EMERGING INFECTIOUS DISEASES®

Zoonoses

December 2013

Paul Gauguin (1848–1903) Black Pigs (1891) Oil on canvas (91 cm × 72 cm) Museum of Fine Arts, Budapest, Hungary

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EMERGING INFECTIOUS DISEASES

December 2013

On the Cover

Paul Gauguin (1848–1903)
***Black Pigs* (1891) (detail)**

Oil on canvas (91 cm × 72 cm)
Museum of Fine Arts,
Budapest, Hungary

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Perspective

Institute of Medicine and National Research Council Recommendations for One Health Initiative 1913

C. Rubin et al.

Review of articles on One Health subjects concludes that future studies and workshops should consider progress made and address remaining gaps.

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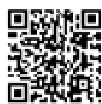


Epidemiological Investigations into Outbreaks of Rift Valley Fever in Humans, South Africa, 2008-2011 1918

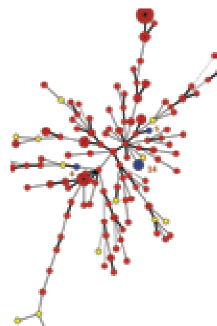
B. Archer et al.

Rift Valley fever continues to pose a notable public health threat to humans.

Potential Role of Deer Tick Virus in Powassan Encephalitis Cases in Lyme Disease–endemic Areas of New York, USA.................. 1926

M.Y.B. Khoury et al.

The epidemiologic pattern and limited laboratory testing indicate that this virus lineage might account for most of these illnesses.



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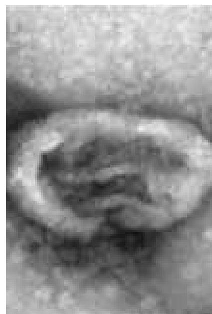

Twenty-Year Summary of Surveillance for Human Hantavirus Infections, United States..................................... 1934

B. Knust and P.E. Rollin

Sin Nombre virus causes most cases, but other viruses must be considered in patients with atypical exposures/clinical presentations.

Research

Spontaneous Generation of Infectious Prion Disease in Transgenic Mice................................ 1938

J.-M. Torres et al.

One amino acid change in bovine prion induced a new infectious prion.

Zoonotic *Chlamydiaceae* Species Associated with Trachoma, Nepal... 1948

D. Dean et al.

Zoonotic transmission of species other than Chlamydia trachomatis probably plays a role in ocular trachomatous disease.

Guillain-Barré Syndrome Surveillance during National Influenza Vaccination Campaign, New York, USA, 2009........................ 1956

G.P. Giambrone et al.

A Clinical Network provided timely data, but such data were less complete than traditional hospital discharge data.

Antiviral Susceptibility of Highly Pathogenic Avian Influenza A(H5N1) Viruses Isolated from Poultry, Vietnam, 2009–2011.......................... 1963

H.T. Nguyen et al.

Monitoring antiviral drug susceptibility of HPAI (H5N1) viruses in poultry is crucial to pandemic preparedness.

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1972 **Novel Reassortant Influenza A(H1N2) Virus Derived from A(H1N1)pdm09 Virus Isolated from Swine, Japan, 2012**
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1975 **Myocarditis after Trimethoprim/Sulfamethoxazole Treatment for Ehrlichiosis**
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1978 **Distinct Lineage of Vesiculovirus from Big Brown Bats, United States**
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1981 **Acute *Toxoplasma gondii* Infection among Family Members in the United States**
D.G. Contopoulos-Ioannidis et al.

1985 **Novel Orthoreovirus from Mink, China, 2011**
H. Lian et al.

1989 **Outbreak of Human Infection with *Sarcocystis nesbitti*, Malaysia, 2012**
S. AbuBakar et al.

1992 **Transmission of Brucellosis from Elk to Cattle and Bison, Greater Yellowstone Area, USA, 2002–2012**
J. C. Rhyan et al.

1996 **Concomitant Human Infections with 2 Cowpox Virus Strains in Related Cases, France, 2011**
C. Ducournau et al.

2000 **Zoonotic *Onchocerca lupi* infection in Dogs, Greece and Portugal, 2011–2012**
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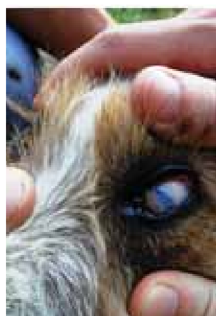
2004 **Novel Cause of Tuberculosis in Meerkats, South Africa**
S.D.C. Parsons et al.



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V. Ntoukas et al.

2012 **Powassan Virus in Mammals, Alaska and New Mexico, USA, and Russia, 2004–2007**
E.R. Deardorff et al.

2017 **Reemergence of Vaccinia Virus during Zoonotic Outbreak, Pará State, Brazil**
F.L. de Assis et al.

2021 **Novel Variants of Clade 2.3.4 Highly Pathogenic Avian Influenza A(H5N1) Viruses, China**
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2025 **Rift Valley Fever in Namibia, 2010**
F. Monaco et al.

2028 **Hepatitis E Virus Variant in Farmed Mink, Denmark**
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2031 **Historical Prevalence and Distribution of Avian Influenza Virus A(H7N9) among Wild Birds**
S.H. Olson et al.

2034 **Lack of MERS Coronavirus Neutralizing Antibodies in Humans, Eastern Province, Saudi Arabia**
S. Gierer et al.

2037 **Peste des Petits Ruminants Infection among Cattle and Wildlife in Northern Tanzania**
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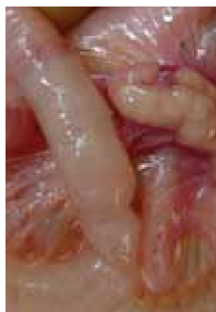

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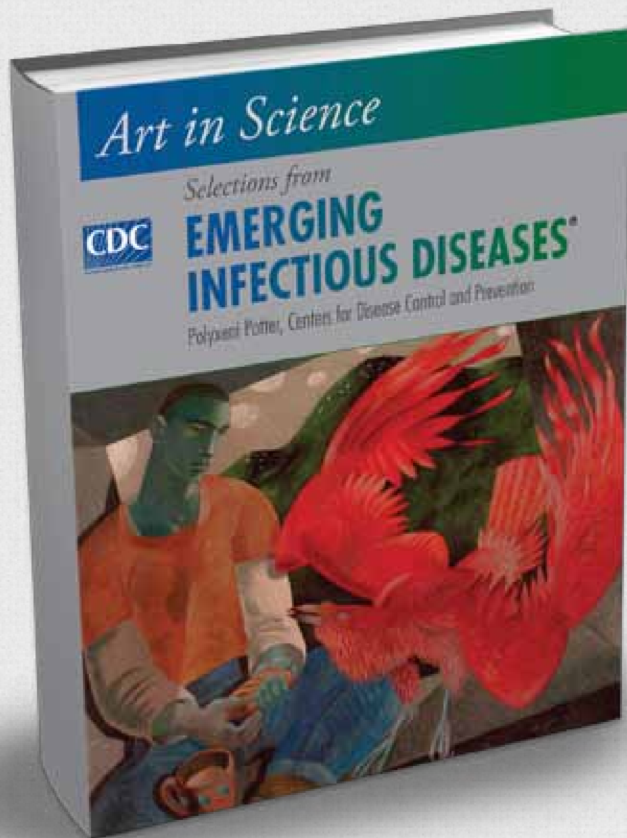

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This collection of 92 excerpts and covers from **Emerging Infectious Diseases** will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.

Review of Institute of Medicine and National Research Council Recommendations for One Health Initiative

Carol Rubin, Tanya Myers, William Stokes, Bernadette Dunham, Stic Harris, Beth Lautner, and Joseph Annelli

Human health is inextricably linked to the health of animals and the viability of ecosystems; this is a concept commonly known as One Health. Over the last 2 decades, the Institute of Medicine (IOM) and the National Research Council (NRC) have published consensus reports and workshop summaries addressing a variety of threats to animal, human, and ecosystem health. We reviewed a selection of these publications and identified recommendations from NRC and IOM/NRC consensus reports and from opinions expressed in workshop summaries that are relevant to implementation of the One Health paradigm shift. We grouped these recommendations and opinions into thematic categories to determine if sufficient attention has been given to various aspects of One Health. We conclude that although One Health themes have been included throughout numerous IOM and NRC publications, identified gaps remain that may warrant targeted studies related to the One Health approach.

Over the past decade, animal and human health leaders have begun to consider the benefit of collaboration, prompted by recognition that highly specialized practices of veterinary and human medicine are missing inextricable links between human health, animal health, and the viability of ecosystems. The 2008 Final Report of the American Veterinary Medical Association (AVMA) One Health Initiative Task Force defined One Health as the collaborative effort of multiple disciplines—working locally, nationally, and globally—to attain optimal health for people, animals and our environment. The report included the recommendation that the AVMA, the American Medical Association, and other interested parties should "plan a study on One Health to be conducted by the National Academy of Sciences and secure the necessary funding to underwrite this effort" (*1*). In 2009, the Institute of Medicine (IOM) and National Research Council (NRC) co-hosted the One Health Commission Summit, described as "a forerunner to an IOM study on One Health…[that will be] used to develop a strategic roadmap for public and private policies and initiatives that will, in turn, be instrumental in shaping the implementation of the One Health vision, both domestically and internationally" (*2*). The study was slated to begin in 2010; however, funding required to initiate it has not yet been committed.

A review of existing IOM publications for One Health–related consensus recommendations or individual opinions is a critical step in assessing whether to move forward with a general, or more refined, focus that will complement the existing body of IOM/NRC reports. We sought to complete such a review, and to fit the findings into a framework that would facilitate a data-driven assessment of how to move forward in possibly seeking an IOM/NRC review of One Health.

Methods

The National Academies and Their Reports

A primary function of the National Academies is to provide unbiased and timely expert advice to policy makers and the general public. The National Academies include the National Academy of Sciences, the IOM, the NRC, and

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the National Academy of Engineering. Their operations are independent from the US federal government and not funded by direct appropriation, although studies are often mandated by Congress in the interest of seeking expert counsel. Studies can be requested by federal agencies or by independent organizations.

At the National Academies there is a vast difference in the weight ascribed to consensus committee recommendations, compared to the individual opinions that are collected in a workshop summary. A consensus committee, typically including 10–15 members, is carefully chosen to represent a range of specific disciplines and experiences. Consensus committees are carefully structured to ensure that all members are independent of the sponsoring agencies (*3–5*). The committees operate under a set of rigorous rules pertinent to Section 15 of the Federal Advisory Committee Act. Each committee member undergoes an extensive bias and conflict of interest review, and their names are posted online for public comment. The committee collects information from presentations, literature reviews, and other means; the committee's recommendations are then designed in a very structured way. When a draft report is compiled, it is submitted to a review committee with a similar mix of disciplinary expertise. The entire process is overseen by the overarching National Academies Report Review Committee (RRC).

In strict contrast, a workshop summary is not allowed to contain anything that could be interpreted as a consensus conclusion or recommendation. It is not reflective of a Federal Advisory Committee Act process and the RRC is minimally involved in most instances. To separate the workshop summary from the report of the persons who designed the workshop, the summary is always written by an appointed reporter rather than a workshop planning committee member . The goal is to ensure that the workshop report is not seen as the product of a committee process, but as a collection of opinions expressed by workshop participants. The standard of peer review for a workshop is very different from that described above for a consensus study. A much smaller group of reviewers is involved, and the objective of the review is to ensure that the report is an accurate and clear description of what happened, not what should have happened. Because there is some overlap in content between the IOM/NRC consensus reports and IOM workshop summaries in terms of coverage of health-related issues, we included both types of reports in this review.

Selection of Reports for Review

Titles and objectives of NRC/IOM reports during 1991–2013 were reviewed to identify content addressing interactions among humans, animals, and the environment. By using this process, 20 reports (Table) (*6–25*) were judged most likely to contain multiple recommendations or opinions related to One Health concepts. Although it is likely that additional reports may contain One Health concepts, this review was constructed to provide a starting point to inform those considering how future studies of One Health by the National Academies could be constructed.

Defining One Health Concepts

For the purposes of this review, a consensus recommendation or workshop opinion was deemed related to One Health concepts if it included any aspect of the relationships between humans, animals, and the ecosystems in which they coexist and interact. Although this definition may be viewed as broad, it was chosen intentionally to prevent bias for or against any particular component of One Health.

