares, all from the same supplier). Because raw meat consumption has been associated with *B. suis* infections in dogs (*6*,*7*), the feed was considered a potential source of infection. In December 2016, the index dog owner provided leftovers of the commercial mixed raw feed, which we tested by PCR for the presence of porcine DNA and *Brucella* spp.; results for both were negative. The investigators visited the raw feed supplier and sampled a (not yet marketed) 30,000-kg batch of hare carcasses imported from Argentina. Of 40 representative

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

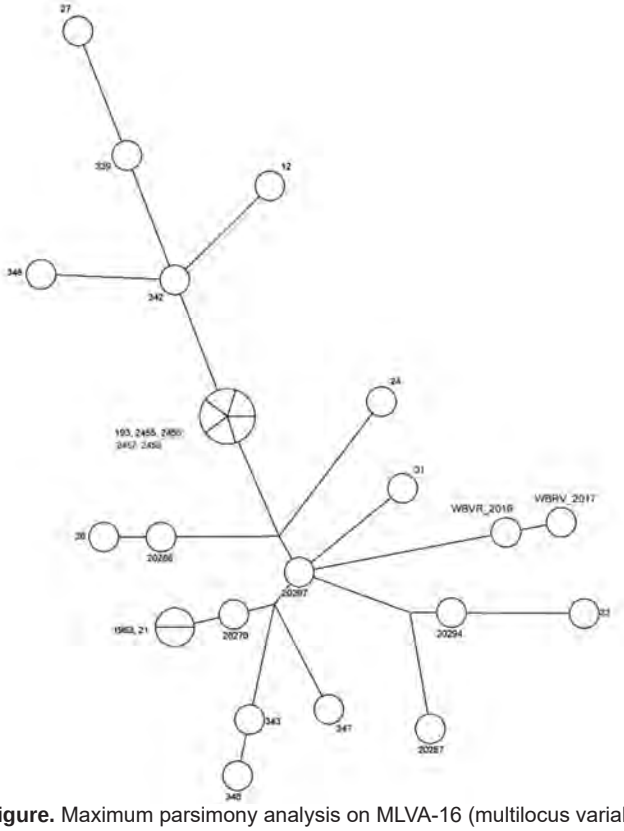
samples, 2 yielded a positive PCR result for *Brucella* spp. and were subsequently cultured. Colonies from 1 sample were confirmed by MALDI-TOF mass spectrometry (with an in-house extended database) to be *B. suis* biovar 1. One isolate was sequenced and molecularly characterized in silico by MLVA and MLST (ST14) (*2*–*4*). The isolates from the index dog and from the batch of hare carcasses showed high similarity (only 1 locus difference in the MLVA Ms07: 4 repeats dog isolate; 6 repeats hare isolate). Similarity with 24 closely related reference isolates from a public database (http://microbesgenotyping.i2bc.paris-saclay.fr/) was much lower (Figure).

## Conclusions

This *B. suis* biovar 1 infection in a dog in the Netherlands was linked to its commercial raw meat–based diet. Canine infections with this biovar have been documented in *B. suis* biovar 1–endemic areas (e.g., Australia and Latin America), mostly associated with exposure to feral pigs or consumption of raw feral pig meat (*6*,*7*). In the case we report, the *B. suis* biovar 1 infection most likely originated from hare carcasses imported from Argentina into the Netherlands. *B. suis* biovar 1 is endemic to Latin America and has been isolated from hares (*7*–*9*). The dog showed clinical signs ≈4 months after it had been fed raw hare heads from a supplier of commercial raw feed. The presence of *B. suis* biovar 1 in another batch of hare carcasses from the same supplier makes foodborne transmission highly likely. The genotypic similarity between the isolates from the dog and the feed and the fact that the supplier imported multiple batches from the same slaughter plant in Argentina during the preceding months confirms the feed as the most probable source of infection.

This report illustrates possible implications of the global trade of raw meat. Importation of hare carcasses, whether or not approved for human consumption, from countries outside the European Union into the European Union is legal. Because the aforementioned batches of hare carcasses from Argentina were approved for human and animal consumption, humans and other animals were potentially at risk when handling or consuming meat products from these batches.

Medical microbiologists of the Municipal Health Service assessed the zoonotic risks for all persons who had come in contact with the dog or with samples from the dog or hare carcasses. Five laboratory technicians who had been exposed to pure cultures (before bacterial identification) were given postexposure prophylaxis and tested for




| Key | Strain ID | Host | Isolated in | Year |
|---|---|---|---|---|
| 12 | REF 1330 | Swine | USA | NA |
| 21 | BCCN#87-67a | Unknown | China | 1987 |
| 22 | BCCN#87-68 | Unknown | Portugal | 1987 |
| 24 | BCCN#87-72 | Unknown | Denmark | 1987 |
| 26 | BCCN#87-87 | Unknown | Poland | 1987 |
| 27 | BCCN#87-88 | Unknown | Poland | 1987 |
| 31 | BCCN#98-43 | Swine | Argentina | 1998 |
| 193 | BCCN#03-27 | Horse | Croatia | 2003 |
| 339 | S-88 | Horse | Croatia | 2003 |
| 340 | S-90 | Wild boar | Croatia | 1992 |
| 342 | S-94 | Wild boar | Croatia | 2003 |
| 343 | S-97 | Swine | Croatia | 1994 |
| 347 | S-104 | Hare | Croatia | 1980 |
| 348 | S-105 | Hare | Croatia | 1980 |
| 1963 | N11 | Cattle | China | 2009 |
| 2455 | INRA03-26 | Swine | France | 2003 |
| 2456 | AFSSA-03/3081-2 | Caprid | Croatia | 2003 |
| 2457 | AFSSA-04/115 | Hare | France | 2004 |
| 2458 | AFSSA-04/3025-3 | Swine | Croatia | 2004 |
| 20257 | B05-1335_IA_SwineD | Swine | USA | 2016 |
| 20270 | B11-0438_FL_Cow | Cattle | USA | 2016 |
| 20286 | B13-0198_TX_Feralpig | Feral pig | USA | 2016 |
| 20294 | B94-0444_TX_Swine | Swine | USA | 2016 |
| 20297 | B95-0512_OH_Dog | Dog | USA | 2016 |
| WBVR_2016 | WBVR_2016 | Dog | Netherlands | 2016 |
| WBVR_2017 | WBVR_2017 | Hare | Netherlands | 2017 |

**Figure.** Maximum parsimony analysis on MLVA-16 (multilocus variable-number tandem-repeat analysis) of genotypes from 2 recent *Brucella suis* biovar 1 isolates from the Netherlands (WBVR2016 from a dog and WBVR2017 from hare carcasses) in conjunction with *B. suis* biovar 1 strains of the highest similarity from a public database (http://microbesgenotyping.i2bc.paris-saclay.fr/) with 521 entries of *B. suis*. NA, not available.

seroconversion to *B. suis* (postexposure weeks 2, 4, 6, and 24) according to national guidelines (*10*). To our knowledge, no human infections were linked to this case.

*B. suis* biovar 1 is a potential threat to the pig farming industry because introduction of *B. suis* into pig herds can have substantial economic consequences (*11*). A striking detail is that the last *B. suis* infection in pigs in the Netherlands (1969) was associated with swill feeding of hares imported from Argentina (*12*).

In response to our findings, preventive measures were implemented (e.g., sampling of imported raw meat and communication of risk to international authorities and raw-feed suppliers). This case stresses the microbiological risks for humans and animals of feeding raw meat–based diets, which has become increasingly popular among pet owners (*13*). This case also highlights the need for a One Health approach because *B. suis* biovar 1 is a zoonotic agent and can cause severe infections in humans (*14*,*15*).

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# Veal Liver as Food Vehicle for Human *Campylobacter* Infections

**Colette Gaulin, Danielle Ramsay, Réjean Dion, Marc Simard, Céline Gariépy, Éric Levac, Karon Hammond-Collins, Maude Michaud-Dumont, Mélanie Gignac, Marc Fiset**

A matched case–control study in Quebec, Canada, evaluated consumption of veal liver as a risk factor for campylobacteriosis. *Campylobacter* was identified in 28 of 97 veal livers collected concurrently from slaughterhouses and retailers. Veal liver was associated with human *Campylobacter* infection, particularly when consumed undercooked.

Recent investigations conducted in Quebec, Canada, after an increased number of sporadic campylobacteriosis illnesses suggested that consumption of veal liver may be a risk factor for campylobacteriosis. Many of the persons infected reported eating veal liver, and many of those had eaten it pink or undercooked. The association between campylobacteriosis and the consumption of meat products, including chicken liver and offal from different animal species, has been previously described (*1*–*5*). We designed an epidemiologic study to examine the relationship between veal liver consumption and campylobacteriosis.

## The Study

We conducted a matched case–control study to examine a potential association between veal liver consumption and campylobacteriosis, using salmonellosis cases as controls. The study began in September 2016 and continued for 9 months. Salmonellosis and campylobacteriosis cases are reportable in Quebec; we selected all subjects from the provincial reportable disease registry. We used a systematic sampling method to select every fifth reported campylobacteriosis case-patient ≥45 years of age. We paired each campylobacteriosis case-patient with 1 salmonellosis case-patient by age group (45–64 and ≥65 y) and sex; both infections were confirmed by fecal culture. We matched case-patients if the salmonellosis sample was collected within a window of 7 days before to 60 days after the campylobacteriosis sample was collected. Inclusion criteria for cases and controls were infection that was sporadic and domestically acquired. Exclusion criteria were co-infection with another pathogen, being part of a recognized outbreak, or contact with a gastroenteritis case-patient ≤10 days before illness.

We administered a structured questionnaire by telephone to collect information on exposures in the 7 days before illness onset. Exposures were consumption of meat and unpasteurized milk products, contact with animals, drinking and recreational water exposures, and occupational exposures. In particular, we investigated consumption of a variety of livers and the degree to which they were cooked. We conducted matched univariate and multivariate analysis to estimate odds ratios (OR) for each exposure.

In addition, we collected samples of veal, chicken, pork, and beef livers from slaughterhouses and retail stores in Quebec between October 2014 and March 2017. We tested each liver specimen for the presence of *Campylobacter*, *Salmonella*, and *Escherichia coli* O157:H7 by using standardized methods (*6*,*7*).

We matched a total of 112 campylobacteriosis cases to salmonellosis cases. We found no significant statistical difference in the age or sex distribution of retained cases or controls and the excluded patients. The species of *Campylobacter* were *C. jejuni* (79.5%), *C. jejuni/coli* undifferentiated (3.6%), *C. coli* (0.9%), other (1.8%), and not identified (14.3%). Among campylobacteriosis case-patients, 42 (37.5%) consumed veal liver and 29 (69.0%) ate it undercooked.

Only the consumption of veal liver and having contact with farm animals were statistically significantly associated with campylobacteriosis (Table 1). After applying the Bonferroni correction to adjust for multiple comparisons (0.05 level of significance divided by 45 variables tested yields $\alpha = 0.001$), only veal liver consumption remained as a statistically significant exposure (matched OR 9.50, 95% CI 3.39–26.62; $p = 0.000001$).

Among veal liver consumers, adequate cooking (e.g., well-cooked vs. pink or rare, on the basis of the participant's subjective observation) was protective. Specifically, 13 (30.2%) of 43 case-patients versus 6 (85.7%) of 7 controls

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**Table 1.** Results of univariate matched analysis for patients with campylobacteriosis or salmonellosis, Quebec, Canada, September 2016–May 2017*

| Exposures | No. pairs | | | | | Matched odds ratio† | | p value§ |
|---|---|---|---|---|---|---|---|---|
| | e | f | g | h | Total | Estimate | 95% CI‡ | |
| Liver | | | | | | | | |
| Chicken liver | 0 | 4 | 1 | 104 | 109 | 4.00 | 0.45–35.79 | 0.2 |
| Pork liver | 0 | 2 | 5 | 102 | 109 | 0.40 | 0.08–2.06 | 0.3 |
| Beef liver | 0 | 3 | 3 | 105 | 111 | 1.00 | 0.20–4.96 | 1.0 |
| Veal liver | 4 | 38 | 4 | 62 | 108 | 9.50 | 3.39–26.62 | 0.000001 |
| Lamb liver | 0 | 3 | 0 | 109 | 112 | Undefined | Undefined | 1.0 |
| Poultry | | | | | | | | |
| Breaded chicken | 10 | 15 | 18 | 64 | 107 | 0.83 | 0.42–1.65 | 0.6 |
| Ground chicken | 0 | 3 | 10 | 95 | 108 | 0.30 | 0.08–1.09 | 0.07 |
| Whole chicken | 44 | 35 | 22 | 8 | 109 | 1.59 | 0.93–2.71 | 0.09 |
| Pork | | | | | | | | |
| Ham | 27 | 26 | 26 | 29 | 108 | 1.00 | 0.58–1.72 | 1.0 |
| Bacon | 8 | 23 | 27 | 50 | 108 | 0.85 | 0.49–1.49 | 0.6 |
| Ground pork | 4 | 12 | 14 | 76 | 106 | 0.86 | 0.40–1.85 | 0.7 |
| Beef | | | | | | | | |
| Ground beef | 47 | 25 | 24 | 9 | 105 | 1.04 | 0.60–1.82 | 0.9 |
| Roast beef | 3 | 22 | 15 | 66 | 106 | 1.47 | 0.76–2.83 | 0.3 |
| Beef steak | 20 | 21 | 25 | 39 | 105 | 0.84 | 0.47–1.50 | 0.6 |
| Veal | | | | | | | | |
| Ground veal | 0 | 8 | 13 | 86 | 107 | 0.62 | 0.26–1.49 | 0.3 |
| Veal escalope | 0 | 8 | 2 | 98 | 108 | 4.00 | 0.85–18.84 | 0.08 |
| Unpasteurized milk products | | | | | | | | |
| Raw milk | 0 | 2 | 1 | 107 | 110 | 2.00 | 0.18–22.06 | 0.6 |
| Raw-milk cheese | 0 | 10 | 4 | 93 | 107 | 2.50 | 0.78–7.97 | 0.1 |
| Water exposures | | | | | | | | |
| Drinking water from source other than aqueduct | 3 | 12 | 12 | 81 | 108 | 1.00 | 0.45–2.23 | 1.0 |
| Animal exposures | | | | | | | | |
| Dog or cat | 24 | 23 | 22 | 38 | 107 | 1.05 | 0.58–1.88 | 0.9 |
| Farm animal | 0 | 10 | 1 | 100 | 111 | 10.00 | 1.28–78.12 | 0.03 |
| Work in contact with animals | 0 | 8 | 2 | 102 | 112 | 4.00 | 0.80–38.67 | 0.07 |

*Results by matched analysis: e, campylobacteriosis case exposed, salmonellosis case exposed; f, campylobacteriosis case exposed, salmonellosis case not exposed; g, campylobacteriosis case not exposed, salmonellosis case exposed; h, campylobacteriosis case not exposed, salmonellosis case not exposed.
†By McNemar method.
‡Lower and upper limits determined by McNemar or exact method.
§By Wald or Fisher exact test, bilateral.

ate their veal liver well-cooked (unmatched OR 0.07, 95% CI 0.002–0.72; p = 0.02). Multivariate analysis using logistic regression confirmed that a statistically significant association between the consumption of veal liver and campylobacteriosis remained when all other exposures were included as covariates. Although we conducted this study among persons ≥45 years of age, it is reasonable to assume that eating veal liver, especially undercooked, would also carry risk for younger persons.

We sampled 339 veal, pork, chicken, and beef livers collected from 138 retailers and 16 slaughterhouses. When we evaluated all livers collected at these locations, we detected *Campylobacter* in 28.0% of veal livers, 22.2% of pork livers, 36.8% of chicken livers, and 19.1% of beef livers (Table 2). We detected *Salmonella* more frequently in chicken livers (22.1%) and pork livers (19.1%) than in veal livers (3.1%); we did not detect *Salmonella* in beef livers. We rarely identified *E. coli* O157:H7 in livers of any kind. The proportion of contaminated livers differed between animal species and also with respect to location of sampling. A higher proportion of veal livers (35.7%) collected from retailers were contaminated by *Campylobacter*, compared with veal livers collected from slaughterhouses (16.2%). We observed the reverse for chicken and pork livers. The reason for these variations is unclear at this time, but this finding may be an artifact resulting from the relatively small number of samples taken at each location.

Cattle are a well-known reservoir for a variety of *Campylobacter* species, such as *C. jejuni*, *C. coli*, and *C. fetus* (*8*,*9*). *Campylobacter* species have been isolated from beef intestinal contents and also from beef bile, bile ducts, gallbladder, and liver (*10*–*14*). The gallbladder and bile contain substances that have a chemoattractant effect on *C. jejuni*, which explains the presence of *Campylobacter* within the biliary tract (*10*,*15*). Liver contamination varies between animal species (*10*–*14*). Chicken liver, for example, can be contaminated by *Campylobacter* and S*almonella* and has been the source of several outbreaks (*3*,*4*,*11*,*13*). Because few case-patients consumed livers from other animal species during our study, we were not able to identify any substantial risks associated with those exposures.