Grouping of Recommendations

All consensus reports and some workshop summaries and workshop reports included summary or overview chapters containing an aggregated view of consensus report recommendations or themes emerging from the workshop. However, in cases in which reports lacked such an organized set of recommendations, the full report was reviewed to determine whether any pertinent information was conveyed within individual chapters. Recommendations or opinions found to be related to One Health were compiled for each individual report; then the aggregated list was reviewed to identify common themes. Finally, we sought to identify examples of completed or ongoing activities that address recommendations and opinions.

Results

Of the 20 publications that were reviewed in depth for this article, 8 were consensus reports. More than 50 recommendations and opinions were extracted, covering a broad array of topics ranging from a specific disease, system, or policy improvement, to general statements encouraging expansions of partnerships and broad investments in infrastructure for surveillance systems. As expected, the strongest and most specific recommendations were captured in consensus reports.

We grouped the recommendations and opinions into 7 topical categories: Surveillance and Response, Governance and Policy, Laboratory Networks, Training Needs, Research Needs, Communication Needs, and Partnerships. Online Technical Appendix Table 1 (wwwnc.cdc.gov/EID/article/19/12/12-1659-Techapp1.pdf) displays a paraphrased listing of the recommendations by category. The list of recommendations was circulated among the authors and other subject matter experts in an attempt to identify ongoing activities or programs that appear to address gaps identified in the IOM and NRC reports. Online Technical

Table. Listing of Institute of Medicine/National Research Council reports included in review, 1991–2013*

Date	Title	Type
1991	**Animals as sentinels of environmental health**	**Report**
1992	**Emerging infectious diseases: Microbial threats to health in the United States**	**Report**
1999	**The use of drugs in food animals: Benefits and risks**	**Report**
2001	Emerging infectious diseases: From the global to the local perspective	WS
2002	The emergence of zoonotic diseases: Understanding the impact on animal and human health	WS
2003	**Microbial threats to health: Emergence, detection and response**	**Report**
2005	**Animal health at the crossroads: Preventing, detecting, and diagnosing animal diseases**	**Report**
2005	**Critical needs for research in veterinary science**	**Report**
2006	Addressing foodborne threats to health: Policies, practices, and global coordination	WS
2006	The impact of globalization on infectious disease emergence and control	WS
2007	Global infectious disease surveillance and detection: Assessing the challenges – finding solutions	WS
2008	Vector-borne diseases: Understanding the environment, human health and ecologic consequences	WS
2009	**Sustaining global surveillance and response to emerging zoonotic diseases**	**Report**
2010	Antibiotic resistance: Implications for global health and novel intervention strategies	WS
2010	Infectious disease movement in a borderless world	WS
2011	**Climate change, the indoor environment, and health**	**Report**
2011	Critical needs and gaps in understanding prevention, amelioration, and resolution of Lyme and other tick-borne diseases	WS
2011	Fungal diseases: an emerging threat to animal, human and plant health	WS
2011	The causes and impacts of neglected tropical and zoonotic diseases: Opportunities for integrated intervention strategies	WS
2012	Improving food safety through a One Health approach	WS

* Report and bold text indicates recommendations from consensus reports, NRC committee reports, or IOM consensus reports; IOM, Institute of Medicine; NRC, National Research Council; WS, IOM workshop summary or workshop report.

Appendix Table 2 lists all of the recommendations, the exact references that support each recommendation, and examples of activities that appear to respond to specific recommendations.

Discussion

On the basis of the list compiled from the 20 reviewed reports, we found that the principles of One Health have, to varying extents, been included in many of the NRC/IOM recommendations and IOM workshop summaries. All of the reviewed reports had at least 1 recommendation related to an aspect of One Health. This sample was, admittedly, skewed toward those reports most likely to include recommendations, but we were impressed with the quantity identified in this review. Although even the earliest (1991) consensus report reviewed contained recommendations, a deeper review including reports from earlier dates would likely find additional recommendations linked to One Health concepts. As might be expected, One Health (or a related term) was not used in all instances as a descriptor for recommendations or opinions that fit within the definition of One Health activities used for this review; in fact, many recommendations that by today's understanding are clearly related to One Health were not tagged as such.

The quantity of recommendations and workshop opinions related to One Health concepts suggests that a reasonable level of attention has been given to the One Health movement in the past 2 decades of IOM/NRC publications. However, level of coverage does not necessarily translate into sufficient consideration of all aspects of a One Health approach, nor does it indicate adequate consideration of current understandings of One Health concepts. Apportioning our findings into thematic categories let us create a framework for evaluation of breadth of coverage. We found that some categories have received more attention than others. For example, the Surveillance and Response category had 16 recommendations or opinions that originated from 14 individual reports; and the Governance and Policy category had 10 recommendations or opinions from 8 reports. By contrast, 4 recommendations or opinions were identified in the Partnership category, and 3 were identified in the category of Communication Needs.

Most of the examples of implementation of One Health concepts that are described in the Technical Appendix are not directly associated with specific IOM or NRC recommendation. In contrast, recommendations from the United States Agency for International Development (USAID)-supported IOM report "Sustaining Global Surveillance and Response to Emerging Zoonotic Diseases" (2009) were translated into One Health activities under USAID's Emerging Pandemic Threats (EPT) Program. Progress in One Health activities may be a result of explicit recommendations from IOM and NRC reports, or simply be occurring because of increasing awareness of One Health concepts.

Examples of Progress

In the Surveillance and Response category, there is good evidence that progress has been made in addressing

some of the One Health–related recommendations generated from IOM and NRC studies. Several recommendations in this category address the need for integrated surveillance systems that capture information from multiple sectors. An excellent example of such integrated surveillance is the National Antimicrobial Resistance Monitoring System, which became operational in 1996 as a collaborative effort of the Centers for Disease Control and Prevention (CDC), the US Food and Drug Administration, and the US Department of Agriculture. The National Antimicrobial Resistance Monitoring System tracks antimicrobial susceptibility among enteric bacteria from humans, retail meats, and food animals (*26–28*) and provides timely integrated surveillance information that has enhanced the effectiveness of response to outbreaks of enteric disease. Although many of the recommendations regarding surveillance and response have been addressed in part, this particular area may provide an opportunity for a more focused IOM study group to evaluate how existing systems could be linked or merged to provide a sustainable, integrated surveillance system that addresses the needs of multiple sectors.

Recommendations in the Governance and Policy category appear not to have been specifically addressed and may represent a gap that needs to receive more attention. However, a One Health website, www.onehealthglobal.net, was released in mid-April 2012. The portal is intended to be a network-of-networks that speaks to One Health governance and that may serve as a mechanism that facilitates the recommendations within the Governance and Policy category (*29*). The portal, Operationalizing "One Health": A Policy Perspective—Taking Stock and Shaping an Implementation Roadmap is a product of the One Health Global Network Work Group that was formed as an outcome of the "Stone Mountain" meeting organized by CDC in collaboration with the World Organisation for Animal Health (OIE), the United Nations Food and Agriculture Organization (FAO), and the World Health Organization (WHO) (*30*).

Laboratory network recommendations have been addressed on several national fronts, including planning for a National Bio and Agro-Defense Facility (*31*) and the flourishing National Animal Health Laboratory Network (*32*). Internationally, OIE, FAO, and WHO have received USAID EPT funds to improve networking among human and animal laboratories (*33*). As mentioned previously, this EPT funding occurred after a 2009 Consensus Report, demonstrating direct actions to enhance laboratory capabilities in response to recommendations made within an IOM report (*18*)

Within the Training category, some recommendations are being addressed by the Stone Mountain Meeting Training Workgroup, grantees from 1 of 4 EPT projects named RESPOND, and the University of Minnesota with Rockefeller Foundation funding (*34*). These 3 groups work independently and also collaboratively to define One Health Core Competencies for varying levels of practitioners. They also develop course catalogs that capture existing training opportunities and identify needed training materials. In April 2012, the University of Florida announced that it will offer 2 new One Health degree programs, including a PhD in Public Health with a One Health concentration. "The One Health concentration is a research-oriented health degree that emphasizes working across public health, veterinary health, and environmental health disciplines to tackle difficult health problems" (*35*).

Similar selected examples of programs and projects that address the IOM recommendations can be identified for the categories of Research Needs (e.g., National Institutes of Health [NIH] Centers of Excellence for Influenza Research and Surveillance program, EPT PREDICT projects and the NIH-NSF Ecology and Evolution of Infectious Diseases Program: A Joint Program for Multidisciplinary Research [*36*]), Communication Needs (e.g., formation of One Health Offices at USDA and CDC), and Partnerships (e.g., US Interagency One Health Working Group, inclusion of veterinarians in CDC Field Epidemiology and Laboratory Training Programs). Although these examples are excellent steps in the right direction, they do not respond to the majority of the recommendations. In particular, recommendations that point toward collaboration, resource sharing, coordinated research, and strengthened lines of communication deserve greater attention.

Conclusions

The quantity of recommendations found suggests that, on a relatively consistent basis, One Health concepts have been considered to be part of working group deliberations, and of IOM and NRC studies, although there is no single entity or process for tracking progress on the recommendations of the National Academies' studies related to One Health. The examples we provide of completed, ongoing, and planned activities that address the recommendations are not intended to be comprehensive; however, the examples demonstrate that the One Health approach is making inroads. If additional IOM or NRC studies addressing One Health do go forward, we suggest that progress to date be considered and that the questions posed by the National Academies be carefully targeted to address remaining gaps.

Acknowledgment

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Epidemiologic Investigations into Outbreaks of Rift Valley Fever in Humans, South Africa, 2008–2011

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Release date: November 21, 2013; Expiration date: November 21, 2014

Learning Objectives

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

- Evaluate the general epidemiology and clinical consequences of Rift Valley fever (RVF)
- Assess temporal trends and demographics for infections with RVF
- Distinguish the most common exposure among patients with RVF in the current study
- Analyze specific risk factors for RVF associated with animal care.

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Rift Valley fever (RVF) is an emerging zoonosis posing a public health threat to humans in Africa. During sporadic RVF outbreaks in 2008–2009 and widespread epidemics in 2010–2011, 302 laboratory-confirmed human infections, including 25 deaths (case-fatality rate, 8%) were identified. Incidence peaked in late summer to early autumn each year, which coincided with incidence rate patterns in livestock. Most case-patients were adults (median age 43 years), men (262; 87%), who worked in farming, animal health or meat-related industries (83%). Most case-patients reported direct contact with animal tissues, blood, or other body fluids before onset of illness (89%); mosquitoes likely played a limited role in transmission of disease to humans. Close partnership with animal health and agriculture sectors allowed early recognition of human cases and appropriate preventive health messaging.

Rift Valley fever (RVF) is an emerging arboviral zoonosis, endemic to Africa. During periods of anomalous heavy and prolonged rainfalls that favor the breeding of competent mosquito vectors, Rift Valley fever virus (RVFV) can cause widespread epidemics in livestock in the absence of high vaccination coverage. These large outbreaks are associated with high rates of abortion and death among domestic and wild ruminant animals. Not only do these outbreaks have a substantial socioeconomic effect, but they also pose a public health threat to humans (*1*,*2*).

Numerous routes of transmission of RVFV to humans have been observed during previous epizootics, with varying contributions to the overall epidemiologic profile. These routes include direct contact with infected animal tissues, blood, or other body fluids; inhalation of aerosolized infected fluids; and transmission through bites of infected mosquito vectors (*1–3*). Ingestion of raw and unpasteurized milk has also been epidemiologically associated with RVF disease in humans in previous outbreaks (*4–8*). A causal link between consumption of milk from infected animals and human infection has, however, not been conclusively demonstrated, and laboratory analysis of milk from experimentally infected animals provides conflicting evidence (*9–11*).

Because of the zoonotic nature of the virus, specific occupational groups are at increased risk of infection, such as farm, abattoir (slaughterhouse), veterinary, and allied animal-health workers (*1–3*,*12*,*13*). Most infections with RVFV in humans are asymptomatic or self-limiting, mild, influenza-like illness. However, in a small proportion of patients, severe complications can manifest as hemorrhage, encephalitis, hepatitis, or retinitis (*1*,*2*,*14*). The overall case-fatality rate is estimated to be 0.5%–2.0% (*1*).

Large epidemics were most recently documented in Somalia (2006–2007), Kenya (2006–2007), Tanzania (2007), Sudan (2007–2008), Mayotte (2007–2008), and Madagascar (2008) (*15*). Before 2008, South Africa experienced 2 large epizootics on the interior plateau (Free State, Eastern Cape, and Northern Cape provinces) during 1950–1951 (*16*) and again during 1974–1976 (*17*); however, smaller sporadic outbreaks have been regularly reported since the 1950s. We previously documented the reemergence of RVF in South Africa during 2008, when a cluster of veterinarians and animal farmers became ill after an outbreak among cattle on a dairy farm (*13*). The present study documents the investigation of human RVF cases observed from 2008 to 2011 and describes temporal and spatial trends, demographic characteristics, and exposure to RVFV.

Case Detection and Outbreak Investigations

Following reports of RVF outbreaks in domestic and wild ruminants during 2008, systems for identifying and testing suspected RVFV infection in humans were enhanced. Throughout the study period (2008–2011), health care professionals were encouraged to consider RVF in the differential diagnosis of patients who met the suspected case criteria given below. Health care professionals were reached through various communication methods, including the distribution of guidelines (*18*) to health care facilities throughout the country, provision of regular reports and recommendations in newsletters of the National Institute for Communicable Diseases (NICD) that were widely distributed through health care profession networks, and numerous presentations in South Africa. Site visits and health promotional and enhanced case-finding activities were conducted by local health and veterinary authorities following reports of RVF in animal populations. Symptomatic persons identified during site visits were referred to local health care facilities for further assessment. A suspected RVF case-patient was defined as any person meeting ≥1 of the following criteria: 1) a person belonging to a high risk category who has an influenza-like illness, which could include fever, myalgia, arthralgia, or headache; 2) a person belonging to a high risk category who has signs and symptoms of encephalitis, such as hemorrhage, hepatitis, or ocular pathology/retinitis, with or without fever; or 3) a person with unexplained encephalitis, hepatitis, or hemorrhagic illness who resides in an area where RVF can potentially occur. High risk categories included the following: a) recent close contact with livestock and game animals in or from RVF-affected areas, including slaughtering and butchering (traditional or commercial), disposal of carcasses and fetuses, assisting with birthing or other animal husbandry activities that resulted in exposure to animal blood and body fluids, or veterinary procedures and necropsies; b) residing in an area where RVF is known to occur or has the potential to occur and recent mosquito bites; or c) consuming unpasteurized milk from RVF-affected areas.

Two clotted blood specimens were requested from all persons who met the suspected case definition. Specimens were transported to the Special Pathogens Unit, NICD, where assays were performed in order to detect RVFV-specific nucleic acid and antibodies against RVFV. These assays included a combination of real-time reverse transcription PCR, loop-mediated isothermal amplification assays, virus isolation, hemagglutination-inhibition assays, or IgM ELISA, as per previously published protocols (*19–21*). We also considered cases identified through specimens submitted for routine arbovirus testing. A confirmed case was defined as the detection of live RVFV, RNA, or IGM against-RVF.

Basic patient identification and demographic information accompanied specimens. After identifying a confirmed case, we interviewed the attending health care worker and patient (when possible) to complete a standardized questionnaire. In most instances, interviews were completed by telephone, but occasionally interviews were conducted during field visits. The questionnaire captured the following: demographic details, including patient's age, sex, address and location, and occupation , clinical details, including timing of illness onset, symptoms, sequelae, clinical outcome, hospital admissions, and past medical history (clinical findings to be presented elsewhere); and exposure details. For the latter, we asked whether the case-patient had experienced any of the following exposure categories in the week before illness onset: contact with animal tissues, blood, or body fluid; mosquito bites; drinking unpasteurized milk; or, acquiring, handling, or consuming meat either directly from a farm or from an informal or traditional butcher. These questions were not mutually exclusive, and case-patients could report experiencing >1 exposure category. The questionnaire additionally allowed for comments to describe details about recalled exposure events, which were later coded for data capturing and analysis.

Data from specimen submission records, laboratory reports, and questionnaires were captured, combined, cleaned, and analyzed in a combination of Excel 2003 (Microsoft, Redmond, WA, USA) and EpiInfo v3.5.3 (Centers for Disease Control and Prevention, Atlanta, GA, USA). In this study, we considered all persons with a completed laboratory investigation for RVF in South Africa from 2008 to 2011 and focused subanalyses on confirmed cases only. When date of symptom onset was not available, we estimated the date on the basis of the date of specimen collection. Incidence rates (IR) were calculated using the annual Statistics South Africa midyear population estimates for 2008–2011 (*22*). Spatial analyses were completed in ArcGIS v10.0 (ESRI, Redlands, CA, USA). We geocoded confirmed cases to an administrative local municipality using an address or nearest reported town. The revised South African municipal boundaries, released June 28, 2011, were used for spatial analysis for all years (*23*).

Ethics clearance for essential communicable disease surveillance was granted to the NICD by the Human Medical Research Ethics Committee of the University of the Witwatersrand, Johannesburg (protocol number M060449, reference R14/49 Schoub). This clearance includes outbreak investigations related to notifiable medical conditions under surveillance, including RVF.

Temporal and Spatial Trends

A total of 2,621 specimens were tested for RVFV from 2008 through 2011. We excluded duplicated sets of specimens from patients tested on ≥2 occasions (n = 93), specimens collected from patients outside of South Africa (n = 6), and specimens from those who did not meet the suspected case definition and tested negative for RVFV infection (n = 513). Of the remaining 2,009 suspected cases, 302 cases were laboratory-confirmed (15% detection rate). Interviews were completed for 245 (84%) confirmed cases; however, partial descriptive data were available through specimen submission records for all confirmed cases, and therefore, we considered all confirmed cases in further analyses.

Following the reemergence of RVF in 2008, outbreaks of the disease were noted for 4 consecutive years, and the influx of specimens and incidence of confirmed human cases in late summer to early autumn months increased annually and peaked in March each year (Figure 1). Few or no cases were detected during the colder winter months of each year.

Sporadic RVF outbreaks, of relatively limited spatial extent and magnitude, were observed during 2008 and 2009. A total of 17 confirmed cases (IR 0.03/100,000 persons) were detected during 2008 from 3 of the 4 provinces that reported RVF among animal populations at that time (Table 1, Figure 2). During 2009, a total of 7 confirmed cases (IR 0.01/100,000 persons) were detected following isolated, sporadic outbreaks among animals in KwaZulu-Natal and Northern Cape provinces.

During February 2010, following heavy rainfalls across large parts of the country, an explosive and geographically extensive RVF epizootic occurred (Figures 1, 2). A total of 241 confirmed cases were identified. The outbreak was most concentrated in the interior plateau of South Africa, and the highest rates of human infection were observed in Northern Cape (IR 7.25/100,000 persons) and Free State (IR 4.43/100,000 persons) provinces.

Human RVF cases continued to be detected from December 2010 to May 2011; most were diagnosed in areas bordering Eastern Cape and Western Cape provinces. Thirty-seven human cases were confirmed (IR 0.07/100,000 persons) in 2011. The last confirmed human RVF case reported illness onset on May 21, 2011.

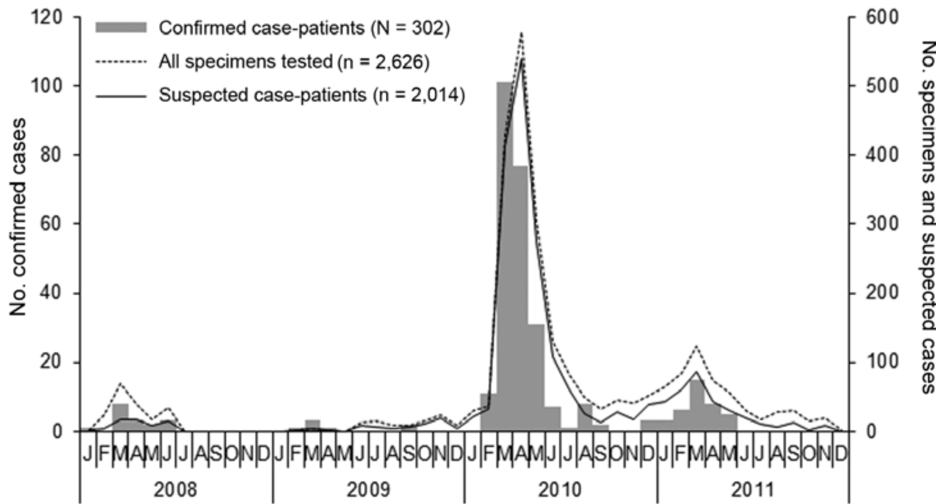

Figure 1. Epidemic curve illustrating the frequency of Rift Valley fever laboratory-confirmed cases, all specimens tested, and suspected cases tested by month of illness onset, South Africa, 2008–2011 (N = 302)

Demographic Description

Table 2 gives a demographic description of the 302 confirmed cases. The ratio of male to female cases was 6.55 to 1, and infection was predominantly among adults (median age 43 years, range 1 to 86 years). Data on occupation were available for 289 (96%) of persons with confirmed cases. Of these, animal farmers and animal farm workers (n = 173, 60%) made up the largest proportion. Infection among persons working in various animal health and veterinary science professions (n = 37, 13%) and persons in a meat-related industry (n = 32, 11%) were also frequently reported. Collectively, 242 (83%) persons with confirmed cases reported working within occupations in which direct contact with animals frequently occurs.

Twenty-five of the confirmed case-patients died from RVF (overall CFR, 8%). All deaths occurred during 2010 (year-specific CFR, 10%). The median age at illness onset among patients with fatal cases was 47 years (range, 15–72 years).

Exposures

Individual exposure history before onset of symptoms was obtained from 284 (94%) confirmed case-patients. Of these, 254 (89%) reported a history of direct contact with animal tissues, blood, or body fluid; 46 (16%) noted being bitten by mosquitoes; 30 (11%) reported drinking unpasteurized milk; and 21 (7%) reported acquiring, handling, or consuming meat directly from a farm or an informal or traditional butcher.

In addition, we considered the frequency of each exposure classification among case-patients reporting only a single event. Of 234 case-patients who met these criteria, 205 (88%) reported only a history of direct contact with animals, 15 (6%) reported only being bitten by mosquitoes, 6 (2%) reported only drinking unpasteurized milk, 3 (1%) reported only acquiring, handling, or consuming meat either directly from a farm or informal or traditional butcher, and 5 (2%) could not recall any of the listed exposure types.

Table 1. Frequency and incidence rate of human laboratory-confirmed Rift Valley fever cases by province where exposed, stratified by year, South Africa, 2008–2011*

	2008		2009		2010		2011		Total
Province	No. (%)	IR	No. (%)	IR	No. (%)	IR	No. (%)	IR	No. (%)
Free State	0	0	0	0	125 (52)	4.43	3 (8)	0.11	128 (43)
Northern Cape	0	0	2 (29)	0.17	80 (33)	7.25	3 (8)	0.27	85 (28)
Eastern Cape	0	0	0	0	16 (7)	0.24	17 (46)	0.25	33 (11)
Western Cape	0	0	0	0	11 (5)	0.21	14 (38)	0.26	25 (8)
Gauteng	9 (53)	0.09	0	0	0	0	0	0	9 (3)
North West	0	0	0	0	8 (3)	0.25	0	0	8 (3)
Mpumalanga	6 (35)	0.17	0	0	0	0	0	0	6 (2)
KwaZulu-Natal	0	0	5 (71)	0.05	0	0	0	0	5 (2)
Limpopo	2 (12)	0.04	0	0	0	0	0	0	2 (1)
Total	17	0.03	7	0.01	240*	37	0.07	301†	

*N = 302; IR, incidence rate/1000,000 persons.
†Province known for 301 (99%) cases; province data not available for 1 case-patient in 2010.