**Table 2.** Animal livers collected from retailers and slaughterhouses and percentage positive for *Campylobacter*, *Salmonella*, and *Escherichia coli* O157:H7, Quebec, Canada, October 2014–March 2017

| | Veal livers | | Pork livers | | Chicken livers | | Beef livers | | |
|---|---|---|---|---|---|---|---|---|---|
| Source and pathogen | No. | % Positive | No. | % Positive | No. | % Positive | No. | % Positive | Total |
| Retailer | 59 | | 27 | | 19 | | 41 | | 146 |
| *Campylobacter* | | 35.7 | | 16.7 | | 10.5 | | 28.2 | |
| *Salmonella* | | 5.1 | | 44.4 | | 32.0 | | 0 | |
| *E. coli* O157:H7 | | 0 | | 0 | | 0 | | 0 | |
| Slaughterhouse | 38 | | 41 | | 58 | | 56 | | 193 |
| *Campylobacter* | | 16.2 | | 25.6 | | 45.6 | | 12.7 | |
| *Salmonella* | | 0 | | 2.4 | | 19.0 | | 0 | |
| *E. coli* O157:H7 | | 0 | | 2.4 | | 0 | | 0 | |
| Total | 97 | | 68 | | 77 | | 97 | | 339 |
| *Campylobacter* | | 28.0 | | 22.2 | | 36.8 | | 19.1 | |
| *Salmonella* | | 3.1 | | 19.1 | | 22.1 | | 0 | |
| *E. coli* O157:H7 | | 0 | | 1.5 | | 0 | | 0 | |

Because livers may be collected from several animals and stored together, they may be contaminated during the evisceration process or by cross-contamination (*11*). Both the external and internal tissue of a liver may be contaminated with *Campylobacter*, and inadequate cooking may not fully inactivate *Campylobacter* and *Salmonella* (*10*,*11*), which is a cause for concern because ≈70% of the patients with campylobacteriosis who consumed veal liver in our study reported eating it undercooked. We did not examine possible cross-contamination of foods and surfaces and the host-related factors that may increase the risk for enteric diseases.

## Conclusions

Our study identified a strong and statistically significant association between the consumption of veal liver and sporadic, domestically acquired campylobacteriosis among persons ≥45 years of age in Quebec. We found that adequate cooking of veal liver mitigates the risk of infection. We detected *Campylobacter* in almost one third of veal livers we sampled from slaughterhouses and retail stores, which supports our finding that veal liver consumption is a risk factor for campylobacteriosis. In light of these results, we recommend the dissemination of safe food handling practices for veal liver and other offal for retailers, food establishments, slaughterhouses, and the general public.

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### About the Author

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# Marburg Virus Infection in Egyptian Rousette Bats, South Africa, 2013–2014[1]

**Janusz T. Pawęska, Petrus Jansen van Vuren, Alan Kemp, Nadia Storm, Antoinette A. Grobbelaar, Michael R. Wiley, Gustavo Palacios, Wanda Markotter**

We detected a high seroprevalence of Marburg virus (MARV) antibodies in fruit bats in South Africa; 19.1% of recaptured bats seroconverted. The MARV RNA isolated closely resembled the 1975 Ozolin strain. These findings indicate endemic MARV circulation in bats in South Africa and should inform policies on MARV disease risk reduction.

As of March 2018, thirteen outbreaks of Marburg virus (MARV) disease (MVD) have been reported, most occurring in sub-Saharan Africa (*1*,*2*). The first recognized outbreak of MVD in Africa occurred in 1975 after a person hitchhiking through Zimbabwe was admitted to Johannesburg Hospital, Johannesburg, South Africa (*3*). The largest MVD outbreak occurred in Angola during 2004–2005 and had a case-fatality rate of 90% (*4*).

Outbreaks of MVD in Africa have been associated with persons entering caves or mines (*5*–*8*), and results of outbreak investigations and ecologic and experimental studies implicate the Egyptian rousette bat (*Rousettus aegyptiacus*) as the prime reservoir host for MARV (*9*–*13*). As part of a biosurveillance program in South Africa investigating the presence of viral zoonotic pathogens in bats, we tested a local population of Egyptian rousette bats for evidence of MARV infection.

## The Study

At monthly intervals during February 2013–February 2014, we captured and sampled Egyptian rousette bats at the entrance to Matlapitsi Cave, also known as Mahune Cave. The cave is located in the indigenous flora of Matlapitsi Valley (24°1′S, 30°10′E) on the northeastern slope of the Wolkberg mountain range, bordering Lekgalameetse Nature Reserve in Limpopo Province, South Africa. This work was done in accordance with approved protocols by animal ethics committees of the National Health Laboratory Service (Johannesburg, South Africa; AEC 137/12) and the University of Pretoria (Pretoria, South Africa; EC054–14). We captured and handled bats using standard procedures (*10*) and determined sex and age by visual evaluation of size, pelage color, and reproductive status. We tattooed and sampled anesthetized bats and processed blood and tissue specimens as described previously (*12*). We collected blood samples monthly from a subset (n = 1,431) of the total population of Egyptian rousette bats sampled during the 13-month biosurveillance program. Blood sample collection varied from 61 samples/month (April 2013) to 197 samples/month (May 2013). In addition, we collected spleen and liver tissues from 159 bats (average collection rate 12 samples/month). During the study period, 63 bats were recaptured (average rate 5 bats/month). We placed blood and tissue specimens in cryovials and transported them in liquid nitrogen to a biosafety level 4 facility for –70°C storage until testing.

We performed serologic, molecular, and virologic testing as described previously (*12*) and used real-time quantitative reverse transcription PCR (qRT-PCR) targeting the MARV L and VP40 genes to identify MARV-positive bats (*12*). When performing qRT-PCR with serum samples, we used pooled samples from 3–5 bats. We attempted virus isolation with samples that were positive for the MARV genome. We sequenced virus genomes using the TruSeq RNA Access Kit (Illumina Inc., San Diego, CA, USA) with enrichment probes designed against multiple MARV strains, including the 1975 Ozolin strain, and sequenced on an Illumina MiSeq (*14*). We performed sequence alignment using MAFFT version 7.222 (https://mafft.cbrc.jp/alignment/software/) and phylogenetic analysis using MrBayes version 3.2.6 (http://mrbayes.sourceforge.net/download.php).

Mating in the Egyptian rousette bat colony at Matlapitsi Cave occurred during June through mid-September. The first neonates were observed in the second half of October. In December and January, almost all female bats captured carried an infant or were pregnant. Neonates were observed occasionally in March and April, outside the birthing season,

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[1]Some preliminary results from this study were presented at the 6th International Symposium on Filoviruses; March 30–April 2, 2014; Galveston, Texas, USA.

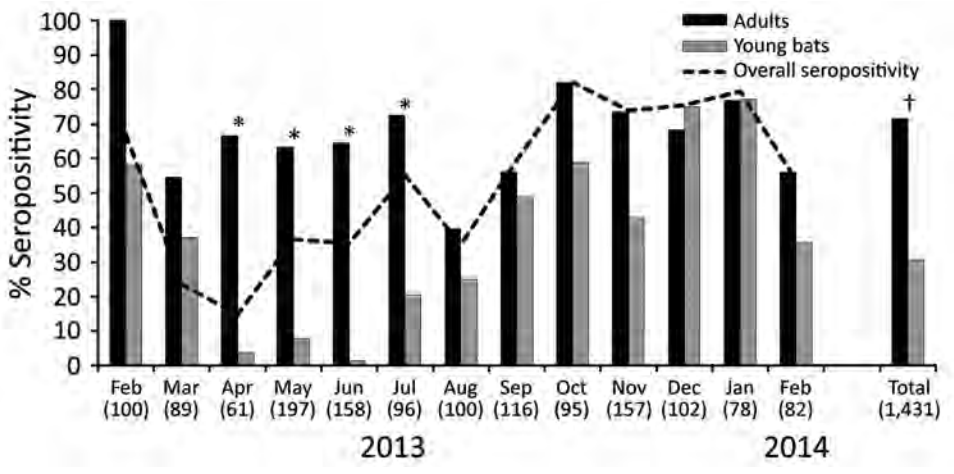


**Figure 1.** Marburg virus seropositivity in Egyptian rousette bats in Matlapitsi Cave, Limpopo Province, South Africa, 2013–2014. Numbers in parentheses indicate number of bats sampled per month. Bats <1 year of age (young bats) represent the new generation of bats born mostly during the October–January birthing peak. Statistically significant differences in seropositivity between adult and young bats are noted over a period of 4 months, April–July 2013. *Significant difference (p = 0.0001) between adult and young bat populations. †Significant difference (p = 0.002) between adult and young bat populations.

suggesting asynchronous births. In previous studies, the Egyptian rousette bat population in Matlapitsi Cave was estimated to fluctuate from 3,270 to 9,000 bats, with the lowest numbers occurring during the winter months (*15*).

Of 1,431 bats tested, 759 (53.04%) were positive for antibodies against MARV; overall seropositivity ranged from 14.75% in April 2013 to 82.1% in October 2013. Seropositivity in adults (n = 784) was 71.56%, ranging from 39.6% in August 2013 to 100% in February 2013. Seropositivity in young bats (n = 647) was 30.6%, ranging from 1.3% in June 2013 to 77.3% in January 2014. Seropositivity was significantly higher in adult than young bats (p = 0.002), especially during April 2013–July 2013 (p = 0.0001) (Figure 1). In total, 45.3% of male bats (n = 592) and 58.5% of female bats (n = 839) were seropositive (p = 0.667). Seroconversion was detected in 12 (19.1%) of 63 recaptured bats. The bats that seroconverted were all juvenile bats on first capture (Table 1).

All serum pools tested by qRT-PCR were negative for both L and VP40 genes. Of the 159 liver-spleen tissue pools tested, 3 (1.89%) were positive for MARV RNA: specimen no. SPU191/13 from a juvenile female bat (no. 2,764) captured in July 2013 (cycle threshold [$C_t$] L 29.84, $C_t$ VP40 31.58); specimen no. SPU249/13 from a juvenile male bat (no. 3,003) captured in August 2013 ($C_t$ L 34.86, $C_t$ VP40 33.85); and specimen no. SPU282/13 from a juvenile male bat (no. 3,092) captured in September 2013 ($C_t$ L 33.04, $C_t$ VP40 34.05). Attempts to culture the virus from qRT-PCR–positive tissue pools were unsuccessful. A similar ecologic study conducted in Uganda obtained identical results from bat tissues with $C_t$s >30 (*11*).

Genomic analysis was performed only with the specimen with the lowest qRT-PCR $C_t$ (bat no. 2,764; SPU191/13). The MARV sequence detected (GenBank accession no. MG725616) was closely related to the 1975 Ozolin strain (99.3% nucleic acid homology, 0%–1.2% amino acid differences) (Table 2; Figure 2). Only 22 aa substitutions were identified between these 2 viruses, which were isolated 38 years apart.

**Table 1.** Marburg virus seroconversion in 12 Egyptian rousette bats recaptured at Matlapitsi Cave, Limpopo Province, South Africa, April 2013–January 2014

| | First capture | | Second capture | | Third capture | |
|---|---|---|---|---|---|---|
| Bat no. | ELISA, % positivity* | Capture date | ELISA, % positivity* | Capture date | ELISA, % positivity* | Capture date |
| 243 | 8.48 | 2013 Mar 13 | 5.26 | 2013 May 13 | 67.15 | 2013 Aug 13 |
| 287 | 13.75 | 2013 Apr 13 | 45.67 | 2013 Nov 13 | | |
| 310 | 7.11 | 2013 Apr 13 | 36.41 | 2013 Jul 13 | | |
| 323 | 11.30 | 2013 Apr 13 | 53.94 | 2013 Feb 14 | | |
| 525 | 6.22 | 2013 May 13 | 42.61 | 2013 Jun 13 | | |
| 542 | 7.19 | 2013 May 13 | 67.97 | 2013 Aug 13 | 100.69 | 2013 Nov 13 |
| 615 | 4.91 | 2013 Jun 13 | 31.68 | 2013 Oct 13 | | |
| 633 | 7.58 | 2013 Jun 13 | 53.93 | 2013 Jul 13 | | |
| 653 | 4.53 | 2013 Jun 13 | 64.19 | 2013 Nov 13 | | |
| 694 | 7.58 | 2013 Jun 13 | 4.24 | 2013 Sep 13 | 42.73 | 2013 Nov 13 |
| 742 | 5.72 | 2013 Jul 13 | 23.82 | 2013 Oct 13 | | |
| 822 | 6.86 | 2013 Jun 13 | 41.97 | 2014 Jan 14 | | |

*Percentage positivity of the internal positive control serum sample; cutoff value of assay is 16.78% positivity (*12*).

**Table 2.** Base pair changes between reference MARV strains and MARV from Matlapitsi Cave, Limpopo Province, South Africa, 2013*

| Sequence type, MARV gene | Strain, no. (%) | | | |
|---|---|---|---|---|
| | Ozolin 1975 | Angola 2005 | Musoke 1980 | Uganda 2009 |
| Nucleotide sequence | | | | |
| Nucleocapsid | 9 (0.43) | 120 (5.8) | 112 (5.4) | 123 (5.9) |
| Viral protein 35 | 4 (0.43) | 56 (5.7) | 37 (3.7) | 58 (5.9) |
| Viral protein 40 | 5 (0.57) | 54 (5.9) | 50 (5.5) | 52 (5.7) |
| Glycoprotein | 17 (0.85) | 198 (9.7) | 185 (9.0) | 190 (9.3) |
| Viral protein 30 | 4 (0.48) | 55 (6.5) | 49 (5.8) | 55 (6.5) |
| Viral protein 24 | 2 (0.26) | 43 (5.7) | 38 (5.0) | 34 (4.4) |
| Polymerase | 43 (0.6) | 469 (6.7) | 425 (6.1) | 479 (6.8) |
| Amino acid sequence | | | | |
| Nucleocapsid | 0 | 11 (1.6) | 12 (1.7) | 11 (1.6) |
| Viral protein 35 | 0 | 4 (1.3) | 1 (0.3) | 4 (1.3) |
| Viral protein 40 | 1 (0.34) | 4 (1.4) | 3 (1.0) | 3 (1.0) |
| Glycoprotein | 8 (1.2) | 60 (8.8) | 61 (9.0) | 63 (9.3) |
| Viral protein 30 | 1 (0.36) | 8 (2.9) | 13 (4.7) | 10 (3.6) |
| Viral protein 24 | 0 | 2 (0.79) | 2 (0.79) | 1 (0.4) |
| Polymerase | 12 (0.52) | 83 (3.6) | 87 (3.7) | 87 (3.7) |

*Matlapitsi Cave MARV sequences were from specimen no. SPU 191/13 from bat no. 2,764 (GenBank accession no. MG725616). MARV, Marburg virus.

## Conclusions

Our findings indicate endemic MARV circulation in bats in Matlapitsi Cave, located 111 km from Polokwane City, South Africa's largest urban center north of Gauteng Province. Matlapitsi Cave, a 400-m hike from the main road running through the rural community of Fertilis, is accessible to humans. The cave was used in the past for religious practices and circumcision rituals, which have since been discontinued. In spite of their discontinuation, we found human shoe prints, litter, and signs of recent fire pit use at the cave entrance during our sampling trips. Informal discussions with persons of the local communities indicated that bats were not being hunted and their meat was not being consumed by local residents. However, uncontrolled migration of persons from neighboring countries where bat meat is consumed and increasing economic pressures, which could force local persons to hunt wildlife, might put the population at increased risk for MVD.

Observations made in this study confirm a distinctly seasonal Egyptian rousette bat reproductive period as previously reported (*15*). Gradual loss of passive immunity increases the number of susceptible bats, thus creating suitable conditions

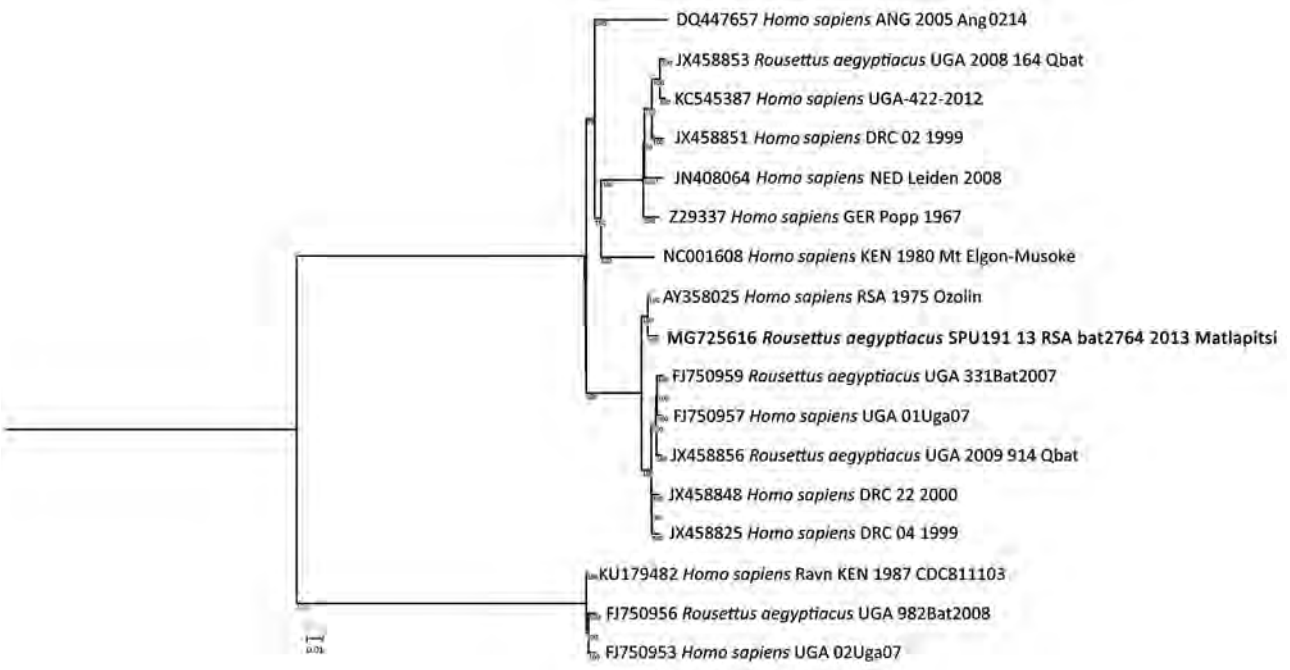


**Figure 2.** Phylogenetic tree of partial (97.5%) Marburg virus nucleic acid sequence detected in Egyptian rousette bats in Matlapitsi Cave, Limpopo Province, South Africa, 2013 (bold; GenBank accession no. MG725616) and complete nucleic acid sequences of representative Marburg virus strains from GenBank. Node values indicate posterior probability percentages obtained from 1,000,000 generations in MrBayes version 3.2.6 (http://mrbayes.sourceforge.net/download.php). Scale bar indicates nucleotide substitutions per site.

for MARV spread in the colony. Results of our study suggest that the single but relatively long birthing season complemented by asynchronous births and potential migration of bats might contribute to sustained annual MARV circulation in this area (*15*). These findings appear to be in contrast with those from the study in Uganda, which indicated that 2 yearly birthing seasons were required to maintain circulation of MARV in Egyptian rousette bats (*11*). The period of lowest seropositivity in young bats (April–July) might indicate a period of increased risk for exposure and shedding. The MARV sequence from the Matlapitsi Cave is phylogenetically most closely related to the Ozolin MARV strain, suggesting this variant has persisted in the southern part of Africa relatively unchanged since first discovered in 1975 (*3*). These findings contribute to our knowledge of MARV ecologic factors that could lead to a zoonotic spillover into humans and, thus, assist in the development of evidence-based policies for MVD risk reduction in South Africa.