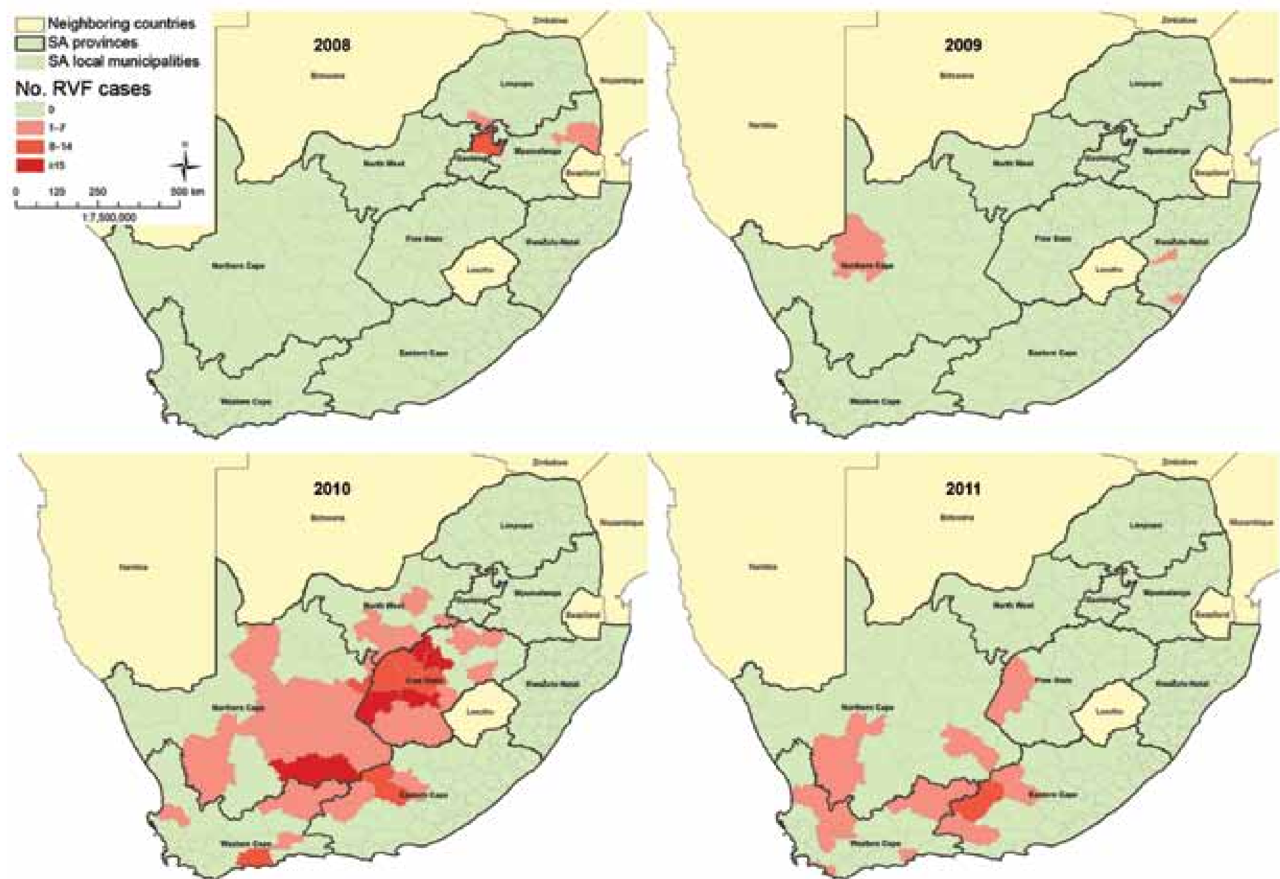

Figure 2. The spatial frequency distribution of human laboratory-confirmed Rift Valley fever cases by administrative local municipality by year, South Africa (SA), 2008–2011 (N = 302).

Of 254 case-patients who reported a history of direct contact with animals, 169 (67%) provided comments that allowed further categorization of animal-related exposures before onset of illness. Comments were open-ended, and >1 activity was often reported by a single case-patient. Of the 169 case-patients who provided additional comments, 136 (80%) reported physical contact with animal carcasses, either during the disposal of animals that died of RVF or during other procedures. Slaughtering of livestock or game animals was documented in 70 (41%) cases, and included reports of animal slaughter on farms (n = 35), in commercial abattoirs (n = 26), while hunting (n = 4), and in unspecified locations (n = 5). The performance of necropsies on animals was documented in 32 (19%) cases, including 26 instances in which necropsies were performed by animal health professionals, and 6 instances in which necropsies were undertaken by animal farmers or animal farm workers. Handling and disposing of fetal material after abortions in pregnant ruminants was documented for 28 (16%) cases. Exposures were, however, not limited to deceased animals but also included physically assisting with the birthing of live animals (n = 16, 9%), unspecified veterinary procedures (n = 8, 4%), and shearing (n = 2, 1%). Eighteen (11%) case-patients reported vaccinating livestock against RVF; of these, 2 case-patients reported needle-stick injuries while administering vaccine.

Conclusions

During sporadic RVF outbreaks in 2008–2009 and extensive epidemics during 2010–2011, a total of 302 laboratory-confirmed human infections were identified. The incidence of human cases peaked in the late summer to early autumn months of each year and was spatially concentrated in the inferior plateau, later extending down to the southern coastal provinces of South Africa. This coincided with epizootics observed following heavy rainfall, and we observed spatial and temporal patterns for human RVF infections similar to those observed in RVF outbreaks reported in domestic livestock (*24*). The sporadic cases in 2008–2009 were attributed to RVFV lineage C, which is distributed widely and has been responsible for epizootics throughout Africa and the Arabian Peninsula (*25*).

Table 2. Frequency distribution of human laboratory-confirmed Rift Valley fever cases by patient characteristic, South Africa, 2008–2011

Characteristic	No. (%); N = 302
Male sex*	262 (87)
Age group, y†	
0–9	1 (<1
10–19	16 (5)
20–29	67 (22)
30–39	47 (16)
40–49	68 (23)
50–59	53 (18)
60–69	30 (10)
≥70	18 (6)
Occupation‡	
Farmer or farm worker	173 (60)
Animal health worker	37 (13)
Abattoir worker, butcher, or hunter	32 (11)
Farm resident (nonworker)	5 (2)
Non–animal related occupation	42 (15)

*Known for 302 case-patients.
†Known for 300 case-patients.
‡Known for 289 case-patients

RVFV lineage H, an apparent antecedent from Namibia in 2004, was responsible for the 2010–2011 South African epizootics (*25*). A degree of spatial overlap was observed when comparing outbreaks in 2010 to 2011; however, in 2011, human infections were primarily observed in areas that were not previously affected. This finding may be explained by accumulated herd immunity in areas affected in 2010, attributable to a combination of natural infections and extensive vaccinations conducted in livestock populations, while neighboring populations remained susceptible to RVFV outbreaks in 2011. Two livestock vaccines were applied during the 2008–2011 outbreaks: inactivated whole RVFV vaccine, which requires a booster vaccination and annual revaccination; and the live-attenuated Smithburn vaccine, which provides lifelong immunity but may cause abortions and fetal malformations when administered to gestating adult animals.

Laboratory-confirmed human cases were typically in men who worked in animal farming, animal health, and meat-related industries. Most (89%) case-patients reported direct contact with animal tissues, blood, or other body fluids, which suggests that this is the most common risk factor and route of transmission to humans in South Africa. Slaughtering animals in commercial and farm settings, conducting necropsies, and handling fetuses of aborted ruminants were frequently documented as specific exposure events. These findings are consistent with risk factor analyses conducted during the 2007 RVF outbreaks in Kenya and Tanzania, in which ≈40% of cases had direct contact with sick animals (*3*,*7*). In the South African outbreaks reported here, however, the role of transmission through direct contact appears to have been even more predominant. During the 2000–2001 outbreaks in Saudi Arabia, researchers showed that in 23% of cases transmission likely occurred through mosquito exposure (*26*). Although we observed that mosquito vectors played a role in establishing RVF in South Africa and amplifying the epizootic among animal populations, this route appears to have a limited role in transmission of RVFV to humans (16% of case-patients reported mosquito bites; 6%reported mosquito bites as the only exposure). Similarly, other possible routes of transmission, such as consuming fresh, unpasteurized milk likely played a lesser role in human infections in South Africa. Indeed, ingesting unpasteurized milk has only been implicated epidemiologically (*4*–*8*); nonetheless, it remains a consideration for health promotional interventions.

Exposure to RVFV during animal vaccinations was the likely route of transmission in a limited number of cases. As livestock vaccines are commonly sold in multidose vials and are administered with automatic syringes with intermittent needle changes, vaccine may inadvertently be given to viremic livestock, with the potential for serial transfer of wild RVFV to other animals or to humans through needle-stick injuries. Reassortant RVFVwas identified in a patient who experienced a needle-stick injury and was potentially exposed to both live vaccine and wild virus supports this assumption (*25*).

We found an overall CFR of 8% for all laboratory-confirmed RVF cases identified in 2008–2011. This rate is substantially higher than the generally accepted death rate (0.5%–2.0%) but lower than that observed in Saudi Arabia (13.9% of confirmed cases), Kenya (26.5%) and Tanzania (47%) (*1*,*7*,*26*,*27*). However, in many RVF outbreak investigations (as in this study), the most clinically severe cases are often those that are detected and confirmed.

Our investigation was limited in its capacity to detect asymptomatic and subclinical RVFV infections. Serologic surveys conducted following outbreaks elsewhere have found most human RVF case-patients remain asymptomatic after infection (*1*). Recall bias may have also affected our findings because of delays incurred between onset of illness, laboratory-confirmation of RVF infection, and completion of interviews. Noteworthy events, such as animal birthing or slaughtering, may have been more easily recalled by patients. Likewise, in some instances interviews were limited to only the clinician treating the patients, who would likely rely on information recorded in a patient's file.

Despite these limitations, this study provides a reliable minimum estimate of the magnitude of human infections during 4 years of heightened RVFV transmission among animal populations. The availability of extensive laboratory capacity for testing all suspected RVF cases afforded a unique opportunity (both for South Africa and the African continent) to support RVF outbreak investigations on such a scale. Close collaboration between field investigators and the laboratory is vital, given the nonspecific nature of mild RVF disease in humans, which can be easily confused

with diverse causes of febrile illnesses. The combination of multiple laboratory assays for direct detection of RVFV, as well as serologic evidence of acute infection, proved successful in identifying cases. Equally important was a close working relationship between health and agriculture sectors, which illustrates the importance of intersectoral responses to zoonotic outbreaks and a successful One Health Initiative approach. In previous outbreaks, detecting severe (usually hemorrhagic) illness in humans was often the catalyst for investigations. In the present study, our investigations were triggered in response to surveillance conducted by agriculture authorities; these early warnings also provided an opportunity for preventative interventions and timely response to human RVF cases in many instances.

When will the next RVF epizootic occur in South Africa? La Niña weather conditions with heavy rain falls were predicted for the early months of 2012; however, further RVF outbreaks were not observed. Extensive livestock vaccination against RVFV during recent years may have resulted in decreased susceptibility of the host populations, reducing the likelihood of further explosive epidemics in the short term. However, we may be entering another interepizootic period of unknown duration. The natural history of the disease in South Africa has been in part attributable to animal vaccination practice; epidemics prompt mass vaccination of livestock, followed by a dramatic drop-off in vaccination coverage rates during extended interepizootic periods, which leads to the accumulation of large numbers of susceptible animals that are not immune and sets the stage for explosive epidemics in concert with conducive climatic conditions. Ongoing preparedness and continued preventative interventions aimed towards population groups at high risk and practices identified by this study will be essential in reducing the effect of future epizootics on human populations. Continued strengthening of surveillance studies in humans, livestock, and wild animals will be critical in enabling a rapid response to RVF epidemics, as well as preventing future widespread epizootics.

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Potential Role of Deer Tick Virus in Powassan Encephalitis Cases in Lyme Disease–endemic Areas of New York, USA

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Learning Objectives

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

- Evaluate the virology of Powassan virus
- Assess the clinical presentation of Powassan virus encephalitis
- Analyze laboratory and radiologic data in cases of Powassan virus encephalitis
- Distinguish clinical outcomes of Powassan virus encephalitis.

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Powassan virus, a member of the tick-borne encephalitis group of flaviviruses, encompasses 2 lineages with separate enzootic cycles. The prototype lineage of Powassan virus (POWV) is principally maintained between *Ixodes cookei* ticks and the groundhog (*Marmota momax*) or

[1]These authors contributed equally to this article.

striped skunk (*Mephitis mephitis*), whereas the deer tick virus (DTV) lineage is believed to be maintained between *Ixodes scapularis* ticks and the white-footed mouse (*Peromyscus leucopus*). We report 14 cases of Powassan encephalitis from New York during 2004–2012. Ten (72%) of the patients were residents of the Lower Hudson Valley, a Lyme disease–endemic area in which *I. scapularis* ticks account for most human tick bites. This finding suggests that many of these cases were caused by DTV rather than POWV. In 2 patients, DTV infection was confirmed by genetic sequencing. As molecular testing becomes increasingly available, more cases of Powassan encephalitis may be determined to be attributable to the DTV lineage.

Powassan virus is a positive-sense RNA virus that belongs to the tick-borne encephalitis group of flaviviruses (1). The first recognized case of Powassan encephalitis in North America was from Canada in 1958 (2); the first case in Russia was from the Primorsky Krai region in 1972 (3). Powassan virus comprises 2 closely related lineages: the Powassan virus prototype (POWV) lineage and the deer tick virus (DTV) lineage. POWV and DTV, which share 84% nucleotide sequence identity and 94% amino acid sequence identity (4), have a common ancestral origin, from which they diverged around 485 years ago (5). Each lineage has separate tick vectors and reservoir hosts in North America (5). POWV is maintained in an enzootic cycle between *Ixodes cookei* as the tick vector and the groundhog (*Marmota momax*) and striped skunk (*Mephitis mephitis*) as the principal reservoir hosts (6). DTV is believed to be maintained between *I. scapularis* ticks and the white-footed mouse (*Peromyscus leucopus*) (7,8).

DTV can be accurately differentiated from POWV only by genetic sequence analysis. Four cases of proven DTV encephalitis have been reported: 1 from Ontario, Canada (*4*,*9*); 2 from New York (*10*,*11*); and 1 from Minnesota (*12*). For clarity, in this article, we will use the term POWV/DTV to designate infection with Powassan virus of undetermined lineage. We present a detailed description of the clinical signs and symptoms, laboratory diagnosis, and outcome of the 14 cases of POWV/DTV encephalitis diagnosed during 2004–2012 in New York. We also provide a review of the literature for epidemiologic evidence suggesting that many of these cases were caused by DTV rather than POWV.

Methods

Case Definition and Study Design

We conducted a retrospective review of the medical records of all POWV/DTV cases that were reported to the New York State Department of Health (NYSDOH) during 2004–2012. During this period, the NYSDOH initiated the use of diagnostic testing methods to detect this virus for all patients for whom arboviral testing was requested. We also reviewed published reports of 6 of these cases for additional details (*10*,*11*,*13*–*15*). POWV/DTV neuroinvasive infection was defined by using the 2011 United States surveillance case definition (*16*), which includes clinical criteria (fever of ≥38°C with any peripheral or central nervous system dysfunction documented by a physician and the absence of another more likely explanation) and ≥1 of the following:

- Isolation of POWV/DTV from, or detection of specific viral antigen or nucleic acid in, tissue, blood, cerebrospinal fluid (CSF), or another body fluid;
- A ≥4-fold change in POWV/DTV-specific quantitative antibody titers in paired serum samples;
- POWV/DTV-specific IgM in serum with confirmatory POWV/DTV-specific neutralizing antibodies in the same or a later serum specimen (POWV/DTV neutralizing antibody was considered specific if the titer was ≥4-fold higher than the corresponding neutralizing antibody titer to West Nile virus [WNV] or Saint Louis encephalitis virus [SLEV]);
- POWV/DTV-specific IgM antibodies in CSF and a negative result for other IgM antibodies in CSF for arboviruses endemic to the region where exposure occurred.

Demographic data were summarized by using descriptive statistics, mean and SD for continuous variables, and numbers and percentages for categorical variables. The study was approved by the Institutional Review Board at New York Medical College.

Laboratory Analyses

Serologic testing for POWV/DTV, performed at the NYSDOH Wadsworth Center in Albany, included a microsphere immunoassay to detect IgM and, separately, total antibodies (IgG + IgA + IgM) against recombinant DTV envelope protein in serum. The microsphere immunoassay also was used to detect IgM antibodies against recombinant DTV envelope protein in CSF. Recombinant DTV envelope protein was produced from the DTV-Ipswich strain, as described (*17*,*18*). Results were assessed as the ratio of the median fluorescence intensity (MFI) for 100 beads that reacted with the patient's serum to the MFI of beads that reacted with a negative control serum specimen. The cutoff for a positive result was a value of 3 SDs above the mean MFI result based on a panel of serum specimens from healthy subjects.

Serum and CSF samples from 2 patients were also tested for POWV/DTV at the Centers for Disease Control and Prevention diagnostic and reference laboratory (Arboviral Diseases Branch, Fort Collins, CO, USA) by using an IgM antibody capture ELISA (MAC-ELISA) and IgG ELISA against POWV envelope protein (LB strain, Canada, 1958). A plaque reduction neutralization test (PRNT) against POWV (LB strain, Canada, 1958) (*2*) was performed at the Wadsworth Center and/or the Arboviral Diseases Branch by using BHK-21 cells. The antibody titer reported is the reciprocal of the dilution of serum that inhibited 90% of the test virus inoculum. PCR for POWV/DTV and genetic sequence analysis were done at the Wadsworth Center, as described (*10*,*11*). Serum samples submitted to the NYSDOH for arboviral screening were also tested for antibodies to WNV by using a MAC-ELISA and for antibodies to SLEV by using an indirect immunofluorescence assay; if these assays were positive, specific WNV and SLEV neutralizing antibodies were measured by using PRNT.

Results

Demographic Characteristics

Fourteen cases of POWV/DTV encephalitis were identified in New York during 2004–2012; geographic locations by case-patient county of residence are shown in Figure 1. Ten (72%) case-patients were from Westchester, Putnam, or Dutchess Counties, which are located in the Lower Hudson Valley (LHV), a highly Lyme disease–endemic region. Three (21%) patients were <10 years of age; 10 (72%) were ≥60 years of age. The male-to-female ratio was 3:1. Eight (57%) patients had symptom onset in June, July, or August (Figure 2). Ten (72%) patients lived in a wooded area or reported outdoor exposure, and 8 (57%) had a pet (mainly a dog or cat). A tick bite was reported for 5 (36%) case-patients before illness onset, but only 3 remembered the exact date; incubation times from the tick bite to the onset of symptoms in these patients were 9, 11, and 32 days.

All case-patients were hospitalized. The mean ± SD time from onset of symptoms to hospitalization for 13 patients was 3.5 ± 1.9 (range 1–6) days. For the remaining patient, time from onset of symptoms to hospitalization was reported to be 2–3 weeks. Seven case-patients had been prescribed an oral antimicrobial drug (amoxicillin or doxycycline) within the few days preceding hospital admission: 4 for a febrile illness with a nonspecific macular or papular rash, 2 for erythema migrans (both lived in the LHV), and 1 for headaches and malaise following a tick bite.

Clinical and Radiographic Data

Fever (100%), generalized weakness (86%), and lethargy (72%) were the most commonly reported signs and symptoms (Table 1); 11 patients (79%) had a temperature >39°C at the time of hospitalization. Results of computed tomography scans of the brain were negative for acute findings for 13 (93%) case-patients (whether intravenous contrast was given is unknown). Results of magnetic resonance imaging (MRI) (with and without contrast for 4 patients, without contrast for 2, unknown for 8) showed acute abnormalities in 13 (93%) case-patients (mean ± SD time to MRI after hospitalization 2.9 ± 2.3 [range 1–10] days). Scattered T2-hyperintense foci predominantly affecting the gray matter and acute ischemic changes were the most common abnormalities found (Table 2). Among the 9 patients for whom electroencephalography (EEG) data were available, epileptic waveforms were documented for 3 (33%) and diffuse or severe slowing for 7 (78%) (mean ± SD time to EEG after hospitalization 9.3 ± 9.5 [range 2–30] days).

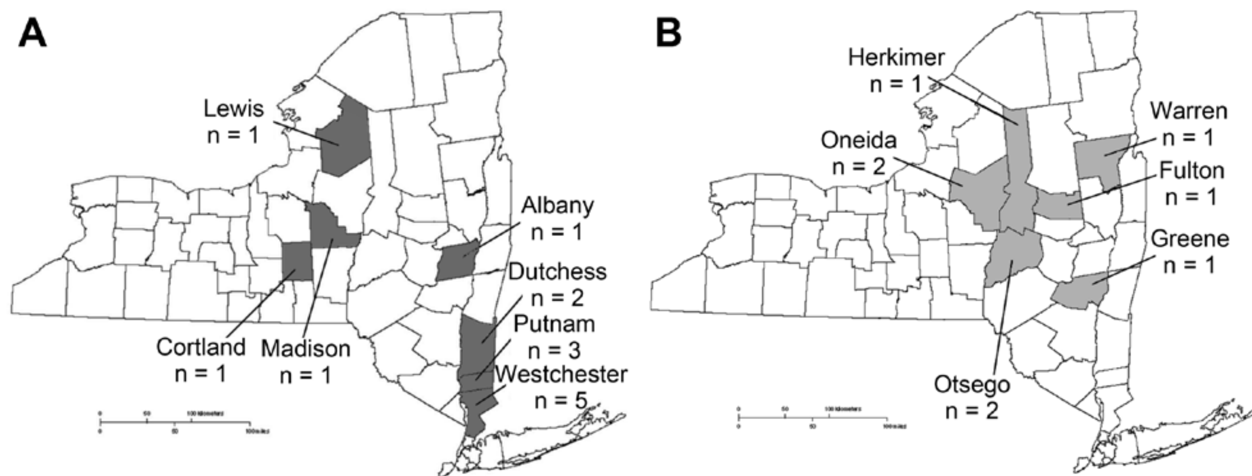

Figure 1. Cases of Powassan/deer tick virus encephalitis, by county, New York, USA, for 2004–2012 (A) and 1958–2003 (B) (*19*). A total of 14 cases occurred during 2004–2012 and 9 cases during 1958–2003. One additional case from 1958–2003 is not shown because the patient had lived in and traveled through multiple counties in the 6 weeks before illness onset (*20*).

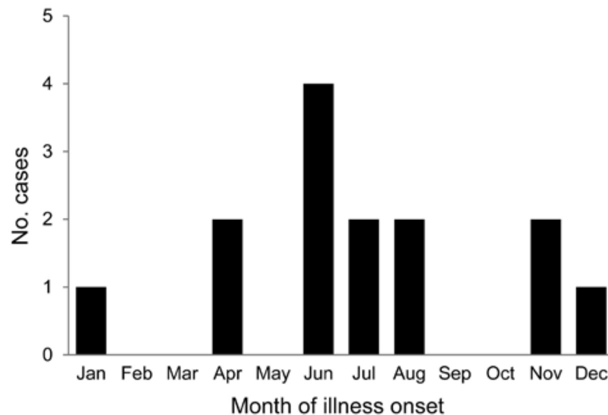

Figure 2. Cases of Powassan/deer tick virus encephalitis, by month of illness onset, New York, USA, 2004–2012.

Laboratory Findings

The mean ± SD hemoglobin level for all case-patients was 12.3 ± 1.62 g/dL; 12 case-patients were anemic based on reference ranges for age and sex. Mean ± SD platelet count was 217 ± 86 × 10^9/L (reference range 172–450 × 10^9/L). With the exception of 1 patient who had chronic lymphocytic leukemia, the mean ± SD leukocyte count at admission was 9.36 ± 3.99 × 10^9 cells/L (reference range 4.4–11.3 × 10^9 cells/L). Aspartate and alanine aminotransferase enzyme levels were within reference ranges.

CSF analysis (mean ± SD time to initial lumbar puncture after hospitalization 3.3 ± 3.9 [range 0–15] days) showed a mean ± SD leukocyte count of 84 ± 88 (range 0–263) cells/mm^3. Of the 13 patients who had CSF pleocytosis, 12 had a predominance of lymphocytes; 1 patient had a CSF leukocyte count of 68 with 65% neutrophils. Although CSF in 1 patient initially showed no leukocytes, repeat CSF testing 2 days later showed 891 leukocytes with 93% lymphocytes, 6% monocytes, and 1% neutrophils. The mean ± SD CSF glucose and protein levels were 86 ± 43 (range 39–179) mg/dL and 76.7 ± 28 (range 52–142) mg/dL, respectively. Results of Gram stain testing and bacterial culture of CSF were negative for all patients.