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# Mixed *Leptospira* Infections in a Diverse Reservoir Host Community, Madagascar, 2013–2015

**Mark Moseley, Soanandrasana Rahelinirina, Minoarisoa Rajerison, Benoit Garin, Stuart Piertney, Sandra Telfer**

We identified mixed infections of pathogenic *Leptospira* in small mammals across a landscape-scale study area in Madagascar by using primers targeting different *Leptospira* spp. Using targeted primers increased prevalence estimates and evidence for transmission between endemic and invasive hosts. Future studies should assess rodentborne transmission of *Leptospira* to humans.

As a result of underreporting and lack of awareness, leptospirosis has been recognized as one of the world's most neglected diseases (*1*). As is the case for other zoonotic pathogens, identifying key maintenance hosts and sources of human infection is essential for designing effective control strategies (*2*). However, leptospirosis epidemiology is complex; 10 pathogenic *Leptospira* species are phylogenetically delineated into 4 subgroups that differ in virulence and transmission (*3*), and multiple potential host species exist (*4*). In most studies, PCR protocols use primers targeting all pathogenic species, and the infecting *Leptospira* are identified on the basis of amplicon DNA sequence differences (*5*) or melt curve analyses (*6*). However, because of PCR primer biases or differences in infection intensities, such approaches probably underestimate mixed infections in areas with high *Leptospira* diversity.

Leptospirosis risk is high on islands in the western Indian Ocean; several *Leptospira* species on these islands are associated with disease (*7*). Studies in Madagascar have revealed acute cases of human leptospirosis and a seroprevalence of 3% (*7*–*9*). Studies of potential reservoirs in the region have revealed contrasting *Leptospira*–host associations. On Mayotte, an island neighboring Madagascar, 4 *Leptospira* species implicated in human disease (*L. interrogans* and *L. kirschneri* [taxonomic subgroup 1], *L. borgpetersenii* and *L. mayottensis* [taxonomic subgroup 2]) (*7*) have been detected in *Rattus rattus* rats (*6*), a highly successful invasive host introduced to the western Indian Ocean islands. However, *Tenrec ecaudatus* tenrecs, a mammal introduced from Madagascar, might also be a host of *L. mayottensis* (*10*). On Madagascar, only *L. interrogans* has been detected in invasive *Rattus* spp. rats (*11*), whereas *L. borgpetersenii*, *L. mayottensis*, and *L. kirschneri* have been detected in hosts endemic to Madagascar (*5*). In these studies, researchers did not attempt to identify mixed infections or sample invasive and endemic hosts from the same location, which would have been needed to fully assess *Leptospira* dynamics, spillover, and the role of hosts with widely different abundances and spatial distributions. Therefore, as part of a large landscape-scale study of *Leptospira* reservoirs in Madagascar, we developed quantitative PCRs (qPCRs) targeting individual *Leptospira* species and tested samples from small mammals to assess whether this approach changed our understanding of the reservoirs and spatial variation of risk.

## The Study

We conducted trapping and sample collection under permits issued by the Madagascar Ministry of Environment and Forests (no. 154/13/MEF/SG/DGF/DCB.SAP/SCB; no. 312/13/MEF/SG/DGF/DCB.SAP/SCB; no. 178/14/MEF/SG/DGF/DCB.SAP/SCB). We conducted this study in accordance with Institut Pasteur animal use guidelines (https://www.pasteur.fr/en/file/2626/download? token=YgOq4QW7); the study was approved by a committee of the Institut Pasteur de Madagascar.

During 2013–2015, we sampled small mammal hosts at 11 sites in Moramanga District, eastern Madagascar. Two sites were within an uninhabited humid forest, and the remaining sites included areas of human habitation and heterogeneous land use. We identified host species on the basis of phenotypic characteristics, external measurements, and craniodental measurements (when appropriate) (*12*). We euthanized and dissected animals and stored kidneys in 95% ethanol.

We extracted DNA from 0.04 g of kidney tissue with DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA,

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USA) using the manufacturer's instructions and an elution volume of 100 µL. We detected *Leptospira* with a TaqMan qPCR assay targeting the 16S rRNA gene (*13*) using the StepOne Real-Time PCR System (Life Technologies, Waltham, MA, USA). Any sample with a cycle threshold <36 in 1 assay or <40 in 2 replicate assays was classified as *Leptospira* positive.

Initial genotyping of positive samples was achieved by amplification and sequencing of ≈300 bp of the *lfb1* gene on an Eco-Illumina qPCR System (Illumina Inc., San Diego, CA, USA) (*6*). To characterize mixed infections, we designed forward primers targeting the *lfb1* locus of 4 *Leptospira* species (*L. interrogans* 5′-CCTCTTACGCACAGATCRGTC-3′, *L. borgpetersenii* 5′-CCAACACTCCCTCCTCTATCAGC-3′, *L. mayottensis* 5′-CGCAGACTAGCAGCCCAACC-3′, and *L. kirschneri* 5′-GACCGCTTACGCACAGATCG-3′) and paired them with the standard *lfb1* reverse primer using the same thermal profile. After sequencing, we retested samples with redesigned primers targeting *Leptospira* spp. not previously identified and sequenced those products (GenBank accession nos. MG759567–664). Each assay included a negative control (sterile water) for every 4 samples and a positive control. We purified PCR products using the QIAquick PCR Purification Kit (QIAGEN) and sent them to Eurofins Genomics GmbH (Ebersburg, Germany) for sequencing. We calculated prevalence and logit CIs using the binom package in R version 3.2.2 (https://cran.r-project.org/package=binom).

We captured 2,847 small mammals across 11 sites; 5 invasive species (*R. rattus* and *R. norvegicus* rats, *Mus musculus* mice, and *Suncus murinus* and *S. etruscus* shrews) accounted for 93% (2,653/2,847) of the captures. Of these, we captured *R. rattus* rats most frequently (n = 2,312) and at all sites, including forest sites. Although we found endemic hosts at all sites, 56% (107/190) were captured at forest sites. We tested 723 captured animals (43–102 animals/site) for *Leptospira*. We tested all endemic host samples and a subset of introduced host samples for each site. Overall prevalence of infection was 26%, ranging from 11% in *Microgale* spp. tenrecs to 48% in *M. musculus* mice (Table).

We genotyped 93 *Leptospira*-positive samples; the prevalence of mixed infections was 19% (95% CI 13%–29%). This value is still likely an underestimate, considering that cross-amplification of *Leptospira* species within the same taxonomic subgroup occurred. Mixed infections comprised *L. interrogans* and either *L. borgpetersenii* (n = 14) or *L. mayottensis* (n = 3); 1 animal was infected with all 3 species. All mixed infections were detected in rodents (order Rodentia): 78% (14/18) in *R. rattus* rats, 11% (2/18) in *R. norvegicus* rats, and the remaining 2 in endemic *Nesomys rufus* mice and *Eliurus minor* rats.

**Table.** Prevalence of *Leptospira* infection in small mammal hosts, Madagascar, 2013–2015

| Host type and species | No. positive/no. tested | Prevalence, % (95% CI) |
|---|---|---|
| Endemic | | |
| *Microgale* spp. tenrecs* | 12/108 | 11 (6–19) |
| *Eliurus* spp. rats* | 6/24 | 25 (12–46) |
| *Hemicentetes semispinosus* tenrec | 6/29 | 21 (10–39) |
| Invasive | | |
| *Rattus rattus* rat | 80/347 | 23 (19–28) |
| *Suncus murinus* shrew | 16/60 | 27 (17–39) |
| *R. norvegicus* rat | 17/36 | 47 (32–63) |
| *Mus musculus* mouse | 57/119 | 48 (39–57) |
| Total | 194/723 | 27 (24–30) |

*Endemic *Microgale* tenrecs and *Eliurus* rats were analyzed at the genus level because these genera were composed of a large number of species.

After characterizing mixed infections, the proportion of *R. rattus* rats infected with *L. borgpetersenii* nearly doubled (Figure), and the number of *L. mayottensis*–infected *R. rattus* rats equaled the number of *L. mayottensis*–infected endemic hosts (n = 4). All of the *L. mayottensis*–infected *R. rattus* rats were captured at sites with human habitation; 75% (3/4) of *L. mayottensis*–infected endemic hosts were captured at forest sites. The *L. interrogans lfb1* genotype most commonly identified was identical to the *lfb1* sequence obtained from a human with a case of leptospirosis contracted in Madagascar (*9*).

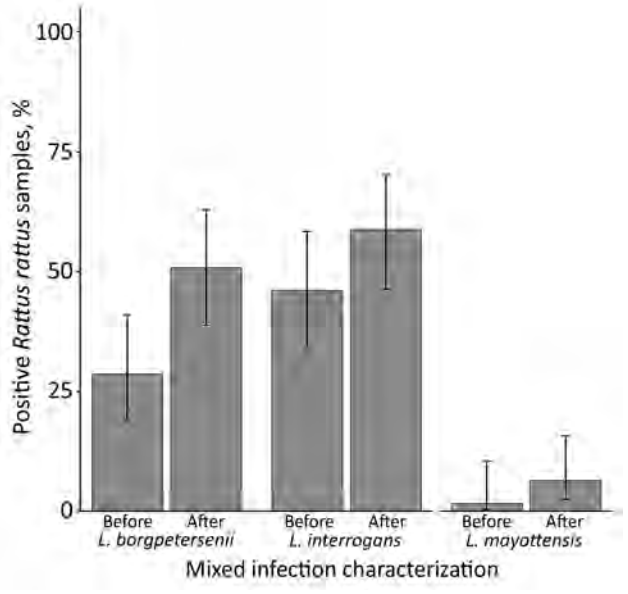


**Figure.** Proportion of *Leptospira*-positive *Rattus rattus* rat samples (n = 63) infected with *L. borgpetersenii*, *L. interrogans*, or *L. mayottensis* before and after characterizing mixed infections, Madagascar, 2013–2015. We initially genotyped *R. rattus* rat samples positive for *Leptospira* 16S rRNA by sequencing ≈300 bp of the *lfb1* gene using standard primers and thermal profile (*6*). To characterize mixed infections, we used forward primers targeting the *lfb1* locus of the different *Leptospira* species and the standard reverse primer and thermal profile. Mixed infections result in the sum of proportions exceeding 100% after characterization. Error bars represent 95% CIs.

## Conclusions

We present definitive molecular evidence that small mammal hosts carry mixed infections of pathogenic *Leptospira* spp. The characterization of mixed infections and testing of sympatric endemic and invasive reservoir hosts has altered our understanding of leptospirosis epidemiology. Previously, only *L. interrogans* was detected in *Rattus* spp. rats in Madagascar (*11*). We show that, when mixed infections are characterized, the prevalence of *L. borgpetersenii* and *L. interrogans* in *R. rattus* rats is similar. Similar to findings from Mayotte (*14*), *R. rattus* rats are a potential source of human infection for 3 of the 4 *Leptospira* species present in Madagascar. Because of the high abundance and widespread distribution of these rats, they could act as a key reservoir for *Leptospira*, including for *L. mayottensis*, which might occur as spillover infections from endemic species.

The high prevalence of mixed *Leptospira* infections also provides a potential explanation for the genetic and serologic diversity of pathogenic *Leptospira* in the region (*5*,*7*), considering horizontal genetic transfer has been implicated in *Leptospira* evolution, including evolution of the locus responsible for serologic classification (*rfb*) (*3*). Further work is needed to better characterize the evolutionary and landscape-scale epidemiologic consequences of mixed infections. Moreover, the prevalence of infection and the identification of an *lfb1* genotype in *Rattus* spp. rats identical to that in a human case (*9*) suggests that rodentborne transmission of *Leptospira* might be an underreported health problem in Madagascar. Studies on human exposure are urgently needed.

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# Mosquitoborne Sindbis Virus Infection and Long-Term Illness

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An unexpected human outbreak of the mosquitoborne Sindbis virus occurred in a previously nonendemic area of Sweden. At follow-up, 6–8 months after infection, 39% of patients had chronic arthralgia that affected their daily activities. Vectorborne infections may disseminate rapidly into new areas and cause acute and chronic disease.

Mosquitoborne viruses such as chikungunya virus, Ross River virus, and Sindbis virus (SINV) are members of the genus *Alphavirus* (family *Togaviridae*) and cause human arthritic diseases (*1*). SINV has mainly been reported in northern Europe and South Africa (*1*); Sweden has an average of 3 SINV cases per year, with occasionally more cases in a previously defined endemic region in central Sweden (Figure) (*2*). Birds are the reservoir for SINV, and there is no evidence of human-to-human transmission. SINV infection in humans, called Ockelbo disease in Sweden, causes rash, arthritis, and mild fever (*3*–*5*). Most patients recover within weeks or months, but arthralgia and myalgia can persist for years following infection, suggesting inflammatory response or a persistent infection (*4*–*6*).

In mid-August 2013, several patients with rash, arthralgia, and fever visited the healthcare center in the small village of Lövånger in Västerbotten County, Sweden (Figure). The university hospital laboratory in Umeå received 172 blood samples from patients with suspected SINV infection; 50 patients had SINV-specific IgM and IgG (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/6/17-0892-Techapp1.pdf). SINV infections have been believed to be almost exclusively confined to the central part of Sweden (*2*), but the 2013 outbreak occurred north of the endemic area (Figure). The distribution of verified cases by sex in this outbreak showed a higher proportion of female patients (62%) than male patients (38%) with acute SINV infection (online Technical Appendix Table 1).

Previous reports suggested that joint symptoms might persist for years in SINV infections (*4*–*6*). To evaluate long-term consequences, we contacted 46 SINV patients by telephone 3–4 months, 6–8 months, or in both periods after acute disease (online Technical Appendix Figure 1). Our study was approved by the Regional Ethics Review Board (2014-102-32M), and we obtained written informed consent from all participants. We include details of the study results summarized here in the online Technical Appendix. In total, 18/46 (39%) of the patients reported persistent musculoskeletal pain (arthralgia and myalgia) and restriction in their daily activity 6–8 months after the onset of acute symptoms (online Technical Appendix Table 2). We invited these patients for a standardized examination by a rheumatologist, including a health assessment


**Figure.** Geographic distribution of the SINV outbreak in 2013 and previous occurrence of SINV infections in Sweden. Dark gray indicates the 2 northernmost counties in Sweden where the SINV IgG seroprevalence was 2.9% in 2009. SINV, Sindbis virus.

questionnaire, patient assessments of their pain and their global health using a visual analog scale, and a blood sample (online Technical Appendix). Of 17 symptomatic patients who participated, 1 had arthritis in the ankle, 14 had ≥1 tender joint, and 10 had enthesitis, tendinitis, or tenosynovitis at examination. Large joints (knee, hip, shoulder, wrists, ankles) and small joints (toes, fingers) were affected, with a predominance for the lower extremities, in contrast to other studies in which small and peripheral joints were mainly affected (*4*–*6*). Patients graded their global symptoms as more severe than the examining doctor did, indicating that joint function was only mildly affected whereas the pain was perceived as restricting. Test results did not detect citrulline antibodies, and the single patient with positive rheuma factor had no arthritic symptoms. A notable finding was that 4 patients (24%) had psoriasis, a condition present in <4% of the northern European population (*7*), raising the question whether psoriasis makes SINV patients more vulnerable to long-term arthralgia.

We asked the 28 patients at the 6- to 8-month follow-up who had recovered to complete a questionnaire and donate a blood sample at their local healthcare provider; 23 did so. Symptomatic patients reported more pain and impaired health, compared with patients who were asymptomatic 6–8 months after acute disease. We detected SINV-specific IgM in patients with and without persistent symptoms, as previously reported (*5*).

We isolated a new SINV strain from a mosquito caught in the area during the outbreak; it was most closely related to a SINV strain from Finland (*8*). However, no increased incidence was recorded in Finland either the year before or concomitantly with the Swedish outbreak (*9*). In addition, only 1 case was reported from the endemic area of central Sweden in 2013, suggesting that local factors such as weather conditions may determine an outbreak. June 2013 stood out with a high mean temperature and precipitation, which have been shown to be associated with a high incidence of SINV infection later in summer (online Technical Appendix Figure 2).

A recent study in northern Sweden revealed that in a randomly selected population-based cohort, 2.9% had SINV IgG (Figure), indicating that the virus was present in the region, although not recognized (*10*). More research is warranted regarding the long-lasting joint pain caused by a previous SINV infection; patients with undiagnosed SINV may visit a healthcare facility with such symptoms even several months postinfection. Our report illustrates how a vectorborne zoonotic disease can result in a large, unexpected outbreak. The key factors for outbreaks of SINV or other alphavirus-caused diseases are generally unknown, which warrants further investigations, especially in light of the global emergence of alphaviruses (*1*).

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# *Ehrlichia muris* in *Ixodes cookei* Ticks, Northeastern United States, 2016–2017

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*Ehrlichia muris* is an agent of human ehrlichiosis. To determine its geographic spread in the United States, during 2016–2017, we tested 8,760 ticks from 45 states. A distinct clade of *E. muris* found in 3 *Ixodes cookei* ticks from the northeastern United States suggests transmission by these ticks in this region.

*Ehrlichia muris* was originally isolated from a mouse in Japan in 1983 (*1*). In 2009 in the United States, an *E. muris*–like agent (EMLA) was identified as a causative agent of human ehrlichiosis for 3 symptomatic patients in Wisconsin and 1 in Minnesota (*2*). A retroanalysis of 760 *Ixodes scapularis* ticks collected from 1992 through 1997 in Wisconsin revealed an EMLA infection rate of 0.94%, indicating presence of this pathogen in the upper midwestern region since at least the mid-1990s (*3*). Another study found this infection in 69 patients from 5 states from 2007 through 2013, although all patients had probably been exposed to the ticks in Minnesota or Wisconsin (*4*). In 2017, the *E. muris*–like isolates from the upper midwestern United States were proposed as a taxonomically distinct subspecies, *E. muris* subsp. *eauclairensis* (*5*).

*E. muris* is thought to be transmitted by *Haemaphysalis flava* ticks in Japan, by *I. persulcatus* ticks in eastern Europe, and by *I. ricinus* ticks in western Europe (*5*). Detection of the bacterium in nymphal and adult stages of *I. scapularis* ticks (*2*,*5*,*6*) and in white-footed mice (*Peromyscus leucopus*) suggests that the primary vectors and reservoir hosts of Lyme borreliosis play a major role in the enzootic transmission cycle of *E. muris* in the United States. However, despite the broad distribution of *I. scapularis* ticks and *P. leucopus* mice in North America, to our knowledge, *E. muris* has not been reported outside of Wisconsin and Minnesota (*2*,*7*).

To evaluate the geographic distribution of *E. muris* from May 30, 2016, through October 1, 2017, we used a Taqman real-time PCR to test 8,760 ticks for EMLA, *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato, *B. miyamotoi*, *B. mayonii*, *and Babesia microti*. The EMLA test is a modified version of a multiplex Taqman assay and targets the p13 gene (*8*). The human-biting ticks used for this study were submitted to the TickReport public testing program (https://www.tickreport.com) at the University of Massachusetts (Amherst, MA, USA). We confirmed EMLA positivity of the samples by amplifying and sequencing the EMLA citrate synthase (*gltA*) and heat shock protein (*groEL*) genes (*3*). We confirmed the species of EMLA-positive ticks by amplifying and sequencing a partial tick 16S rRNA gene (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/6/17-1755-Techapp1.pdf). We received 8,760 ticks from 45 states: 243 *Amblyomma americanum*, 2 *A. maculatum*, 7 *Amblyomma* spp., 6 *Dermacentor andersoni*, 3 *D. occidentalis*, 271 *D. variabilis*, 45 *Dermacentor* spp., 14 *I. angustus*, 22 *I. cookei*, 215 *I. pacificus*, 5 *I. ricinus*, 7,800 *I scapularis*, 19 *I. spinipalpis*, 47 *Ixodes* spp., and 7 *Rhipicephalus sanguineus*; 54 ticks were unidentifiable.

We found DNA specific for EMLA in only 2 species of *Ixodes* tick: *I. scapularis* and *I. cookei*. The overall prevalence of EMLA was very low. Only 5 (0.057%) ticks were positive for *E. muris*–specific DNA. Although we tested 7,800 *I. scapularis* ticks from 33 states in the northeastern, midwestern, and southeastern regions, we found only 2 (2/7,800, 0.026%) EMLA-positive *I. scapularis* ticks (1 from Laporte, MN, and 1 from Eleva, WI). These 2 ticks were co-infected with *B. burgdorferi* s. l. and *B. microti*. However, no DNA from *B. miyamotoi*, *B. mayonii*, or *A. phagocytophilum* was detected in these 2 ticks.

The prevalence of EMLA in *I. cookei* ticks was much higher than that in *I. scapularis* ticks. Of the 22 *I. cookei* ticks tested, 3 (3/22, 13.64%) were positive for EMLA (from Holden, ME; Littleton, ME; and Salamanca, NY). Co-infections were not detected in these 3 ticks.

To determine the identity of these EMLA isolates, we examined *gltA* and *groEL* gene sequences of isolates from the 2 *I. scapularis* ticks and found them to be identical. Phylogenetic analysis showed that they clustered with *E. muris* subsp. *eauclairensis*. The *gltA* and *groEL* gene sequences from the 3 *I. cookei* ticks were also identical but formed a new clade between *E. muris* subsp. *eauclairensis* and subsp. *muris* (Figure).

The detection of *E. muris* in *I. scapularis* ticks from the upper midwestern United States corroborates previously reported findings (*2*,*3*,*6*). The detection of a distinct clade of *E. muris* in *I. cookei* ticks from the northeastern United States represents a potential risk to humans or a different enzootic cycle of *E. muris* in the Northeast. As primary vectors of Powassan virus (lineage 1), *I. cookei* ticks are widely distributed in eastern North America and are the second most common species of *Ixodes* ticks found on persons in Maine, USA (*9*). Further study is warranted with regard to the vector competence of *I. cookei* ticks for transmitting *E. muris*, the natural enzootic cycle of *E. muris*, and the transmission potential of the pathogen to humans in this region. Meanwhile, human ehrlichiosis should be considered as a possible diagnosis for persons who have been

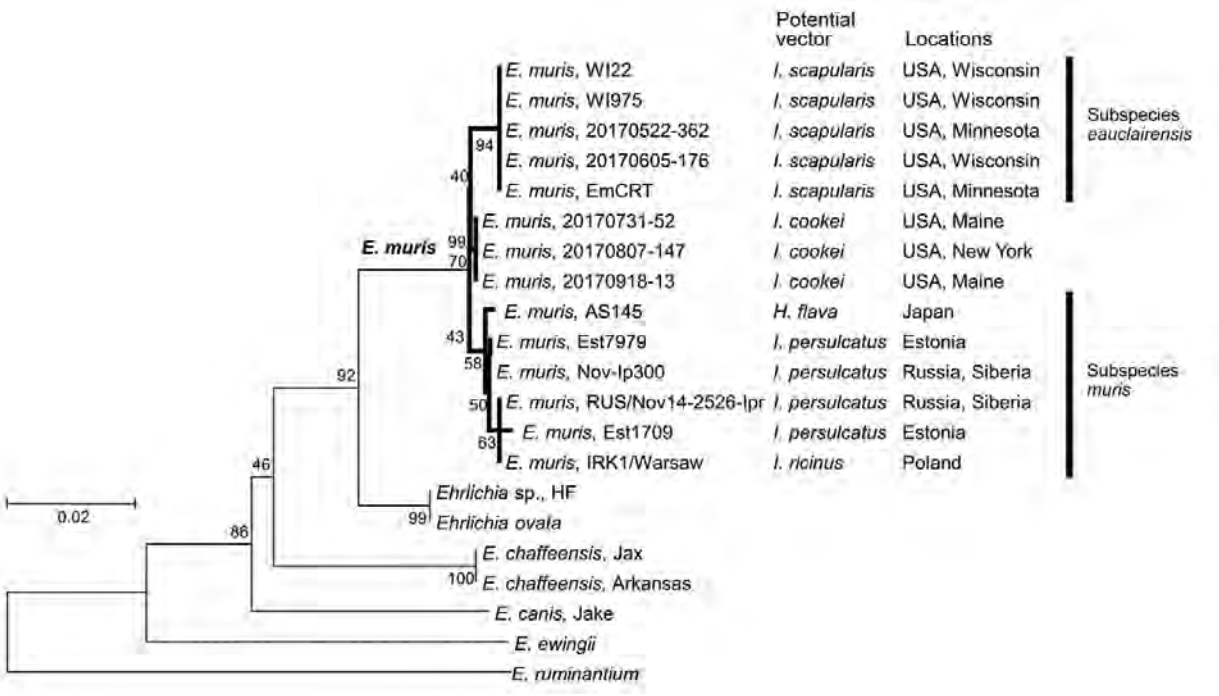


**Figure.** Phylogenetic tree of *Ehrlichia* citrate synthase (*gltA*) and heat shock protein (*groEL*) genes constructed by the maximum-likelihood method of MEGA6 software (http://www.megasoftware.net). The total length of 2 concatenated genes is 1,045 bp. Hasegawa-Kishino-Yano with invariable sites was selected as the best model based on Bayesian information criterion scores. Numbers on the branches represent bootstrap support with 500 bootstrap replicates. Scale bar indicates nucleotide substitutions per site.

exposed to *I. scapularis* and *I. cookei* ticks in the upper midwestern and northeastern United States, respectively.


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# Human *Pasteurella multocida* Infection with Likely Zoonotic Transmission from a Pet Dog, Spain

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We report a case of urinary tract infection caused by an unusual genotype (sequence type 211) of *Pasteurella multocida* associated with human infection. Molecular genetic analysis of *P. multocida* isolates obtained from the human patient and his pet strongly suggests a zoonotic transmission of this bacterium.

The bacterium *Pasteurella multocida* is one of the most frequent commensal and opportunistic pathogens found in domestic and wild animals worldwide (*1*). *P. multocida* is commonly cultured from the oropharynx of cats and dogs, and most human infections are associated with animal exposure, mainly from cats and dogs, and usually involve soft-tissue sites after animal bites or scratches (*1*). Among the wide clinical spectrum of invasive and noninvasive infections caused by *P. multocida*, urinary tract infections (UTIs) are rarely diagnosed, with <20 cases reported in the literature, most related to underlying diseases or urologic abnormalities (*2*,*3*). Here we present a case of UTI caused by an unusual genotype of *P. multocida*.

An 83-year-old man was referred to a primary healthcare center with urinary complaints and fever without his general condition being impaired. The patient had previously had prostatic adenoma and inguinal hernia diagnosed. Since 2013, he had suffered recurrent UTIs, which were treated empirically with oral ciprofloxacin, resulting in favorable clinical progression. In the last episode, urine analysis revealed the presence of proteins, nitrites, blood (10–25 cells/×400 microscope field), and abundant leukocytes (>100 cells/×400 microscope field). We sent a urine sample to the clinical microbiology laboratory of Hospital Universitario Central de Asturias and cultured in BBD CHROMagar Orientation Medium (Becton Dickinson, Heidelberg, Germany). We recovered bacterial counts (>$10^5$ CFU/mL) of an oxidase-positive gram-negative coccobacillus producing small (≈1 mm) white colonies in pure culture. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Microflex; Bruker Daltonik GmbH, Bremen, Germany) identified the bacterium as *P. multocida* (score >2), and this finding was confirmed by 2-strand sequencing of the 16S ribosomal RNA gene (*4*). We performed antimicrobial drug susceptibility testing by using the NegCombo Type 44 MicroScan panel (Beckman Coulter, Brea, CA, USA) and interpreted the results according to Clinical and Laboratory Standards Institute guidelines (*5*). The isolate was susceptible to all antimicrobial drugs tested (β-lactams, β-lactams plus β-lactamase inhibitors, quinolones, colistin, tetracycline, tigecycline, chloramphenicol, trimethoprim/sulfamethoxazole, fosfomycin, and nitrofurantoin) except aminoglycosides. We administered oral ciprofloxacin (500 mg every 12 h for 1 wk) to the patient, who had an excellent outcome, including bacteriuria eradication.

Further questioning of the patient indicated that he had a dog at home. We placed gingival swabs obtained from the animal in Amies transport medium and sent them to the hospital's clinical microbiology laboratory, where *P. multocida* was recovered. The animal isolate exhibited an antimicrobial drug susceptibility pattern identical to that of the patient isolate. Links between *P. multocida* human infections and pets are, in most cases, based on the information given by the patients indicating they have dogs or cats at home, but molecular studies aimed to associate *P. multocida* human infections with animal sources have rarely been conducted (*2*,*6*). To determine the source of the UTI, we subjected the patient and dog isolates to molecular typing. We determined the capsular types and genetically characterized the isolates by using multilocus sequence typing (*7*) and pulsed-field gel electrophoresis with *Apa*I and *Sma*I restriction enzymes (*8*,*9*). Both isolates belonged to capsular type A and to sequence type 211, a genotype that has been previously isolated only from avian wound infections (https://pubmlst.org/pmultocida). Moreover, both isolates exhibited indistinguishable pulsotypes with the 2 enzymes used (Figure). These facts, together with the identical antimicrobial drug susceptibility pattern, demonstrate that they are the same strain, thus establishing a definitive epidemiologic link between the patient and his dog.

The patient denied any history of recent bites or scratches, but *P. multocida* infections without a bite history can occur in patients with certain co-occurring conditions (*10*). The patient in this case had several predisposing factors, including a prostatic adenoma, which might

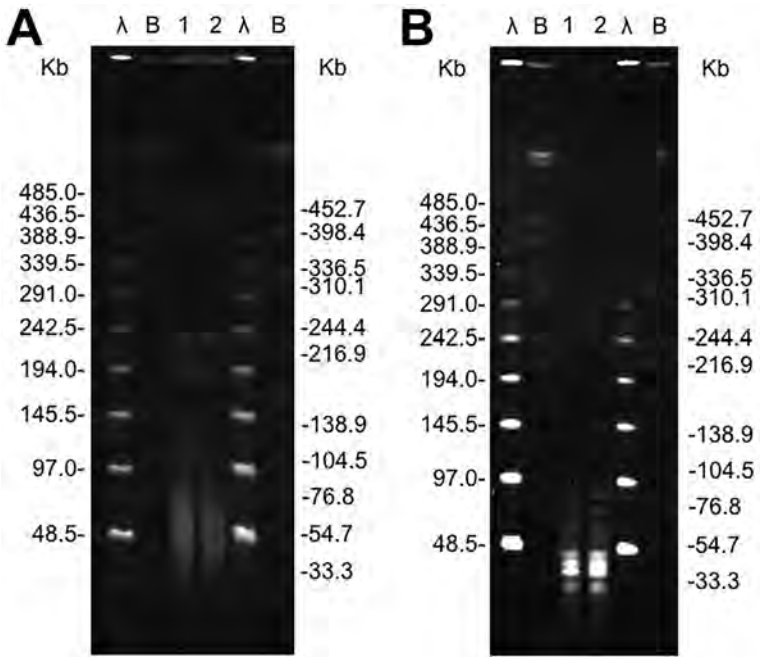


Figure. Pulsed-field gel electrophoresis profiles of *ApaI* (A) and *SmaI* (B) digested genomic DNA of *Pasteurella multocida* isolates from an 83-year-old man with a urinary tract infection (lane 1) and his pet dog (lane 2). Lanes λ (Lambda Ladder PFGE marker [New England BioLabs, Ipswich, MA, USA]) and lanes B (DNA from *Salmonella enterica* serovar Branderup H9812 digested with *XbaI*) used as molecular size standards.

have favored the infection by *P. multocida* because of mechanical alteration of the urinary tract. However, the specific route by which *P. multocida* reached the bladder could not be established. Although the possibility of a small scratch that had gone unnoticed cannot be ruled out, an alternative explanation could be a periurethral contamination of the patient after handling his dog, followed by colonization of the urethra and subsequent migration of the bacteria to the bladder. Although the specific route of transmission could not be elucidated in this case, pet owners and physicians should keep in mind that companion animals could be the source of infection by a wide range of opportunistic pathogens.

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# Reassortant Clade 2.3.4.4 of Highly Pathogenic Avian Influenza A(H5N6) Virus, Taiwan, 2017

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A highly pathogenic avian influenza A(H5N6) virus of clade 2.3.4.4 was detected in a domestic duck found dead in Taiwan during February 2017. The endemic situation and continued evolution of various reassortant highly pathogenic avian influenza viruses in Taiwan warrant concern about further reassortment and a fifth wave of intercontinental spread.