For 2 case-patients who were residents of Putnam County, diagnosis was confirmed by detection of a conserved region in the POWV nonstructural protein 5 gene by real-time reverse transcription PCR. A positive result was obtained from brain tissue (from an autopsy specimen) from 1 patient, collected on day 17 after hospitalization, and from a CSF sample from the other patient, obtained on day 9 after hospitalization. Genetic sequencing analysis demonstrated that both of these patients were infected with DTV (Table 3). These 2 patients were previously reported (*10*,*11*). For 5 other case-patients for whom CSF was available for molecular testing, viral RNA was not detected by the same method.

For 12 case-patients, the diagnosis of POWV/DTV infection was made by serologic testing alone. Of these, 11 had a positive test for IgM in serum to recombinant DTV envelope protein and evidence of neutralizing antibodies against POWV; case-patient 8 had a 4-fold increase in neutralizing antibodies against POWV between acute and convalescent serum samples, without detectable IgM. In total, 8 (67%) of the 12 patients had a ≥4-fold increase in POWV PRNT titers between acute and convalescent serum samples (Table 3). Four patients had weak-positive test results for serum antibodies against SLEV, but serum PRNT titers for this virus were negative for 3 patients and at least 4-fold lower than the PRNT titer for POWV/DTV in 1 patient. The 2 patients with erythema migrans had detectable antibodies to *Borrelia burgdorferi*, the causative agent of Lyme disease, by standard 2-tier testing.

Histologic examination of brain tissue was available for 2 patients, 1 from a brain biopsy and 1 from a specimen obtained at autopsy. In both cases, hematoxylin and eosin stained sections revealed a reactive gliosis, increased numbers of microglial cells, and necrotizing inflammation with a lymphocytic infiltrate, predominantly affecting the gray matter, consistent with acute meningoencephalitis. For 1 of the patients, who had confirmed DTV infection by molecular analysis, a detailed histopathologic analysis showed lymphocytic infiltrates, which in the leptomeninges and perivascular spaces contained predominantly CD4+ helper T cells and in the brain parenchyma predominantly CD8+ cytotoxic T cells (*10*).

Molecular testing for 12 viruses besides POWV/DTV, including enteroviruses, herpesviruses, and arboviruses, was performed on CSF to investigate other potential

Table 1. Clinical signs and symptoms of 14 hospitalized patients with Powassan/deer tick virus encephalitis, New York, USA, 2004–2012

Sign or symptom	No. (%) patients
Fever	14 (100)
Generalized weakness	12 (86)
Lethargy	10 (72)
Confusion	8 (57)
Seizure	6 (43)
Headache	6 (43)
Rash	6 (43)
Nonneurologic symptoms other than rash*	6 (43)
Vomiting	5 (36)
Focal deficit	5 (36)
Neck stiffness	5 (36)
Aphasia	3 (21)
Tremor	2 (14)
Dizziness	2 (14)
Dysarthria	1 (7)
Balance disturbances	1 (7)
Myoclonus	1 (7)

*Other symptoms included dyspnea in 2 (14%), abdominal pain in 3 (21%), diarrhea in 1 (7%), dysuria in 1 (7%), body aches in 3 (21%), and rhinorrhea in 1 (7%)

Table 2. Brain areas affected in 13 hospitalized patients with Powassan/deer tick virus encephalitis, New York, USA, 2004–2012

Areas affected	No. (%) patients
Regions	
Cerebral cortex	7 (54)
Basal ganglia	7 (54)
Brain stem	4 (31)
Cerebellum	3 (23)
Thalamus	3 (23)
Meninges	2 (15)
Sides	
Left	9 (69)
Right	2 (15)
Bilateral	2 (15)

viral causes of encephalitis for the 14 patients (*21*). CSF real-time PCR testing showed Epstein-Barr virus infection (EBV) at 36.2 cycle threshold (C_t) (weak signal) and human herpesvirus 6 (HHV-6) at 29.62 C_t in case-patient 3 and herpes simplex virus 1 at 37.25 C_t (weak signal) in case-patient 11.

Hospital Course

All patients received intravenous antimicrobial drugs during the first week of hospitalization (ceftriaxone [92%], vancomycin [67%], ampicillin [42%], and acyclovir [86%]). Case-patient 13 received oral ribavirin and subcutaneous pegylated interferon α for 2 weeks (*11*). Of the 14 patients, 5 (36%) received intravenous corticosteroids during illness (dexamethasone 0.5–1.0 mg/kg/d for 10 days or methylprednisolone 500–1,000 mg/d for 3–5 days). Time of initiation of systemic corticosteroid therapy ranged from 1 to 45 days after hospitalization.

Twelve (86%) patients were admitted to the intensive care unit (ICU) during hospitalization (mean ± SD time from hospitalization to ICU admission [n = 11] 2 ± 1.6 [range 1–6] days). Seven patients (50%) required endotracheal intubation and mechanical ventilation (mean ± SD time from hospitalization to intubation 4.6 ± 1.8 [range 3–7] days), and 5 (36%) required tracheostomy and gastric feeding tube placement.

Outcomes

Patients were followed for a mean ± SD time of 66 ± 67 (range 10–240) days after the date of hospitalization. The mean ± SD hospital stay was 33 ± 22 (range 9–90) days. For patients admitted to the ICU for whom length of stay was available (n = 10), the mean ± SD ICU stay was 22 ± 17 (range 5–58) days.

Five (36%) patients died. One patient died in the hospital after withdrawal of life support according to the family's wishes; 4 died after discharge from the hospital (mean ± SD time to death from the date of hospitalization, 116 ± 94 [range 13–240] days). Although none of the

Table 3. Diagnostic evaluation and outcome of POWV/DTV encephalitis, by county and date of illness, New York, USA, 2004–2012*

Category	Data by case no.													
	1	2	3	4	5	6	7	8	9	10	11	12	13†	14
Patient age, y	91	83	70	5	62	77	81	81	9	4	76	73	77	32
Date of illness	Jun 2004	Aug 2005	Jun 2007	Aug 2007	Jun 2007	Nov 2007	Nov 2007	Jul 2007	Jul 2008	Apr 2009	Jan 2009	Jun 2009	Dec 2010	Apr 2012
County	Alb	West	Cort	Lewis	Put	West	West	West	West	Dutch	Mad	Put	Put	Dutch
LHV	No	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
POWV MIA														
Acute	Pos	Neg	Pos	Pos	ND	Pos	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Pos
Conv	Pos	Pos	Pos	Pos	ND	Pos	Pos	Ind	Pos	Pos	Pos	Pos	Pos	Pos
PRNT titer														
Acute	40,960	320	320	20	ND	320	1,280	<10	10	320	<10	2,560	5,120	1,280
Conv	40,960	1,280	320	80	ND	320	5,120	40	160	2,560	40	20,480	5,120	320
PCR for POWV/DTV NS5 gene	ND	ND	ND	ND	Pos (brain)	ND	ND	Neg	ND	Neg	Neg	Neg	Pos (CSF)	Neg
Lineage	U	U	U	U	DTV	U	U	U	U	U	U	U	DTV	U
Outcome	LADL	LADL	Died	LADL	Died	Died	LADL	Died	LADL‡	LADL	LADL	LADL‡	Died	LADL
WNV testing														
ELISA	IgM–, IgG+	Neg	Neg	IgM–, IgG+	ND	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
PRNT	Neg	Neg	NA	Neg	NA	Neg	NA	NA	NA	Neg	NA	NA	Neg	Neg
SLEV PRNT														
Acute	Neg	10	Neg	NA	NA	Neg	NA	NA	NA	Neg	NA	NA	Neg	Neg
Conv	Neg	80	Neg	NA	NA	Neg	NA	NA	NA	Neg	NA	NA	Neg	Neg

*All tests were done on serum samples except as indicated. POWV, Powassan virus prototype strain; DTV, deer tick virus; Alb, Albany; West, Westchester; Cort, Cortland; Put, Putnam; Dutch, Dutchess; Mad, Madison; LHV, Lower Hudson Valley, MIA, microsphere immunoassay; ND, not done; Pos, positive; Neg, negative; Conv, convalescent; Ind, indeterminate; PRNT, plaque reduction neutralization test; NS5, nonstructural protein 5 gene; CSF, cerebrospinal fluid; U, Unknown; LADL, limited activity of daily living; WNV, West Nile virus; SLEV, Saint Louis encephalitis virus; NA, not available.
†Acute-phase CSF POWV IgM+ by MIA performed at Wadsworth Laboratory, New York State Department of Health; convalescent-phase CSF POWV IgM+ and IgG+ by ELISA performed at the Centers for Disease Control and Prevention (PRNT titer 8,192).
‡Imbalance.

postdischarge deaths could be directly attributed to POWV/DTV infection, they did appear to be related to the severely impaired health status caused by the infection. All patients who died were >60 years of age. Both patients who had DTV infection confirmed by genetic sequencing died. All 5 patients treated with corticosteroids survived; 5 (71%) of the 7 patients who did not receive corticosteroids died. (Information on corticosteroid use was unavailable for 2 patients, both of whom survived.) All of the patients who were discharged from the hospital (n = 13) had neurologic deficits at the time of discharge (Table 4).

Table 4. Neurologic deficits at the time of discharge in hospitalized patients with Powassan/deer tick virus encephalitis, New York, USA, 2004–2012*

Neurologic deficit	No. (%) patients
Significant limitation in ADL, n = 13	11 (85)
Cognitive deficit, n = 11	6 (55)
Bed bound, n = 13	7 (54)
Focal deficit, n = 10	4 (40)
Quadriplegia, n = 9	3 (33)
Ventilator dependence, n = 11	3 (27)
Aphasia, n = 11	3 (27)
Imbalance, n = 11	2 (18)
Headache, n = 11	2 (18)
Ophthalmoplegia, n = 9	1 (11)

*n, no. patients evaluable; ADL, activities of daily living.

Discussion

Since the initial recognition of POWV/DTV as a cause of viral encephalitis in 1958, only ≈80 cases of POWV/DTV encephalitis have been reported (*6*,*9–15*,*22–26*). In this study, we describe 14 patients with POWV/DTV encephalitis in New York during 2004–2012. Ten of the 14 cases occurred in residents of 3 counties in the LHV, a highly Lyme disease–endemic area. Although 9 cases of POWV/DTV infections were reported in New York before 2004 (*19*,*20*,*22*), none of those cases occurred in patients from the LHV (Figure 1).

Distinguishing between POWV and DTV infection provides epidemiologically relevant information from a public health perspective. Two patients had confirmation of DTV lineage by genetic sequencing; both lived in the LHV. For several reasons, we suspect that the other 8 patients from this region were also infected with DTV. Bites by *I. cookei* ticks are rare in the LHV, whereas *I. scapularis* tick bites are common. Among 126 ticks collected from tick bite victims and submitted to the Westchester County Health Department in 1985, a total of 96 (76.2%) were identified as *I. scapularis*; none was *I. cookei* (*27*). In addition, of the 5,738 ticks submitted to the NYSDOH from persons in the LHV with tick bites during 2004–2011, only 52 (1.2%) were identified as *I. cookei*; 4,225 (72%) were identified as *I. scapularis* (NYSDOH, unpub. data). DTV is also well documented in *I. scapularis* ticks from this region and from multiple other geographic areas, whereas numerous studies have not detected the prototype POWV lineage in *I. scapularis* ticks (*7*,*8*,*28–32*), even though these ticks are vector competent for POWV (*33*). In a field investigation conducted during 2007–2012 and involving >13,500 nymphal and adult ticks of 7 species (including >6,100 *I. scapularis* questing ticks) collected throughout the LHV, DTV, but not prototype POWV, was detected exclusively in *I. scapularis* ticks, and adult ticks from the LHV had infection rates of up to 6% (*34*).

Further lending support to our hypothesis, of the 8 POWV/DTV encephalitis case-patients from the LHV for whom virus sequence data were not available, 2 (25%) had evidence of Lyme disease: erythema migrans in conjunction with seropositivity for antibodies to *B. burgdorferi*. *B. burgdorferi* is not transmitted by *I. cookei* ticks (*35*). In a study of adult *I. scapularis* ticks collected in 2008 from Westchester County, 2 (29%) of 7 ticks infected with DTV were co-infected with *B. burgdorferi*; a third tick was co-infected with *Anaplasma phagocytophilum* (*28*). The frequency of co-infection in human cases may be lower than the co-infection rates in ticks because DTV can be transmitted within 15 minutes after onset of tick feeding, as compared with *B. burgdorferi*, which typically takes at least 48 hours (*29*). This short time required for transmission of DTV can also help explain why up to 50% of the cases from the LHV occurred during spring and fall, when adult *I. scapularis* ticks are more active; these ticks are less likely to go unnoticed than nymphs and thus may not remain attached long enough to transmit *B. burgdorferi*.