Since 1996, H5 A/goose/Guangdong/1/1996 (Gs/GD) lineage of highly pathogenic avian influenza viruses (HPAIVs) originating in Asia have caused outbreaks in Asia, Europe, Africa, and North America (*1*). The H5N1 Gs/GD lineage of HPAIV has evolved into 10 genetically distinct virus clades (0–9) and multiple subclades, including novel H5 clade 2.3.4.4 viruses, which emerged in China (*2*) and have evolved into 4 distinct genetic groups (2.3.4.4A–D) (*3*). Four 4 intercontinental waves of Gs/GD lineage HPAIV transmission have occurred: clade 2.2 H5N1 in 2005, clade 2.3.2.1c H5N1 in 2009, clade 2.3.4.4A H5Nx in 2014, and clade 2.3.4.4B H5Nx in 2016 (*4*). The clade 2.3.4.4A and B H5N8 viruses spread intercontinentally; clade 2.3.4.4A caused outbreaks in Asia, Europe, and North America during 2014–2015, and clade 2.3.4.4B H5N8 caused outbreaks in Asia, Europe, and Africa during 2016–2017 (*1*,*5*). In fall 2016, clade 2.3.4.4C H5N6 viruses caused outbreaks in South Korea and Japan (*6*). Six distinct genotypes of clade 2.3.4.4C H5N6 viruses (designated as C1–C6) were identified in South Korea and Japan during these outbreaks; these genotypes contain different polymerase acidic and nonstructural genes from low pathogenicity influenza viruses from Eurasia (*7*,*8*).

We report HPAIV H5N6 detection from a meat-type duck in Taiwan in February 2017. One dead young Pekin-type domestic duck was found on a country road near the Xiuguluan River in Hualien County during wild bird and habitat surveillance for HPAIV by the Wild Bird Society of Taipei; the carcass was forwarded to the national laboratory of the Animal Health Research Institute (online Technical Appendix 1 Figure 1, https://wwwnc.cdc.gov/EID/article/24/6/17-2071-Techapp1.pdf). We conducted complete genome sequencing and comparative phylogenetic analysis of the detected virus, A/duck/Taiwan/1702004/2017(H5N6) (Dk/Tw/17), to trace the origin and understand its genetic features.

We detected Dk/Tw/17 virus by using reverse transcription PCR and isolated the virus by using egg inoculation as described previously (*9*). We conducted an intravenous pathogenicity index test according to the World Organisation for Animal Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (http://www.oie.int/en/international-standard-setting/terrestrial-manual). We performed full-length genome sequencing by using reverse transcription PCR amplification and Sanger sequencing (*9*). We estimated maximum-likelihood phylogenies by using RAxML (*10*) and constructed a median-joining phylogenetic network of the hemagglutinin gene by using NETWORK 5.0 (online Technical Appendix).

We classified Dk/Tw/17 as an HPAIV on the basis of the amino acid sequence at the hemagglutinin cleavage site (PLRERRRKR/G) and its high lethality in chickens (intravenous pathogenicity index 3.0). Necropsy and histologic examination revealed virus-specific necrotic and inflammatory lesions in the pancreas, heart, and brain (online Technical Appendix 1 Figure 2). Phylogenetic analyses suggested that the Dk/Tw/17 virus belongs to clade 2.3.4.4C genotype C5 that was found in China, South Korea, and Japan during 2016–2017 (Figure, panel A; online Technical Appendix 1 Figure 1). This virus genotype acquired its polymerase acidic gene of low pathogenicity influenza viruses from Eurasia; its other genes originated in the G1.1.9 and G1.1-like lineages of H5N6 viruses from China (*7*,*8*). All 8 gene segments shared high levels of nucleotide identity (99.3%–99.9%) with H5N6 viruses identified from wild birds in Japan and South Korea in November 2016, including A/whooper swan/Korea/Gangjin 49-1/2016 (H5N6), A/spot billed duck/Korea/WB141/2016 (H5N6), and A/teal/Tottori/2/2016 (H5N6) (online Technical Appendix 1 Table). These viruses consistently clustered together with high bootstrap value (>70) in maximum-likelihood phylogenies across all 8 gene segments (online Technical Appendix Figures 3–10).

The genotype C5 comprises 17 H5N6 HPAIVs identified from wild waterfowl in China, Japan, and South Korea during November–December 2016; a virus identified from a chicken farm (A/chicken/Korea/H23/2016 [H5N6]) in South Korea in November 2016; and the Dk/Tw/17 virus. Genotype C5 is phylogenetically distinct from viruses that caused outbreaks in poultry farms in Japan and South

[1]These authors contributed equally to this article.

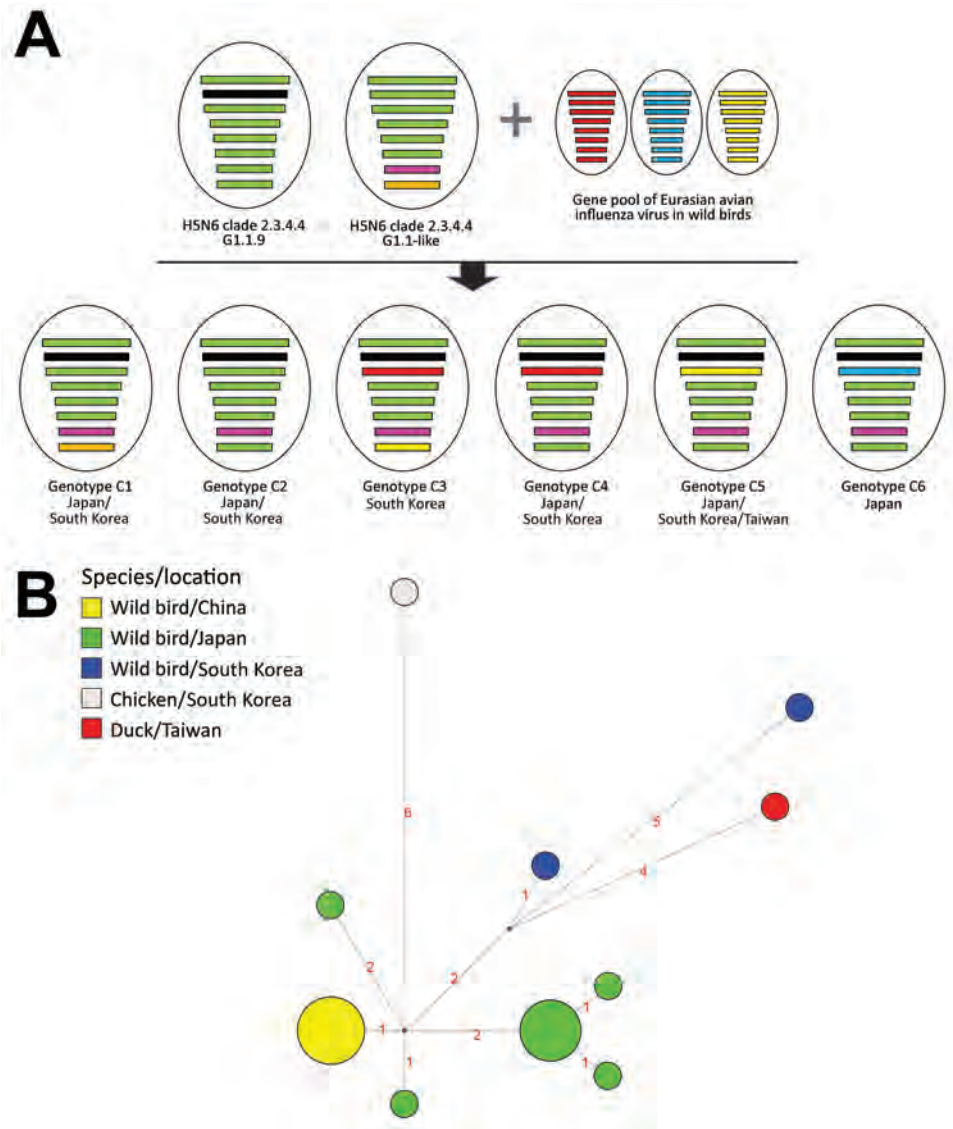


**Figure.** Genome constellation of influenza A(H5N6) viruses identified in East Asia during 2016–2017 and median-joining phylogenetic network of genotype C5. A) Viruses are represented by ovals containing horizontal bars that represent 8 gene segments (top to bottom: polymerase basic 2, polymerase basic 1, polymerase acidic, hemagglutinin, nucleoprotein, neuraminidase, matrix, and nonstructural). B) Median-joining phylogenetic network of genotype C5 constructed from the hemagglutinin gene and includes all the most parsimonious trees linking the sequences. Each unique sequence is represented by a circle sized relative to its frequency in the dataset. Branch length is proportional to the number of mutations.

Korea during 2016–2017. This genotype has independently evolved and been maintained in wild bird populations in the bird flyway of East Asia, highlighting how wild waterfowl play an important role in the maintenance and dissemination of this HPAIV. In addition, the median-joining phylogenetic network analysis suggests that the A/chicken/Korea/H23/2016 (H5N6) is not the direct ancestor of the Dk/Tw/17 virus, which was likely caused by separate introduction from wild birds (Figure, panel B).

The site where the dead duck was collected is adjacent to a river and located near many ponds used for duck farming. After identification of Dk/Tw/17, intensified active surveillance conducted over 3 months detected additional clade 2.3.4.4C H5N6 HPAIVs from 12 farms in 4 counties (online Technical Appendix 1 Figure 1). Clade 2.3.4.4A H5Nx HPAIVs, mainly H5N2 and H5N8, have caused outbreaks in the poultry industry of Taiwan since January 2015 (*9*). In 2017, clade 2.3.4.4A H5Nx and 2.3.4.4C H5N6 HPAIVs were detected in domestic poultry. The endemic situation and continued evolution of various reassortant HPAIVs in domestic poultry warrants concern about further reassortment. Enhanced active surveillance in domestic and wild waterfowl is required to monitor the spread and onward reassortment in Taiwan and to inform the design of improved prevention and control strategies.

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# Reemergence of Human Monkeypox in Nigeria, 2017

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In Nigeria, before 2017 the most recent case of human monkeypox had been reported in 1978. By mid-November 2017, a large outbreak caused by the West African clade resulted in 146 suspected cases and 42 laboratory-confirmed cases from 14 states. Although the source is unknown, multiple sources are suspected.

Human monkeypox is a rare zoonotic infection caused by an orthopoxvirus and characterized by smallpox-like signs and symptoms (*1*). The disease is endemic to the Democratic Republic of the Congo. Reported outbreaks have occurred mainly in rural rainforest areas of the Congo basin and West Africa, caused by the Central and West African clades of the virus, respectively (*1–6*). The West African clade is associated with milder disease, fewer deaths, and limited human-to-human transmission. Since 1970, only ≈10 cases in West Africa had been reported; in 2003, a total of 81 cases (41% laboratory confirmed) were reported in the United States (*2*,*7*,*8*). In Nigeria, a case of human monkeypox in a 4-year-old child in the southeastern part

of the country was reported in 1971 (*4*,*5*); no more cases in Nigeria had been reported since 1978 (*2*,*6*). We provide a preliminary report of a large outbreak of human monkeypox in Nigeria caused by the West African clade of monkeypox virus in 2017.

On September 22, 2017, the Nigeria Centre for Disease Control (NCDC) was notified of a suspected case of monkeypox; the patient had been admitted to the Niger Delta University Teaching Hospital, Bayelsa State, in the South South region of Nigeria. Outbreak investigations commenced immediately; isolation of the suspected case-patient, laboratory testing, and contact tracing were conducted.

The patient was an 11-year-old boy with an 11-day history of fever, generalized rash, headache, malaise, and sore throat. Physical examination revealed generalized well-circumscribed papulopustular rashes on the trunk, face, palms, and soles of the feet and subsequent umbilication, ulcerations, crusting, and scab formation. The patient had associated oral and nasal mucosal lesions and ulcers and accompanying generalized lymphadenopathy. Similar signs and symptoms, with varying degrees of severity, developed in 5 other family members living in the same household. The index case-patient and 2 of his siblings reported a history of having had contact with a neighbor's monkey 1 month earlier, but it cannot be ascertained if the monkey was the source of their infection; the monkey had no known history of illness.

After identifying these cases as being suspected monkeypox, the NCDC immediately deployed epidemiologists to Bayelsa State to support detailed outbreak investigations. Health authorities in all states of the country were notified to establish enhanced surveillance based on a standardized case definition. As notification of suspected cases from other states increased, on October 9, 2017, the NCDC activated a national Emergency Operations Centre to coordinate the response to an unusual evolving outbreak. All relevant stakeholders (e.g., ministries of health, agriculture and animal health, and information) were mobilized for a robust response. The NCDC rapidly developed interim guidelines and protocols; disseminated them to all states; and implemented intensive surveillance, public sensitization, community mobilization, and case management accordingly across all states.

Laboratory diagnosis (by real-time PCR, IgM serology, and genomic sequencing) were initially undertaken at Institut Pasteur (Dakar, Senegal), Redeemer's University Laboratory (Ede, Nigeria), and the US Centers for Disease Control and Prevention (Atlanta, GA, USA). Further diagnostics took place later at the NCDC National Reference Laboratory with technical support from the US Centers for Disease Control and Prevention.

On October 13, 2017, the NCDC received laboratory confirmation of a human monkeypox outbreak in Nigeria. As of November 17, 2017, a total of 146 suspected cases had been reported from 22 of the 36 states in Nigeria (Figure). Of the 134 cases for which samples were available (blood, lesion swab, and crust) collected during the reporting period, 107 were tested, and 42 samples from 14 states were laboratory confirmed as the West African clade of the monkeypox virus (Figure). Most (62%) of the laboratory-confirmed cases were in adults (21–40 years of age; median 30 years of age); the male:female ratio was 2:1. A 46-year-old male patient with confirmed monkeypox and a history of immunosuppressive illness died. For some patients with suspected (but ultimately deemed negative) cases of monkeypox, chickenpox (wild-type virus) was confirmed. Further analysis of the monkeypox-negative samples is ongoing.

Although detailed epidemiologic investigations to ascertain the source and route of transmission are ongoing, 3 family clusters were found, which might suggest some level of human-to-human transmission in this outbreak. For 1 of the families, the secondary attack rate was 71%. However, most patients had no obvious epidemiologic linkage or person-to-person contact, indicating a probable multiple-source outbreak or possibly previously unrecognized endemic disease. The zoonotic source(s) of the outbreak are currently unknown, and it is unclear what, if any, environmental or ecologic changes might have facilitated its sudden reemergence.

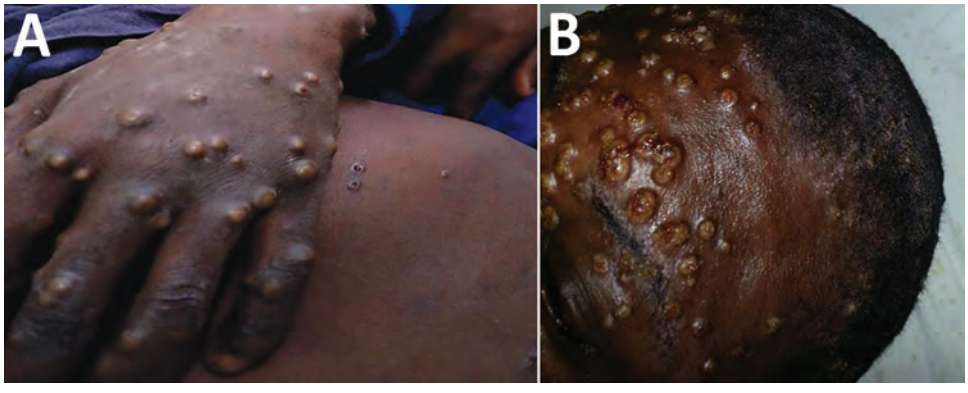


**Figure.** Papulopustular rash on hand (A) and face (B) of patient with monkeypox.

This large outbreak of West Africa clade human monkeypox (*3*,*8*,*9*) mostly affected adults. The NCDC continues response activities and investigations in collaboration with national and international partners. Further findings from our epidemiologic investigations and laboratory diagnostics, including genome sequencing, will add to the existing knowledge of West African monkeypox and help unravel uncertainties in the outbreak.

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# *Mycobacterium bovis* Infection of Red Fox, France

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*Mycobacterium bovis* infection in wild red foxes was found in southern France, where livestock and other wildlife species are infected. Foxes frequently interact with cattle but have been underestimated as a reservoir of *M. bovis*. Our results suggest a possible role of the red fox in the epidemiology of bovine tuberculosis.

*Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex (MTBC), is the main etiologic agent of bovine tuberculosis (TB). In France and other countries in Europe, this ancient zoonotic disease is regarded not just as a problem of cattle but as a concern for multihost communities that include wildlife species such as wild boar (*Sus scrofa*), red deer (*Cervus elaphus*), and badgers (*Meles meles*) (*1*). The red fox (*Vulpes vulpes*) is usually considered a spillover host of TB. Sparse numbers of infected foxes had been found in highly prevalent TB regions, such as Great Britain (*2*). Until recently, the number of infected foxes in TB-endemic zones in France was low (1/68 in Brotonne Forest, Normandy, northwestern France, and 2/61 in Côte d'Or, Burgundy, central-eastern France) (*3*). However, recently an increasing number of reports have described the presence of a substantial number of infected red foxes in Mediterranean habitats in Spain (*4*) and Portugal (*5*), where the disease is highly prevalent in wildlife and livestock. In these cases, the prevalence was high (14% and 26.9%, respectively). Strikingly, most animals did not have TB-like visible lesions in any of these reports.

In a recent study, Payne demonstrated that in Burgundy, France, a region where TB is prevalent in cattle and

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wildlife, the red fox is the species that most often visits cattle environments (*3*). In 2015, we conducted a small-scale investigation on trapped red foxes (n = 6) in a municipality of Dordogne, a TB-endemic region in southwestern France, where multihost cycles involving cattle, badgers, wild boar, and even red deer and roe deer (*Capreolus capreolus*) exist (*6*). The necropsy examination included detailed macroscopic inspection of lymph nodes and abdominal and thoracic viscera (*6*). We found no TB-like visible lesions; pooled tissue samples (retropharyngeal, tracheobronchial, mediastinal, and mesenteric lymph nodes) were submitted for bacterial culture and molecular diagnosis (*6*); urine, feces, and oropharyngeal swabs were taken, and DNA was extracted as previously described (*7*). We performed molecular diagnostic testing using MTBC PCR (IS*6110* and IS*1081*) and the regions of difference and through spoligotyping (*6*), the latter 2 enabling differentiation of the MTBC members when used on highly concentrated DNAs. Of the 6 red foxes, 4 were bacteriology positive; molecular tools enabled identification of *M. bovis* spoligotype SB0120 and MTBC or *M. bovis* excretion in feces of all infected animals (Table). One red fox (RN5) also gave positive results on oropharyngeal swab and urine samples, suggesting *M. bovis* excretion through several routes.

Although molecular detection does not prove that bacilli are viable, this method is particularly useful for monitoring shedding in environmental samples—especially fecal material—where bacterial culture has poor sensitivity. Molecular quantification on these samples seems to correlate strongly with the animal's infection level (*7*).

Fecal excretion may result from digestive infection (*8*). Infection in mesenteric lymph nodes, observed in other carnivores, suggests that the primary route of transmission of infection is through the digestive tract and strongly suggests fecal excretion (*5*). Fox RN5 may be considered a supershedder or super-excretor (*9*) because shedding of the tuberculous bacillus by several routes was observed (e.g., oropharyngeal swab samples, feces, and urine). Super-shedders have been described as responsible for a disproportionately large amount of *M. bovis* excretion from the infected animal with a substantial role in the transmission and maintenance of TB in multihost pathogen systems (*9*), raising the question of the role of the fox in the epidemiology of TB.

Infection in foxes has been suggested to result from scavenging on infected wild ungulate carcasses (*4*). Alternatively, because foxes may inhabit disused badger setts and vacant parts of occupied setts, they could acquire infection from the contaminated environment (*10*). This possibility may constitute a risk regarding TB because the fox has been described as the most frequently observed species directly interacting with cattle and visiting farm facilities (*3*).

The presence of visible lesions alone does not appear to be a good indicator of *M. bovis* infection in carnivores such as foxes, which often lack macroscopic lesions. The nature of *M. bovis* infection and the host response are likely to vary widely among species, making simple generalizations about pathology difficult to determine (*2*). The prevalence of kidney lesions has been reported to be low in wildlife species, which may be explained by the difficulty of detecting TB lesions in organs with a large parenchyma or the presence of microscopic lesions often missed by gross pathology (*3*,*8*). Further studies are needed to investigate urinary excretion and the prevalence of kidney TB lesions in red fox and in wildlife in general. Together with this study's results, these observations highlight that the role of red fox in the epidemiology of TB needs further investigation.

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**Table.** Results of direct *Mycobacterium bovis* detection on organs and molecular detection on excretory samples of red foxes, France, 2015*

| | Direct detection on organs | | Molecular detection on excretory samples | | |
|---|---|---|---|---|---|
| Fox | Bacteriology | Molecular diagnostics | Oropharyngeal swab | Feces | Urine |
| RN2 | Negative | Negative | Negative | Negative | Negative |
| RN3 | Negative | Negative | Negative | Negative | No sample |
| RN4 | *M. bovis* SB0120 | MTBC | Negative | MTBC | No sample |
| RN5 | *M. bovis* SB0120 | MTBC | MTBC | MTBC | *M. bovis* SB0120 |
| RN6 | *M. bovis* SB0120 | *M. bovis* SB0120 | Negative | MTBC | Negative |
| RN7 | *M. bovis* SB0120 | Negative | Negative | *M. bovis* (partial spoligotype profile) | Negative |

*MTBC, *Mycobacterium tuberculosis* complex.

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# *Angiostrongylus cantonensis* Infection of Central Nervous System, Guiana Shield

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We report a case of eosinophilic meningitis complicated by transverse myelitis caused by Angiostrongylus cantonensis in a 10-year-old boy from Brazil who had traveled to Suriname. We confirmed diagnosis by serology and real-time PCR in the cerebrospinal fluid. The medical community should be aware of angiostrongyliasis in the Guiana Shield.

In September 2017, a previously healthy 10-year-old boy from Brazil came to the emergency department of Andrée Rosemon Hospital in Cayenne, French Guiana, a French territory that forms the Guiana Shield together with Guyana (formerly British Guiana), Suriname, and the Brazil state of Amapá. He related a 4-day history of helmet headache, repeated vomiting, and hyperthermia (38.5°C). The patient had lived in Saint-Laurent-du-Maroni, a city on the French Guiana border with Suriname, for 5 years and had recently returned from a 3-day trip in Suriname. He had no memory of ingesting slugs, snails, or uncooked vegetables, but he reported playing with snails during the rainy season (April–August).

At admission to the pediatric department, he was afebrile with a good state of consciousness (Glasgow coma score 15). Our physical examination revealed a stiff neck, with positive Kernig and Brudzinski signs but no focal deficits. Hematology revealed a leukocyte count of 12.30 $\times 10^9$ cells/L (reference range 4–14.5 $\times 10^9$ cells/L) with 5.49 $\times 10^9$ eosinophils/L (reference range 0.05–0.85 $\times 10^9$

eosinophils/L). C-reactive protein was <3 mg/L; liver and renal function tests were normal. Computed tomography of the head showed unremarkable results. We performed a lumbar puncture; cerebrospinal fluid (CSF) analysis revealed $8.7 \times 10^6$ leukocytes/L (30% neutrophils and 70% lymphocytes), protein 0.43 g/L, glucose 4.2 mmol/L, and lactates 2.2 mmol/L. Gram stain result was negative for bacteria. Results of India ink test and microscopic examination of CSF were negative for *Cryptococcus* spp. We saw no helminth larvae in the CSF. Serologic test results for *Treponema pallidum*, *Borrelia burgdorferi*, *Leptospira* spp., *Mycoplasma pneumoniae*, *Chlamydiophila pneumoniae*, *Brucella* spp., herpes simplex virus, and HIV were all negative. Microscopic examinations of 3 fecal specimens using the concentration method and Baermann technique showed negative results. We began empiric treatment with intravenous cefotaxime (300 mg/kg/d).

On day 6 of hospitalization, paraparesis of the lower limbs (more marked on the left) and dysuria appeared; meningeal syndrome persisted. A cerebromedullary magnetic resonance imaging (MRI) scan revealed myelitis lesions through a marrow signal abnormality ranging from T2 to T10 and a discrete signal enhancement after gadolinium injection (Figure, panel A). Electroencephalography results were unremarkable. We performed a second lumbar puncture on day 7; CSF showed $5.5 \times 10^6$ leukocytes/L with 92% eosinophils, protein 0.42 g/L, glucose 2.80 g/L, and lactates 2.7 mmol/L. Results of CSF bacterial cultures and PCRs for herpes simplex virus and enterovirus were negative. Serologic testing by Western blot was negative for *Gnathostoma* spp. nematodes but positive for *Angiostrongylus* spp. roundworms by detection of the specific 31-kDa antigenic band (*1*). Diagnosis of angiostrongyliasis was confirmed by *A. cantonensis* DNA detection in the CSF by real-time PCR performed by the US Centers for Disease Control and Prevention (Atlanta, GA, USA) (*2*).

We treated the patient with oral ivermectin (200 μg/kg/d for 10 days) in combination with intravenous methylprednisolone (30 mg/kg/d for 5 days), followed by oral prednisolone (2 mg/kg/d), which was gradually discontinued over 1 month. The patient's condition improved noticeably, with progressive disappearance of headaches, dysuria, and paraparesis in the following weeks. A cerebromedullary MRI performed on day 38 after admission showed almost complete recovery from the anomalies detected previously and did not report new anomalies (Figure, panel B). Three months after the onset of the disease, the patient had recovered completely without any sequelae.

Our findings demonstrate the presence of *A. cantonensis* roundworms in the Guiana Shield, in the context of a recent emergence of angiostrongyliasis in Brazil (*3*), the Caribbean region (including other French territories of the Americas) (*4*,*5*), and the southern United States (*6*,*7*). The frequency of *A. cantonensis* infections in humans in the Guiana Shield is probably underestimated as a result of the spontaneous course of recovery for most cases (*8*), lack of knowledge of the parasite by health professionals, limited availability of laboratory diagnostic tools, and the absence of national surveillance. Although the disease usually resolves spontaneously, case-fatality rates can reach 5% (*9*). The lack of clinical suspicion for angiostrongyliasis

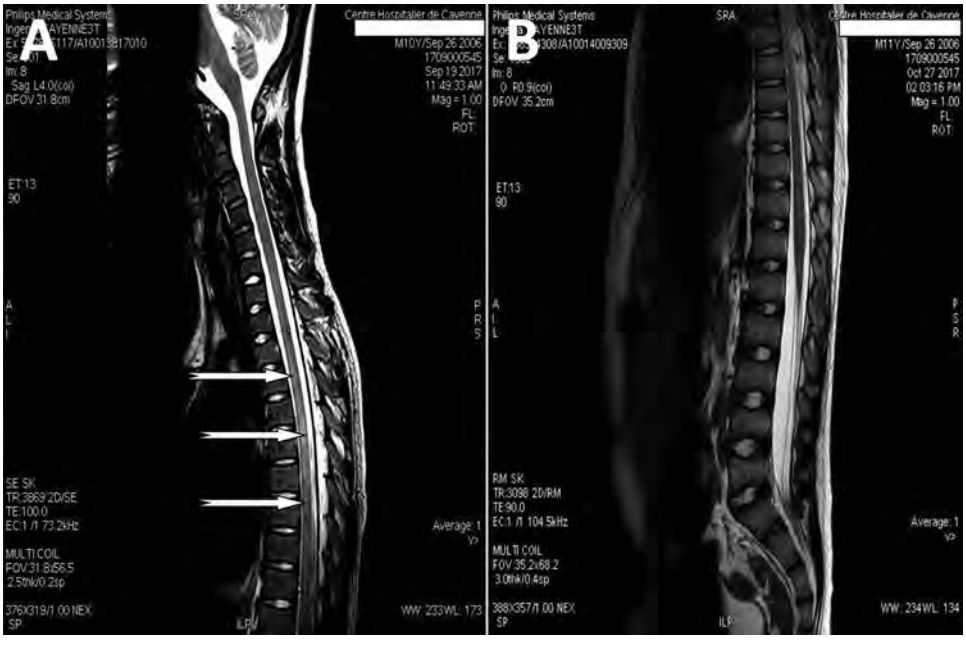


**Figure**. Magnetic resonance imaging (MRI) of the spine in a 10-year-old boy from Brazil with *Angiostrongylus cantonensis* infection. A) MRI before treatment showing myelitis; sagittal T1 postcontrast sequences show intramedullary enhancement in the thoracic spinal cord T2–T10 with diffuse leptomeningeal enhancement (arrows). B) Normal MRI 1 month after treatment.

on the basis of signs and symptoms and delay in initiation of treatment may lead to adverse neurologic outcomes, especially in young children (*10*). Because the patient in this study had traveled to Suriname shortly before symptom onset, the country of origin of the infection could not be determined. The likely route of transmission was contact with a contaminated mollusk, such as the giant African snail *Achatinafulica fulica*, which is a new and invasive species in Latin America and a known vector for *A. cantonensis* roundworms. Our case illustrates the necessity for healthcare providers to consider angiostrongyliasis in cases of eosinophilic meningitis in the Guiana Shield, especially in young children.

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The authors have obtained a written consent to publish from the child's parents.

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# etymologia

## *Angiostrongylus* [an"je-o-stron'jĭ-ləs]

**Ronnie Henry**

From the Greek *angeion* ("vessel") + *strongylos* ("round"), *Angiostrongylus* is a genus of parasitic nematodes (roundworms) in the family *Angiostrongylidae*, 2 species of which are known to parasitize humans. *A. cantonensis* (commonly known as rat lungworm) was first described in 1935 (as *Pulmonema cantonensis*) from rats in Canton, China. It is the most common cause of eosinophilic meningitis in Asia and the Pacific Basin, but cases have been reported in many parts of the world. *A. costaricensis* roundworms were first described in 1971 in Costa Rica from surgical specimens from children with eosinophilic infiltration in the abdominal cavity. The distribution of this species ranges from the southern United States to northern Argentina.

There is still debate about what taxonomic name should be used. *A. cantonensis* remains in general use, but some researchers suggest it should be changed to *Parastrongylus cantonensis* on the basis of the morphology of the adult male bursa and the definitive host being rats.

Adult *Angiostrongylus cantonensis* nematode recovered from rat lung. Image from Enzootic *Angiostrongylus cantonensis* in Rats and Snails after an Outbreak of Human Eosinophilic Meningitis, Jamaica, John F. Lindo et al, Emerging Infectious Diseases, Vol. 8, No. 3, March 2002.

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## Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

# Molecular Diagnosis of *Taenia saginata* Tapeworm Infection in 2 Schoolchildren, Myanmar

**Eun Jeong Won, Bong-Kwang Jung, Hyemi Song, Mi-Seon Kim, Hyun-Seung Kim, Keon Hoon Lee, Min-Jae Kim, Myung Geun Shin, Jong Hee Shin, Soon-Pal Suh, Sung-Jong Hong, Woon-Mok Sohn, Thi Thi Htoon, Htay Htay Tin, Jong-Yil Chai**

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*Taenia saginata* is the most common human tapeworm worldwide but has been unknown in Myanmar. In 2017, fecal examination in Yangon, Myanmar, revealed eggs of *Taenia* species in 2 children from a monastic school. Several proglottids expelled after medication with praziquantel were morphologically and molecularly confirmed to be *T. saginata* tapeworms.

Human taeniasis is a parasitic infection caused by tapeworm species including *Taenia saginata*, *T. solium*, and *T. asiatica* (*1*). *T. saginata* tapeworm infection is acquired through ingestion of raw or undercooked beef; pork is the infection source for *T. solium* and *T. asiatica* tapeworms (*1*). Because of differences in the life cycle, geographic distribution of these parasites can be affected by regional lifestyle, including dietary habit. Little is known about taeniasis in Myanmar. We report 2 cases of taeniasis caused by *T. saginata* tapeworms in Myanmar.

In June 2017, the Korea Association of Health Promotion, in cooperation with the National Health Laboratory, Myanmar, conducted a survey of intestinal parasitic infections near the Yangon region of Myanmar. The Institutional Review Board of the Ministry of Health and Sports, Myanmar (Ethical Review Committee no. 005117) approved the study. A total of 467 fecal samples were obtained from school-age children living in the district of Shwe Pyi Thar, Myanmar. In fecal examination using the Kato-Katz thick-smear technique, we found the eggs of *Taenia* tapeworms in 2 brothers, 8 and 10 years of age (Figure, panel A). They had never traveled out of Myanmar,

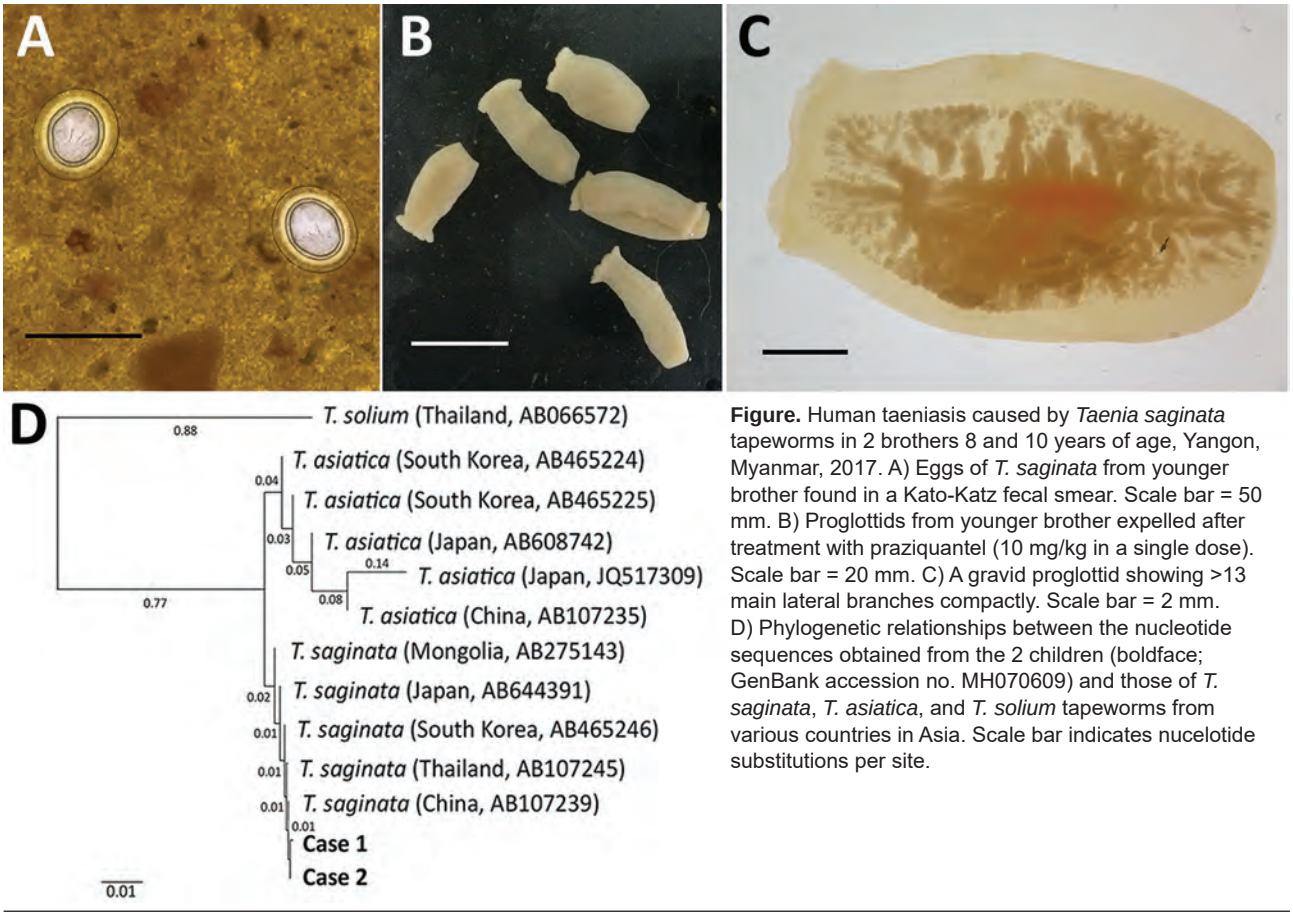


**Figure.** Human taeniasis caused by *Taenia saginata* tapeworms in 2 brothers 8 and 10 years of age, Yangon, Myanmar, 2017. A) Eggs of *T. saginata* from younger brother found in a Kato-Katz fecal smear. Scale bar = 50 mm. B) Proglottids from younger brother expelled after treatment with praziquantel (10 mg/kg in a single dose). Scale bar = 20 mm. C) A gravid proglottid showing >13 main lateral branches compactly. Scale bar = 2 mm. D) Phylogenetic relationships between the nucleotide sequences obtained from the 2 children (boldface; GenBank accession no. MH070609) and those of *T. saginata*, *T. asiatica*, and *T. solium* tapeworms from various countries in Asia. Scale bar indicates nucelotide substitutions per site.

and there was no possibility of consumption of imported beef. The younger boy had no specific gastrointestinal symptoms, but actively moving tapeworm segments had been found in his feces a year earlier. The older boy also had no special gastrointestinal symptoms.