An accurate estimate of human deaths attributable to POWV/DTV infection remains unknown. Unlike the all-cause mortality rate of 36% that we observed, previous studies have reported fatality rates of <20% for POWV/DTV neuroinvasive infection (*9*,*13*,*22*). Differences in the length of follow-up might partly explain this difference; our 30-day fatality rate was also <20%. Several factors may have been associated with poor outcomes in this case series. Among the 5 patients who died, 4 were from the LHV, 2 with genetic sequencing–confirmed DTV infection and 1 with Lyme disease co-infection. This finding indicates a notable proportion of probable DTV-related deaths in our cohort. The 5 patients who died were >60 years of age, and 3 had brain stem or cerebellar involvement on MRI. In contrast, only 1 (11%) of the 9 survivors had brain stem or cerebellar involvement on MRI. Thus, age >60 years and involvement of the rhombencephalon may be poor prognostic indicators for POWV/DTV encephalitis.

No effective therapy is available for POWV/DTV encephalitis; current guidelines recommend supportive therapy (*36*). The role of antiviral therapy remains unclear. The case-patient in our series who was treated with ribavirin plus interferon 3 weeks into his illness (which may

be late) did not improve and eventually died (*11*). In contrast, all of the patients who received corticosteroids during their illness survived; therefore, future studies are needed to evaluate the potential role of systemic corticosteroids in the treatment of POWV/DTV encephalitis.

Although all of the case-patients in this study tested positive for POWV/DTV, HHV-6 DNA (29.62 C_t) and low levels of EBV DNA (36.2 C_t) were detected in the CSF of 1 patient. A high rate of detection of HHV-6 in CSF from healthy adults has been described; therefore, the clinical significance of detection of HHV-6 in CSF in immunocompetent hosts remains unclear (*36*). EBV is also occasionally detected in CSF at low levels and may be of no clinical significance (*37*). Similarly, PCR for HSV had a low-positive result (37.25 C_t) in the CSF of a patient who did not have the characteristic temporal lobe involvement of HSV encephalitis on EEG or on MRI (*38*) and did not respond to acyclovir therapy. The diagnosis of POWV/DTV encephalitis in this patient was made on the basis of a positive test result for serum IgM against POWV/DTV and a 4-fold increase in specific neutralizing antibodies against POWV. Thus, if this patient were co-infected with HSV and POWV/DTV, the latter may have contributed to the disease severity, but to what extent remains unclear. If, in fact, the correct diagnosis was HSV encephalitis alone, then the proportion of POWV/DTV encephalitis cases from the LHV was actually higher than we have reported, since this patient was from another part of New York.

In conclusion, we describe 14 cases of POWV/DTV encephalitis from New York diagnosed during 2004–2012. Ten (72%) of the case-patients were from the LHV, a highly endemic area for Lyme disease. We suspect that the cases occurring in the LHV may have been caused by DTV. Given the high rate of severe illness and death associated with these infections and the evolving epidemiology, molecular analysis is essential in the evaluation of POWV/DTV infections.

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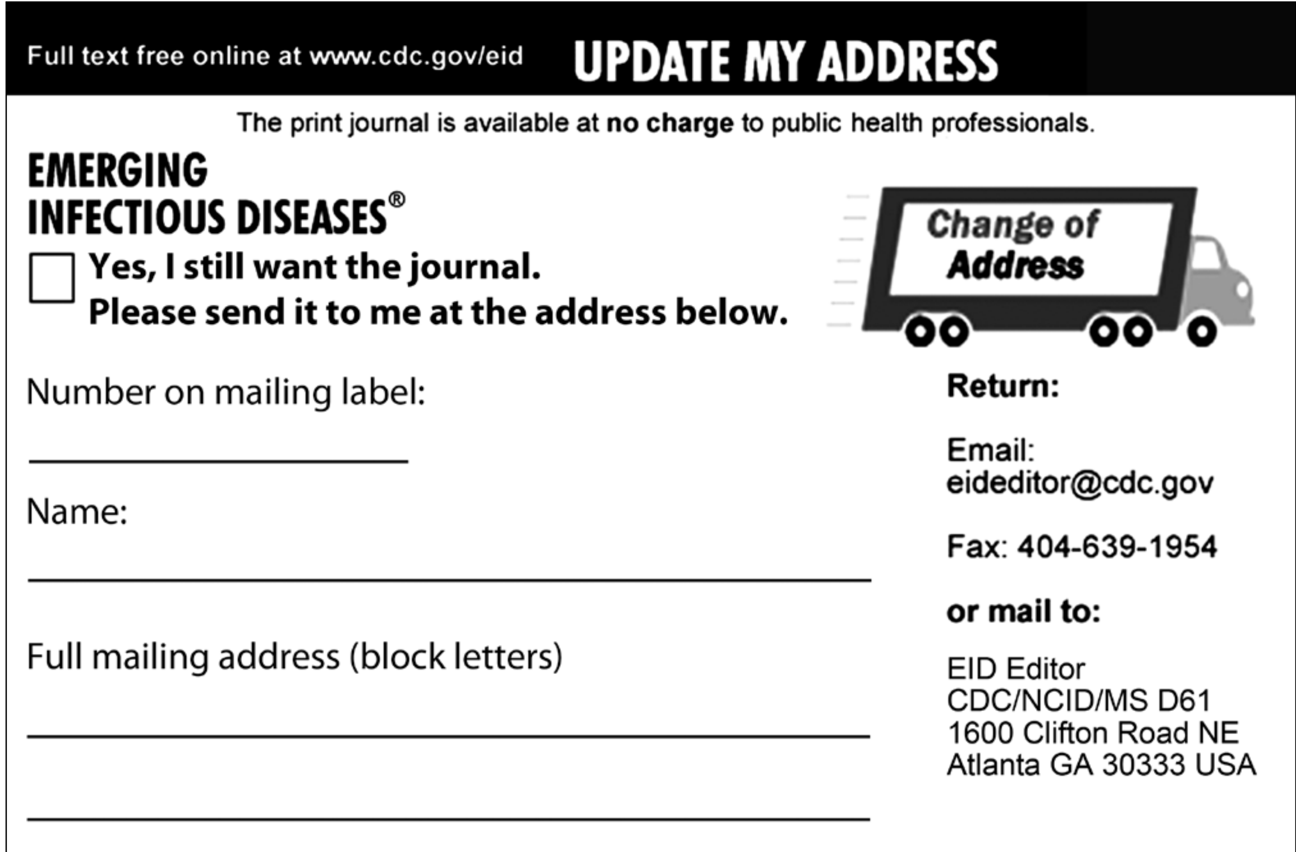

Twenty-Year Summary of Surveillance for Human Hantavirus Infections, United States

Barbara Knust and Pierre E. Rollin

In the past 20 years of surveillance for hantavirus in humans in the United States, 624 cases of hantavirus pulmonary syndrome (HPS) have been reported, 96% of which occurred in states west of the Mississippi River. Most hantavirus infections are caused by Sin Nombre virus, but cases of HPS caused by Bayou, Black Creek Canal, Monongahela, and New York viruses have been reported, and cases of domestically acquired hemorrhagic fever and renal syndrome caused by Seoul virus have also occurred. Rarely, hantavirus infections result in mild illness that does not progress to HPS. Continued testing and surveillance of clinical cases in humans will improve our understanding of the etiologic agents involved and the spectrum of diseases.

In 1993, an outbreak of severe respiratory illness in the Four Corners region of the United States (defined by the shared borders between the states of New Mexico, Arizona, Colorado, and Utah) made national headlines. The subsequent discovery of a new disease, hantavirus pulmonary syndrome (HPS) (*1*), its etiologic agent, Sin Nombre virus (SNV) (*2*), and its rodent reservoir, the deer mouse (*Peromyscus maniculatus*) (*3*), were among the most prominent findings in a flood of new revelations about hantaviruses in the Americas. Reliable and rapid diagnostic tests coupled with national surveillance created conditions whereby patients were tested, relevant information was gathered regarding rodent exposures, and potential rodent virus hosts were caught and tested. Within a few years, 4 additional disease-associated hantaviruses indigenous to the United States were described: Bayou, Black Creek Canal, New York, and Monongahela viruses. We now understand that several hantaviruses are endemic to North America, and infections in humans continue to occur where humans come into contact with infected rodents. Through a review of literature and data from the hantavirus surveillance registry of the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, we have summarized the spectrum and distribution of human hantavirus infections in the United States through July 9, 2013.

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Hantaviruses (family *Bunyaviridae*, genus *Hantavirus*) are rodent- and insectivore-borne viruses that are distributed in all continents except Antarctica (*4*). It is not known if all hantaviruses cause disease in humans. Humans become infected through direct contact with infected rodents or inhalation of virus that has been shed in rodent excreta, blood, and saliva and then become aerosolized. Old World hantaviruses that are known human pathogens (e.g., Hantaan, Dobrava, Seoul, and Puumala viruses) cause hemorrhagic fever and renal syndrome (HFRS), in which the primary organ affected is the kidney. Symptoms include fever; myalgia; and gastrointestinal, urinary, cerebral, and conjunctival hemorrhage. Acute renal failure with oliguria caused by HFRS often lasts for several days before spontaneously resolving (*5*). All known New World hantaviruses pathogenic to humans, including SNV and Andes virus, cause HPS, in which the primary organ affected is the lungs. Symptoms include a prodrome of fever, myalgia, and gastrointestinal symptoms followed by a rapid onset of pulmonary edema (*1*,*6*,*7*). Because cardiac insufficiency, leading to cardiac failure and death, can be prominent in severe cases, many investigators and clinicians refer to the disease as hantavirus cardiopulmonary syndrome (*8*,*9*). Thrombocytopenia, left shift, and hemoconcentration are typical abnormal laboratory findings (*10*). The only hantavirus species with evidence of human-to-human transmission is Andes virus, which is endemic to South America (*5*). In the United States, ≈90% of hantavirus infections are acquired through household or occupational exposures (*11*), although a 2012 outbreak among Yosemite National Park visitors is a noteworthy example of recreational hantavirus exposure (*12*).

National surveillance for hantavirus infections in the United States began in 1993, and HPS became nationally notifiable in 1995 (*13*). The clinical case definition, as

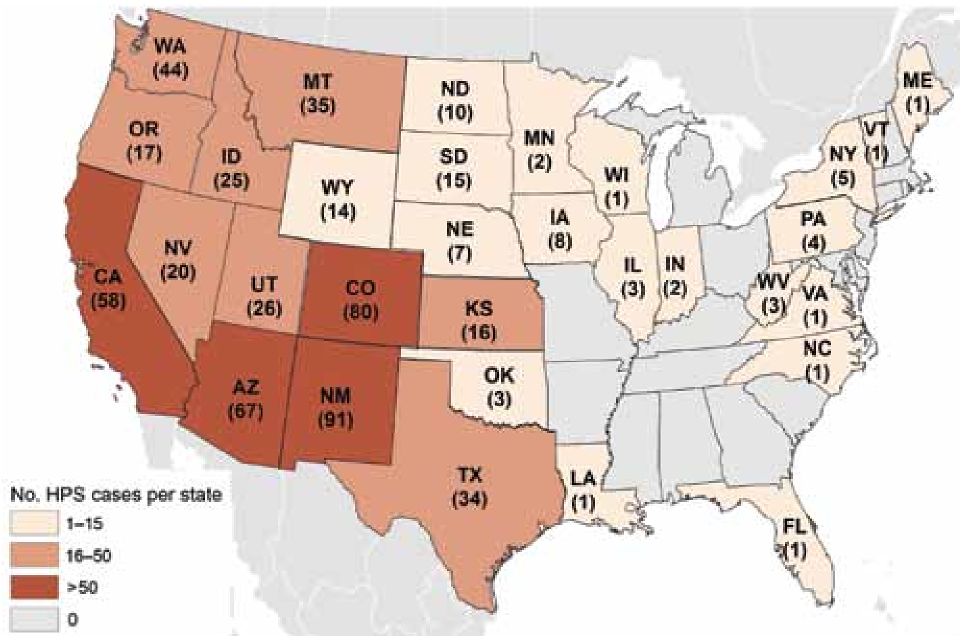

Figure. Hantavirus pulmonary syndrome (HPS) cases by state of exposure, United States, 1993–2013. A total of 624 cases occurred in 34 states; the state of exposure was unknown for another 28 cases. The cumulative case count for each state was current as of July 9, 2013.

approved by the Council of State and Territorial Epidemiologists, includes fever and pulmonary symptoms (bilateral diffuse interstitial edema, clinical diagnosis of acute respiratory distress syndrome, or radiographic evidence of noncardiogenic pulmonary edema) or unexplained respiratory illness resulting in death and an autopsy examination demonstrating noncardiogenic pulmonary edema without identifiable cause (*14*). Clinically compatible HPS cases are confirmed by laboratory testing (serologic analysis, PCR, or immunohistochemical analysis) with results positive for hantavirus infection. Laboratory confirmation is required for a case to be reported through the Nationally Notifiable Diseases Surveillance System.