The children were orphans who lived in Shan State and moved to a monastic school in the Shwe Pyi Thar area in 2015. They relied on traditional food donated from villagers, but food history related to raw beef or pork with their origin (imported or not) was unclear. However, the children reported that the Shan population usually enjoys traditional cuisine, fermented with rice and raw beef or pork.

A sample of whole feces was collected from each child 1 day after treatment with praziquantel (10 mg/kg single oral dose). Seven tapeworm segments were recovered in feces (Figure, panel B), and >13 uterine segments filled with eggs were observed (Figure, panel C). We extracted genomic DNA from segments by using the DNeasy Blood &Tissue Kit (QIAGEN, Hilden, Germany) as recommended by the manufacturer. The mitochondrial cytochrome c oxidase 1 (*cox1*) gene was targeted in PCR amplification and sequencing. The PCR amplification was performed with primers T1F (5′-ATA TTT ACT TTA GAT CAT AAG CGG-3′) and T1R (5′-ACG AGA AAA TAT ATT AGT CAT AAA-3′), and conditions according to a previous study (*2*). Sequencing of the 502 bp *cox1* gene showed 98.8%–99.6% nt identity with *T. saginata*, but 93.8%–94.4% with *T. asiatica*, and 87.8%–88.0% with *T. solium*. These specimens were molecularly close to *T. saginata* tapeworms reported from various Asian countries but far from *T. asiatica* or *T. solium* tapeworms (Figure, panel D). Our results demonstrate that *T. saginata* tapeworms caused the taeniasis in these 2 children.

*T. saginata* tapeworms have a global distribution and are known to be endemic to Southeast Asia. Recently, epidemiologic studies of taeniasis in Myanmar have been performed; however, they focused on *T. solium* cysticercosis in pigs in Nay Pyi Taw area and seropositivity of refugee camp residents on the Thailand–Myanmar border (*3*,*4*). A report of *T. saginata* in Myanmar described only an experimental infection in animals, not human infections (*5*). Fecal examination might not be helpful in cases of taeniasis because *Taenia* spp. eggs are not differentiated morphologically. Instead, taeniasis can be diagnosed through the morphology of gravid proglottids or by immunologic or molecular techniques. In the cases we reported, a history of active movement of proglottids and >13 uterine branches in recovered segments indicated *T. saginata* rather than

*T. solium* tapeworm infection, but *T. asiatica* tapeworms could not be fully ruled out. Although *T. asiatica* tapeworms can differ morphologically from *T. saginata* tapeworms (*6*), the distinctions could not always be found in each strobila; thus, molecular analyses were required to clearly distinguish them (*2*,*7*). We analyzed mitochondrial *cox1* of the *Taenia* tapeworm specimens and showed that the sequences clustered with *T. saginata* tapeworms reported from several Asia countries, but far from those of *T. asiatica* and *T. solium* tapeworms. Recently, human infections caused by hybrid infection with *T. saginata* and *T. asiatica* tapeworms in Laos were determined by sequencing the DNA polymerase delta region (*8*). Thus, for further studies, it may be useful to analyze not only the mitochondrial gene but also nuclear DNA.

Although epidemiologic surveys of *T. saginata* tapeworms have not been conducted in Myanmar, there is a strong possibility of the domestic occurrence of human taeniasis from consumption of undercooked beef or pork. Our report suggests that surveys of the prevalence and associated factors of human taeniases are urgently needed in Myanmar.

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# Strengthening of Surveillance during Monkeypox Outbreak, Republic of the Congo, 2017

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Reports of 10 suspected cases of monkeypox in Likouala Department, Republic of the Congo, triggered an investigation and response in March 2017 that included community education and surveillance strengthening. Increasing numbers of outbreaks suggest that monkeypox virus is becoming a more prevalent human pathogen. Diverse approaches are necessary for disease control and prevention.

On January 27, 2017, the Republic of the Congo Division of Disease Control was notified of 2 suspected human cases of monkeypox (MPX) in Likouala Department, in the northern part of the country, which prompted a local investigation. In March 2017, after 8 additional suspected cases were reported, the Republic of the Congo Division of Disease Control joined with external partners (World Health Organization, United Nations High Commissioner

for Refugees, US Centers for Disease Control and Prevention) and 2 Field Epidemiology and Laboratory Training Program trainees from the neighboring Democratic Republic of the Congo (DRC) to investigate suspected cases and strengthen epidemiologic surveillance in the region.

Although human cases of MPX are routinely reported in the DRC (*1*,*2*), cases are only sporadically reported in the Republic of the Congo; large outbreaks previously occurred in 2003 and 2011 in Likouala Department (*3*,*4*). Poor transportation and communication infrastructure in the region, in addition to competing public health priorities, have contributed to a paucity of knowledge among healthcare workers (HCWs) about MPX case recognition, notification, and reporting. Local HCWs unofficially report MPX, but inconsistent and incomplete case notifications continue to be a challenge.

MPX, a zoonotic orthopoxvirus, is a public health priority in regions of endemicity in West and Central Africa because of its clinical severity and potential for epidemic spread (*1*). The virus is a member of the same genus as variola virus, and the clinical presentation of MPX resembles that of smallpox, with the addition of lymphadenopathy (*5*). Symptoms include an initial febrile prodrome (1–4 days), followed by a disseminated vesiculopustular rash, which includes the palms of the hands and soles of the feet (*6*). Transmission occurs through contact with infectious lesions, contaminated fomites, or respiratory droplets (believed to be most common for human-to-human transmission) (*6*). When human-to-human transmission occurs, identification of persons who have had extensive contact with a MPX patient is critical to limit the spread of disease and prevent outbreaks.

During March 15–22, 2017, a total of 139 HCWs were trained in 7 towns throughout the study region. HCWs received training in MPX clinical characteristics and case recognition; case management; surveillance; and infection prevention and control, including donning and removal of personal protective equipment. Content for the training materials was derived from a 2000 World Health Organization MPX manual with contributions from subject matter experts, further revised after a similar training was conducted in DRC in 2010 (*7*). In addition, HCWs were provided with MPX investigation kits that included surveillance manuals, MPX-specific case investigation forms (which collect demographic, clinical, and exposure information), personal protective equipment, and sample collection supplies to enhance laboratory-based surveillance.

A nongovernmental organization (International Communication and Education Foundation, Homestead, FL, USA) provided community outreach and education. Educators from this organization held screenings of short films in Lingala (the local language) featuring families who had experienced MPX and local public health officials. The educational films were designed to be interactive in nature; community members were encouraged to discuss, debate, and ultimately develop prevention mechanisms/lifestyle changes that will result in zoonotic disease prevention. Educators held screenings in 14 villages in Likouala Department and educated >1,160 community members.

During January–December 1, 2017, a total of 81 suspected MPX cases, 7 laboratory-confirmed cases, and 6 deaths from this disease were reported in Likouala Department. Outbreaks of measles and infection with varicella zoster virus, which are often confused with MPX virus infections, were reported in the region before and during the investigation period. Thus, it was difficult to determine if this is a true increase, an artifact of strengthened surveillance in March, or merely the endemic rate of MPX in the region.

Although enhancing disease surveillance was a priority during the outbreak, there remain numerous challenges to consistent MPX reporting. The Republic of the Congo lacks specific programs to adequately train and support HCWs, and capacity is hindered by the need to cover vast, inaccessible areas that have underdeveloped infrastructure and limited resources. Leveraging resources and reinforcing HCW capacity through ongoing training at the local level will be vital for improving surveillance and effectively responding to outbreaks in the area. Implementation of a surveillance program modeled in a manner similar to other MPX-endemic countries (such as the DRC) could be useful (*8*). In the absence of consistent laboratory diagnostics, detection of endemic MPX cases will require a more specific surveillance case definition (*9*). In addition, investing in training programs, such as the Field Epidemiology and Laboratory Training Program, could provide increased support. Finally, standardizing a multifaceted response that includes community education, for other countries where MPX outbreaks are most likely to occur, such as the DRC, Cameroon, and most recently, Nigeria, could be extremely useful.

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# Zoonanthroponotic Transmission of Drug-Resistant *Pseudomonas aeruginosa*, Brazil

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We recovered VIM-2 carbapenemase-producing *Pseudomonas aeruginosa* isolates from an infected dog, its owner, and the domestic environment. Genomic investigation revealed household transmission of the high-risk hospital clone sequence type 233 in the human–animal–environment interface. Results suggest zooanthroponotic transmission of VIM-2–producing *P. aeruginosa* in the household following the patient's hospital discharge.

The One Health approach has gained worldwide recognition as a valuable way to address critical public health issues, including the problem of antimicrobial drug resistance at the human–animal–environment interface. Although numerous studies have provided substantial evidence of spread of antimicrobial drug–resistant bacteria from animals to humans, current investigations indicate that humans can transmit resistant pathogens to animals in a reverse zoonotic event, called zooanthroponosis (*1*,*2*). Therefore, epidemiologic studies are needed to provide a better understanding of the dynamics of antimicrobial drug resistance transmission between animals and humans. In this study, we investigated an international hospital-associated clone of carbapenem-resistant *Pseudomonas aeruginosa* sequence type (ST) 233 circulating in the human–animal–environment interface of a household setting.

In December 2016, a 5-year-old male Lhasa apso dog was admitted to a veterinary clinic for treatment of head shaking and right ear pruritus. Severe ear canal inflammation and malodorous purulent discharge were observed during clinical examination. A carbapenem-resistant *P. aeruginosa* isolate was recovered from the infected ear (Table). A detailed account of the medical history revealed that the pet owner, a 50-year-old man, had a recent history of hospitalization (of ≈5 months' duration) for severe traumatic brain

[1]These authors contributed equally to this article.

**Table**. Characteristics of carbapenem-resistant VIM-2 metallo-β-lactamase–producing *Pseudomonas aeruginosa* isolates in a human–animal–environment interface in a household setting, Brazil*

| Characteristic | Isolate† | | |
|---|---|---|---|
| | ICBDVIM-2 | ICBHVIM-2 | ICBSVIM-2 |
| Host/environment | Dog | Human | Household environment |
| Sample | Ear secretion, rectal swab, oral swab | Feces | Sofa swab |
| Isolation date | 2016 Dec 15; 2017 Mar 6 | 2017 Mar 6 | 2017 Mar 6 |
| Resistance profile | AMK, AMC, CAZ, CFO, CIP, CL, CPM, CRO, CTX, GEN, IMP, MER, NAL, PPT, STX, TET, TIC | AMK, AMC, CAZ, CFO, CIP, CL, CPM, CRO, CTX, GEN, IMP, MER, NAL, PPT, STX, TET, TIC | AMK, AMC, CAZ, CFO, CIP, CL, CPM, CRO, CTX, GEN, IMP, MER, NAL, PPT, STX, TET, TIC |
| Carbapenem MIC, μg/mL‡ | >32 | >32 | >32 |
| Resistance genes to: | | | |
| β-Lactams | *bla*$_{VIM-2}$, *bla*$_{PAO}$, *bla*$_{OXA-4}$, *bla*$_{OXA-50}$ | *bla*$_{VIM-2}$, *bla*$_{PAO}$, *bla*$_{OXA-4}$, *bla*$_{OXA-50}$ | *bla*$_{VIM-2}$, *bla*$_{PAO}$, *bla*$_{OXA-4}$, *bla*$_{OXA-50}$ |
| Aminoglycosides | *aadA2, aac(3)-Id, aph(3)-IIb* | *aadA2, aac(3)-Id, aph(3)-IIb* | *aadA2, aac(3)-Id, aph(3)-IIb* |
| Chloramphenicol | *catB7*, *cmlA1* | *catB7*, *cmlA1* | *catB7*, *cmlA1* |
| Sulfonamides | *sul1* | *sul1* | *sul1* |
| Trimethoprim | *dfrB5* | *dfrB5* | *dfrB5* |
| Tetracyclines | *tetG* | *tetG* | *tetG* |
| Fosfomycin | *fosA* | *fosA* | *fosA* |
| Location of *bla*$_{VIM-2}$ | Chromosome | Chromosome | Chromosome |
| MLST,(ST/CC | 233/233 | 233/233 | 233/233 |

*AMK, amikacin; AMC, amoxicillin/clavulanic acid; CAZ, ceftazidime; CC, clonal complex; CFO, cefoxitin; CIP, ciprofloxacin; CL, chloramphenicol; CPM, cefepime; CRO, ceftriaxone; CTX, cefotaxime; GEN, gentamicin; IMP, imipenem; MER, meropenem; MLST, multilocus sequence typing; NAL, nalidixic acid; PPT, piperacillin/tazobactam; ST, sequence type; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline, TIC, ticarcillin.
†Clonally related *P. aeruginosa* strains ICBDVIM-2 (ear secretion), ICBRVIM-2 (rectal swab), and ICBBVIM-2 (oral swab) were isolated from samples collected in the infected dog. ICBDVIM-2 was isolated on December 15, 2016. ICBRVIM-2 and ICBBVIM-2 were isolated on March 6, 2017. All *P. aeruginosa* strains from the infected dog displayed identical resistance profiles and genetic backgrounds.
‡Imipenem and meropenem.

injury from a traffic accident, including a 1-month stay in the intensive care unit because of a brain infection, which was treated successfully with vancomycin. The patient was discharged from the hospital 1 month before the onset of infection in the dog.

We conducted an epidemiologic investigation to establish the dynamic of carbapenem-resistant *P. aeruginosa* isolates in the household setting. The household consisted of a married couple without children and owning 2 dogs. We collected surveillance cultures from the owners (fecal samples, n = 2), healthy and infected dogs (rectal and oral cavity, n = 4), and different household sites (sofa, n = 1; living room, n = 2; kitchen, n = 2; bathrooms, n = 3; bedrooms, n = 2; balcony, n = 1; and water cooler, n = 1). We recovered 6 carbapenem-resistant *P. aeruginosa* isolates from the infected dog (rectal and oral cavity), pet owner (fecal samples), sofa, balcony, and water cooler.

We performed whole-genome sequencing of the *P. aeruginosa* isolates (Table) using an Illumina NextSeq platform (Illumina, San Diego, CA, USA). We identified antimicrobial drug resistance genes and multilocus sequence typing of *P. aeruginosa* strains using bioinformatic tools, available from the Center for Genomic Epidemiology (http://genomicepidemiology.org/). We found that all carbapenem-resistant *P. aeruginosa* isolates were clonally related to the hospital-associated lineage ST233, which has been reported as an international high-risk clone, frequently associated with carbapenemase production, and exhibiting resistance to all antimicrobial drugs (*3*–*5*).

In all *P. aeruginosa* strains, carbapenem resistance was associated with the production of VIM-2 metallo-β-lactamase, which was previously reported among clinical *P. aeruginosa* clustered into ST233/clonal complex (CC) 233 in countries in Europe, North America, and Africa, restricted thus far to human nosocomial infections (*3*–*5*).

Genomic data confirmed the household dissemination of VIM-2–producing *P. aeruginosa* ST233 and intestinal colonization of the human host (who had a recent history of hospitalization with a stay in an intensive care unit), suggesting a zooanthroponotic transmission of this nosocomial-adapted clone after the patient's hospital discharge. A limitation of this study is the lack of data supporting previous episodes of colonization or infection of the pet owner by the VIM-2–producing *P. aeruginosa* during the hospital stay. However, VIM-2–producing *P. aeruginosa* lineages, including ST233, have generally been restricted to human hospital settings (*3*–*6*). In this regard, several studies in hospitalized patients have shown that intestinal colonization with gram-negative bacteria (including carbapenemase producers) persists for ≥3 months after discharge from the hospital, whereas long-term carriage of >3 years is possible (*7*,*8*). Thus, patients can acquire clinically significant antimicrobial drug–resistant bacteria during hospitalization. As a result, patients harboring these bacteria might transmit them after discharge, mainly to household contacts (*7*,*8*).

In veterinary medicine, the occurrence of VIM-type metallo-β-lactamase–producing *P. aeruginosa* has been restricted to a report of livestock colonization (*9*). We report the further occurrence of VIM-2–producing *P. aeruginosa*

in an infected companion animal, showing the emergence of carbapenem-resistant metallo-β-lactamase–producing *P. aeruginosa* in small animal medical care. In this regard, the success of a human hospital-associated lineage of *P. aeruginosa* in animal hosts could be favored by the versatility and adaptation of this opportunistic pathogen, which can survive for long periods in the environment (*10*).

These findings suggest that human hospital-acquired pathogens can colonize and infect companion animals, thus becoming further disseminated in the household environment. Transmission to companion animals could occur not only directly from owners to pets but also from humans to the household environment and then to pets. Because human–pet bonds in household settings could become a critical issue for the transmission of clinically significant multidrug-resistant bacteria, human and veterinary medicine professionals should implement collaborative efforts and health cooperation programs to monitor the spread of such pathogens in the human–animal interface.

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Ms. Fernandes is a PhD student at the Universidade de São Paulo, Brazil. Her primary research interest is microbiology, particularly antibiotic resistance in clinically significant bacteria in the human–animal–environment interface.

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# Symptom-Based Ebola Risk Score for Ebola Virus Disease, Conakry, Guinea

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**To the Editor:** In their article, Oza et al. proposed a score to risk-stratify Ebola virus disease (EVD) suspected cases while patients in an Ebola treatment center await laboratory confirmation (*1*). The Ebola symptom-based risk (ESR) score, consisting of 6 symptoms (conjunctivitis, diarrhea, nausea/vomiting, headache, difficulty breathing, loss of appetite), performed well in internal validation, but no external validation was done.

We evaluated the proposed ESR score on 805 EVD-positive and 1,506 EVD-negative case-patients in the Conakry Ebola Treatment Center (ETC), Conakry, Guinea (*2*). The ESR score yielded an area under the curve of 0.58 (95% CI 0.56–0.61), which is lower than the 0.83 (95% CI 0.79–0.86) Oza et al. reported (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/6/17-1812-Techapp1.pdf). Using the proposed risk thresholds (i.e., low risk if score <0, medium risk if score = 0, and high risk if score >0), 371 (46%) EVD-positive patients of the Conakry ETC were classified as high risk and 647 (43%) EVD-negative patients as low risk. However, negative and positive predictive values were generally low (online Technical Appendix Table). Reasons for poor validation could include differences in applying the general EVD suspect case definition (integration of patients' contact history); in patient characteristics because organization and access to care for EVD and non-EVD illness was different (patients in holding centers or ETC); in the quality of data collection (symptoms are entirely self-reported); and in underlying diseases of EVD-negative patients.

Our findings underline the importance of external validation in various settings before risk scores are applied outside of the setting within which they were developed, as well as the need to incorporate patient contact history into predictive models. Point-of-care EVD diagnostic platforms can perform reliable confirmatory testing within 90 minutes (*3*). We argue that, by integrating rapid confirmatory testing in triage, providers can avoid classifying patients by their likelihood of infection with Ebola virus while waiting for laboratory confirmation.

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# Corrections

## Vol. 23, No. 12

The timing of detection of Crimean-Congo hemorrhagic fever virus in Crimea and the Democratic Republic of the Congo were unclear in Phylogenetic Characterization of Crimean-Congo Hemorrhagic Fever Virus, Spain (E. Ramírez de Arellano et al.). The article has been corrected online (https://wwwnc.cdc.gov/eid/article/23/12/17-1002_article).

Patient data were inaccurate and definition of contact categories unclear in Lack of Secondary Transmission of Ebola Virus from Healthcare Worker to 238 Contacts, United Kingdom, December 2014 (P. Crook et al.). The article has been corrected online (https://wwwnc.cdc.gov/eid/article/23/12/17-1100_article).

## Vol. 24, No. 2

*Amblyomma mixtum* ticks were misidentified as *A. sculptum* in *Rickettsia africae* and Novel Rickettsial Strain in *Amblyomma* spp. Ticks, Nicaragua, 2013 (H. Vogel et al.). The article has been corrected online (https://wwwnc.cdc.gov/eid/article/24/2/16-1901_article).

The affiliation of author Pierre Zalloua was listed incorrectly in Containment of Highly Pathogenic Avian Influenza A(H5N1) Virus, Lebanon, 2016 (Z.E. Farah et al.). He is affiliated with Lebanese American University. The article has been corrected online (https://wwwnc.cdc.gov/eid/article/24/2/17-1276_article).

Some data were inaccurate in the text and figures in Spread of Meropenem-Resistant *Streptococcus pneumoniae* Serotype 15A-ST63 Clone in Japan, 2012–2014 (S. Nakano et al.). The article has been corrected online (https://wwwnc.cdc.gov/eid/article/24/2/17-1276_article).

**Vincenzo Campi (1530/1535–1591), *Kitchen* (*Cucina*), 1580**. Oil on canvas, 57 in × 87 in/145 cm × 220 cm. Pinacoteca di Brera, Milan, Italy.

# A Tale of Two Kitchens, Meals and Microbes

**Byron Breedlove and Martin I. Meltzer**

*Kitchen*, a painting completed in 1580 by Italian artist Vincenzo Campi, celebrates the chaotic workspace that was devoted to keeping a noble family's house supplied with food and drink. The kitchen workers are preparing an assortment and quantity of meats, pies and breads, sauces, and side dishes as a special meal for a celebration or holiday.

Invisible to the viewer and unknown to Campi, his subjects, or his patrons, this kitchen would have been permeated by numerous unwelcome microorganisms that could cause zoonotic foodborne diseases. Such a setting would provide many opportunities for transmission of potentially pathogenic bacteria, viruses, and parasites in the raw meat and poultry, in the blood and viscera spattered on the workers' skin and clothing, on floors and shared work surfaces, knives, and other utensils, and from domestic pets, rodents, and insects.

Campi's *Kitchen* is alive with activity. Near the top of the painting, demonstrating the artist's mastery of the technique of perspective, the viewer sees a dining room containing a long table festooned with a white tablecloth and tended by a young girl. Half a dozen colorfully

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dressed women are busy preparing the food, seemingly using every available surface. An older woman is working on the floor and appears to react negatively to the taste or smell of whatever is covering the bottom of a large pestle. A small child is sitting on a colander, amusing himself by inflating an animal's stomach. On the upper left periphery, a pair of men are butchering a deer carcass, while across the kitchen, a young man is carefully skewering raw, uncooked game birds on a spit. A cat and dog scrap for entrails plucked from the poultry carcass in the foreground, cooking pans dangle near rows of stacked plates in the upper right, and a small fire smolders in the fireplace near the center.

In the second half of the 16th century, Vincenzo Campi and his brothers, Giulio and Antonio, were considered among the finest artists in the northern Italy town of Cremona. Specific details about Campi's homelife and education during the early years of his life are scarce. His father, Galeazzo Campi, also an artist of note, had been a pupil of the painter Boccaccio Boccaccini and helped educate his trio of sons in the arts. Giulio, the eldest brother, was a noted architect and artist, who also instructed his younger siblings.

A short biography from the Museo Del Prado notes that Campi's earliest collaborations with his brothers showed little originality. His initial efforts were chiefly portraits of members of the upper class and various Catholic saints. Although throughout his career Campi continued to paint religious iconography and portraits for wealthy patrons, he is remembered more for his realistic paintings that captured the bustle of everyday life among the lower economic classes, food merchants, poultry and fish vendors, butchers, cooks, and kitchen workers.

Sheila McTighe, senior lecturer at the Courtauld Institute of Art, stated that Campi "is best known for his significant contribution to the birth of northern Italian genre painting. The style appeared quite suddenly between 1580 and 1585 in Cremona and Bologna, and its development was heavily influenced by similar genre paintings by Flemish artists Pieter Aertsen and Joachim Beuckelaer." Wealthy merchants and bankers—some of whom were no doubt the subjects of Campi's portraiture—imported examples of those Flemish genre paintings to northern Italy, and Campi would have had ready access to them. Exactly what drove the sudden demand for representational art is not clear.

Art scholar Deborah Krohn notes that "Kitchens, along with foodstuffs, do not appear as a significant focus in paintings until the middle of the sixteenth century. First in the Low Countries, and then in Italy, we find kitchens as the settings for a variety of activities, from cooking and food preparation, to fighting, eating, flirting, and sleeping, as in Vincenzo Campi's *Kitchen* of the 1580s."

Missing from Campi's detailed painting, however, is any depiction of a bucket, sink, or soap for handwashing and cleaning the utensils, knives, or tables. The provision and frequent use of such cleaning materials would have reduced the risk for infection from foodborne pathogens. If the kitchen workers or their employers experienced gastrointestinal or skin infections, they would have been unlikely to blame their working conditions. They could not have heard about shiga-toxin producing *Escherichia coli*, *Shigella*, *Salmonella*, *Campylobacter*, and *Cyclospora cayetanensis* as causes of foodborne illnesses or infections. It was not until the second half of the 1600s that scientists such as Robert Hooke and Anton van Leeuwenhoek built microscopes and observed and recorded microorganisms. It then took more than 150 years after those observations that causal links were made between such microorganisms and disease.

Contemporary kitchens with their gleaming counters, appliances for storing and cooking food, and sinks and cleaning products are not likely to inspire artists to depict such a rich, colorful scene as the one Campi captured on his canvas. Nonetheless, while we may think our kitchens are free from all of the unseen hazards in Campi's *Kitchen*, we still face the same risks for zoonotic infections innocently depicted by Campi. Even with our modern kitchen appliances and comparatively advanced knowledge regarding risk for disease, it is still possible to become ill from eating contaminated or unsafe foods and by inappropriately storing and preparing food.

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# EMERGING INFECTIOUS DISEASES®

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- Progress in Vaccine-Preventable and Respiratory Infectious Diseases—First 10 Years of the CDC National Center for Immunization and Respiratory Diseases, 2006–2015
- Effects of Sexual Network Connectivity and Antimicrobial Use on Antimicrobial Resistance in *Neisseria gonorrhoeae*
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- Perceptions of Zika Virus Risk during Outbreak, Miami-Dade County, Florida, USA, 2016
- Adenovirus Type 4 Respiratory Infections among Civilian Adults, Northeastern United States, 2011–2015

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## Upcoming Infectious Disease Activities

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**September 23–26, 2018**
**ASM Conference on Rapid Applied Mirobial Next-Generation Sequencing and Bioinformatic Pipelines**
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**Oct 1–3, 2018**
**International Conference on Migration Health**
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**ID Week**
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**Keystone Symposia Conference**
**Hong Kong, China**
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**October 28–30, 2018**
**International Society for Vaccines Annual Congress**
**Atlanta, GA, USA**
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**October 28–30, 2018**
**2018 Annual Congress**
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**November 9–12, 2018**
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## 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 75% passing score) and earn continuing medical education (CME) credit, please go to http://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.

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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 this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@medscape.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 go to https://www.ama-assn.org. 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 AMA PRA CME credit certificate, and present it to your national medical association for review.

## Article Title

# Occupation-Associated Fatal Limbic Encephalitis Caused by Variegated Squirrel Bornavirus 1, Germany, 2013

## CME Questions

**1. Your patient, a zookeeper for small mammals, has become acutely ill. Based on the case report by Tappe and colleagues, which of the following statements about the clinical features of limbic encephalitis (LE) in a zookeeper infected with variegated squirrel bornavirus-1 (VSBV-1) is correct?**

A. The patient had a history of immunosuppression from corticosteroid use
B. Initial symptoms were fever, dysphonia, cough, pharyngitis, vertigo, and paresthesia below her eye
C. The patient never lost consciousness before dying of pituitary gland insufficiency
D. Other zoo workers at her facility were infected with VSBV-1

**2. Based on the case report by Tappe and colleagues, which of the following statements about immunologic and pathologic features of LE in a zookeeper infected with VSBV-1 is correct?**

A. Molecular assays and immunohistochemistry showed VSBV-1 in brain tissue in a limbic distribution
B. The VSBV-1 strain in this patient was phylogenetically identical to that found in a separate cluster of VSBV-1 infection among squirrel breeders
C. This patient did not have detectable antibodies against bornaviruses in the cerebrospinal fluid
D. Brain tissue did not contain Joest-Degen inclusion bodies typical for a bornavirus infection

**3. Based on the case report by Tappe and colleagues, which of the following statements about management of LE and VSBV-1 infection and preventive measures for VSBV-1 infection is correct?**

A. This patient initially responded to ribavirin
B. Intravenous immunoglobulins and other immunosuppressive treatment are known to be clinically beneficial in bornavirus infections
C. The findings do not support testing all exotic squirrels in zoos or breeding facilities for VSBV-1
D. Avoiding direct contact with exotic squirrels is a reasonable precaution for zoo employees and visitors and for private breeders

## Earning CME Credit

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## Article Title

# Bioclinical Test to Predict Nephropathia Epidemica Severity at Hospital Admission

## CME Questions

**1. Your patient is a 35-year-old man with nephropathia epidemica (NE). Based on the multicenter, retrospective cohort study by Hentzien and colleagues, which of the following statements about clinical and laboratory characteristics of NE is correct?**

A. Patients were mostly older women with several comorbidities
B. Time elapsed between first symptoms and hospitalization was approximately 2 weeks
C. Plasma creatinine and proteinuria peaked around 8 days after symptom onset, and mean duration of hospitalization was 7 days
D. Approximately half of patients developed severe NE during hospitalization

**2. Based on the multicenter, retrospective cohort study by Hentzien and colleagues, which of the following statements about bioclinical factors predictive of severe NE is correct?**

A. The final score included nephrotoxic drug intake, visual disorders, white blood cell count, anemia, and hematuria
B. The strongest predictive factor was hematuria
C. Fever was a significant predictor of severe NE and was included in the final score
D. Bioclinical score of 10 or higher identified low-risk patients (3.3%); a score of 20 or higher identified patients at high risk (45.3%) for severe NE

**3. Based on the multicenter, retrospective cohort study by Hentzien and colleagues, which of the following statements about clinical performance and other clinical implications of a bioclinical score predictive of severe NE is correct?**

A. It is easy to use in routine practice and could be helpful in the initial evaluation and subsequent treatment of patients with NE
B. The score should now be routinely used in every patient with NE
C. The score had low to moderate discriminatory ability and internal validity
D. The study proves that albumin level at admission is not a good predictor of NE severity

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# EMERGING INFECTIOUS DISEASES®

## JOURNAL BACKGROUND AND GOALS

### What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

### Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

### What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

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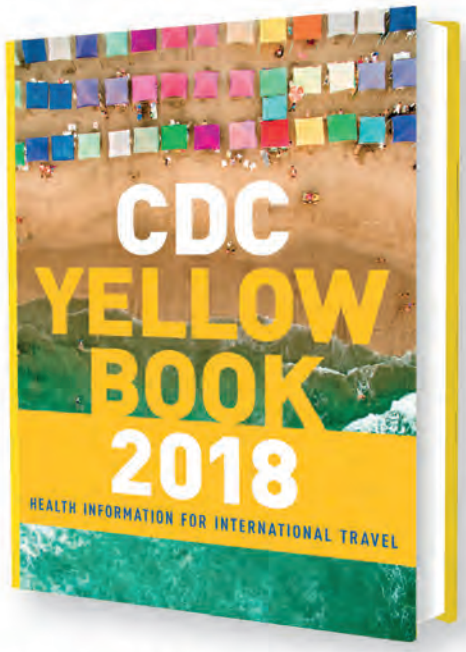


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Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

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.

## Summary of Authors' Instructions

**Authors' Instructions.** For a complete list of EID's manuscript guidelines, see the author resource page: http://wwwnc.cdc.gov/eid/page/author-resource-center.

**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. Set the document to show continuous line numbers. 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 editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpg 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 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

**Synopses.** Articles should not exceed 3,500 words in the main body of the text or include more than 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section comprises case series papers and concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

**Research.** Articles should not exceed 3,500 words and 50 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 50 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.

**Research Letters Reporting Cases, Outbreaks, or Original Research**. EID publishes letters that report cases, outbreaks, or original research as Research Letters. Authors should provide a short abstract (50-word maximum), references (not to exceed 10), and a short biographical sketch. These letters should not exceed 800 words in the main body of the text and may include either 1 figure or 1 table. Do not divide Research Letters into sections.

**Letters Commenting on Articles**. Letters commenting on articles should contain a maximum of 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication.

**Commentaries.** Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

**Another Dimension.** Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

**Books, Other Media.** Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

**Conference Summaries.** Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

**Online Reports**. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

**Photo Quiz.** The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

**Etymologia.** Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

**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.

**Vincenzo Campi (1530/1535–1591), *Kitchen (Cucina)*, 1580.** Oil on canvas, 57 in × 87 in/145 cm × 220 cm. Pinacoteca di Brera, Milan, Italy.


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