As of July 9, 2013, there have been 624 reported cases of HPS in 34 states (Figure), including 31 cases that occurred before 1993 and were retrospectively diagnosed from archived autopsy tissues or convalescent serum samples (*15–17*). Exposure location was determined for 593 cases, and 570 (96%) of these exposures occurred in states west of the Mississippi River. Twelve infections were confirmed by PCR analysis to be caused by hantavirus species other than SNV (Table): 5 infections were caused by Bayou virus, 1 by Black Creek Canal virus, 2 by New York virus, and 4 by Monongahela virus. Because available serologic tests are broadly cross-reactive for all New World hantaviruses (*18*,*19*), virus identification must be performed by PCR with sequencing; this method requires acute specimens to be collected and transported to the laboratory frozen to preserve RNA for analysis. This is not always possible, but attempts to collect specimens suitable for molecular analysis can greatly contribute to our further understanding of the hantaviruses causing disease in the United States.

CDC has recorded a total of 10 laboratory confirmed cases of acute hantavirus infection that did not fit the clinical case definition of HPS, 6 of which clinical case descriptions have been reported previously (*12*,*27*,*28*). Because these patients did not have pulmonary symptoms, they were not included in the national HPS case counts. It is believed that human hantavirus infections infrequently result in mild illness without pulmonary symptoms, an assertion that was strengthened in the wake of the 2012 Yosemite outbreak, where only 2 acute laboratory-confirmed

Table. Summary of laboratory-confirmed hantavirus pulmonary syndrome cases caused by viruses other than Sin Nombre virus, United States, 1993–2007

Virus, reservoir, state	No. cases	Year	References
Bayou, Marsh rice rat (*Oryzomys palustris*)			(*18,20,21*)
Texas	4	1995, 1996, 2001, 2007	
Louisiana	1	1993	
Black Creek Canal, Hispid cotton rat (*Sigmodon hispidus*)			(*19*)
Florida	1	1993	
New York, White-footed mouse (*Peromyscus leucopus*)			(*22,23*)
New York	2	1994, 1995	
Monongahela, Deer mouse (*P. maniculatus*)			(*24–26*)
Pennsylvania	2	1997	
West Virginia	2	2004	

hantavirus infections without pulmonary symptoms were identified, in spite of extensive serologic testing—more than 3,000 samples were tested at 1 commercial laboratory alone (*29*). However, as long as pulmonary symptoms are a required element for reporting of hantavirus cases to CDC (and likely also to state public health authorities), these milder cases of hantavirus infection will continue to go uncounted. This presents a missed opportunity in understanding the full spectrum of hantavirus disease, and reduced awareness of where and how persons are exposed to hantaviruses. In this light, we propose that the clinical case definition should be adjusted so that all laboratory-confirmed hantavirus infections are reportable to health authorities.

Old World hantaviruses have also been found in this country. For example, Seoul virus has been found in Norway rats (*Rattus norvegicus*) and black rats (*Rattus rattus*) in urban areas throughout the United States (*30*,*31*). A series of serosurveys in Baltimore, Maryland, residents demonstrated that humans had serologic evidence of exposure to Seoul virus and identified 3 cases that fit the clinical criteria of acute HFRS (*32*). In 2008, the first domestically acquired Seoul virus infection to be confirmed by PCR was discovered in a Baltimore resident (*33*). An unconfirmed HPS case attributed to domestically acquired Seoul virus infection in Texas in 2010 has also been described (*34*). HFRS should be considered in cases of fever and acute renal failure in persons living or working in environments that may have rats.

Imported cases of hantavirus infection have also occurred in the United States; the first were described in the 1950s when HFRS was detected in 2 military personnel returning from the Korean War (*35*,*36*). More recently, in 2009, HFRS resulting from an imported case of Seoul virus infection developed in a Wisconsin resident shortly after he returned from a visit to China (*37*); in 2010, serologically confirmed HPS resulting from an infection acquired in Brazil developed in a Brazilian visitor to Florida (*38*); and in 2012, HFRS resulting from Puumala virus infection acquired in Germany developed in a German visitor to Florida.

HPS caused by SNV continues to be the predominant form of hantavirus infection in the United States. However, we must continue to consider hantaviruses as a cause of disease in patients with rodent exposures that are outside the western United States, that differ from the usual clinical presentation of pulmonary disease, or that are not associated with deer mouse exposure.

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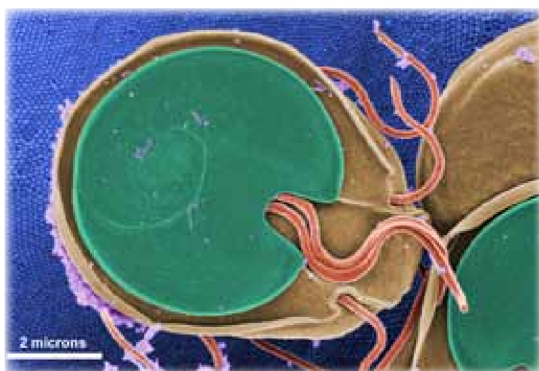

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Spontaneous Generation of Infectious Prion Disease in Transgenic Mice

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We generated transgenic mice expressing bovine cellular prion protein (PrP^C) with a leucine substitution at codon 113 (*113L*). This protein is homologous to human protein with mutation *102L*, and its genetic link with Gerstmann–Sträussler–Scheinker syndrome has been established. This mutation in bovine PrP^C causes a fully penetrant, lethal, spongiform encephalopathy. This genetic disease was transmitted by intracerebral inoculation of brain homogenate from ill mice expressing mutant bovine PrP to mice expressing wild-type bovine PrP, which indicated de novo generation of infectious prions. Our findings demonstrate that a single amino acid change in the PrP^C sequence can induce spontaneous generation of an infectious prion disease that differs from all others identified in hosts expressing the same PrP^C sequence. These observations support the view that a variety of infectious prion strains might spontaneously emerge in hosts displaying random genetic PrP^C mutations.

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative diseases that affect humans and animals and involve pathologic conversion of host cellular prion protein (PrP^C) to a disease-related isoform (PrP^{Sc}), as proposed in the protein-only hypothesis (*1*). Depending on how these encephalopathies originate, TSEs are classified as sporadic, genetic, or infectious. Most have been experimentally transmitted and, with some exceptions, the presence of PrP resistant to proteinase K digestion (PrP^{res}) is related to their infectivity (*2–4*).

Human genetic TSEs are caused by >30 autosomal-dominant point mutations in the human prion protein gene (*Prnp*) and have been classified as Gerstmann–Sträussler–Scheinker syndrome, familial Creutzfeldt–Jakob disease, or fatal familial insomnia (FFI), according to the clinical symptoms. Some of these genetic diseases have been transmitted to primates or rodents, although transmission rates were low in most instances (*5–8*). Regarding TSEs, pathogenic mutations in *Prnp* are believed to predispose mutant PrP^C to convert spontaneously to a pathogenic isoform (*9–11*).

Several transgenic mouse models confirmed that PrP^C with mutations induces a spectrum of neurologic diseases with clinical or histologic features of TSEs (*12–15*). However, the crucial prediction that a disease-associated PrP mutation can spontaneously generate infectivity has only been demonstrated in mice carrying the mutation D177N, the mouse equivalent of the mutation associated with human FFI (*16*). Spontaneous appearance of infectivity has also been reported in transgenic mice expressing a mouse PrP^C with 2 point mutations (170N and 174T) that subtly affect the structure of its globular domain (*17*).

The first described and most common Gerstmann–Sträussler–Scheinker syndrome mutation causing ataxia is P*102L* (*18,19*). Bovine P*113L*, which has a leucine substitution at codon 113, is homologous to human P*102L* and mouse P*101L*. Although bovine PrP^C with the *113L* mutation has not been found in nature, it would be useful to establish whether this mutation could induce spontaneous generation of an infectious prion disease in a bovine PrP context. In this study, we analyzed the phenotype of transgenic mice expressing mutant *113L* bovine prion protein (BoPrP) and the ability of these mice to generate de novo infectious prions in comparison with control mice expressing the wild-type protein.

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Materials and Methods

Ethics

Animal experiments were conducted in strict accordance with recommendations in the guidelines of the Code for Methods and Welfare Considerations in Behavioral Research with Animals (Directive 86/609EC). All efforts were made to minimize detrimental effects on animals. Experiments were approved by the Committee on the Ethics of Animal Experiments of the INIA Institute (permit no. CEEA2009/004).

Transgenic Mice

The open reading frame (ORF) of the bovine *Prnp* gene was isolated by PCR amplification and cloned in a pGEM-T vector as described (*20*). The ORF was mutated by using the QuikChange II-XL Kit (Stratagene, La Jolla, CA, USA) with specific oligonucleotides (5′-CGGTCAGTGGAACAAGCTCAGTAAGCC-GAAAACC-3′ and 5′-GGTTTTCGGCTTACTGAGCTT-GTTCCACTGACCG-3′) according to procedures of the manufacturer. The P*113L*-PrP ORF was excised from the cloning vector by using restriction enzyme *Xho*I and inserted into MoPrP.Xho vector (*21*), which was also digested with *Xho*I. This vector contains the murine PrP promoter and exon-1, intron-1, exon-2, and 3′-untranslated sequences. Transgenic mice were generated by microinjection of DNA according to a published procedure (*22*).

Neuropathologic Studies in Spontaneously Diseased *113L*BoPrP-Tg Mice

Brains were rapidly harvested from the skulls and fixed in 4% paraformaldehyde in phosphate buffer. Coronal slabs were embedded in paraffin and 5-μm sections of cerebrum, cerebellum, brain stem, and spinal cord were obtained by using a sliding microtome. De-waxed sections were stained with hemotoxylin and eosin, Congo red, or thioflavin, or processed for immunohistochemical analysis. Immunohistochemical analysis for detection of glial fibrillary acidic protein (GFAP) and cleaved caspase-3 was conducted by using a modified labeled streptavidin technique (LSAB2-System peroxidase; Dako, Glostrup, Denmark). The rabbit polyclonal antibody to GFAP (Dako) was used at a dilution of 1:600. Cleaved caspase-3 rabbit polyclonal antibody (D175, cell signaling) was used at a dilution of 1:50. Microglial cells were stained with the biotinylated lectin from *Lycopericon esculentum* (L-0651; Sigma, St. Louis, MO, USA) and used at a dilution of 1:100. PrP was immunolabeled with monoclonal antibody (mAb) 6H4 (Prionics, Schlieren, Switzerland) and used at a dilution of 1:30 in sections pretreated with 35% HCl for 2 min at 100°C and then with 96% formic acid for 10 min at room temperature. These procedures have been detailed elsewhere (*23*).

Mouse Transmission Studies

For transmission studies, we used the Tg110 mouse line (*20*) that expresses wild-type bovine-PrP^C in a mouse *Prnp* null background. Inocula were prepared from brain tissues as 10% (wt/vol) homogenates. Individually identified 6–10-week-old mice were anesthetized and inoculated with 2 mg of 10% brain homogenate in the right parietal lobe by using a 25-gauge disposable hypodermic needle. Mice were observed daily and neurologic status was assessed weekly. When progression of TSE was evident or evident at 650 days postinoculation (dpi), animals were euthanized for ethical reasons. Once mice were euthanized, brains were collected, frozen, and analyzed by Western blotting. Samples fixed in buffered 10% formalin underwent histologic analysis, immunohistochemical analysis, or histoblotting. Spleens were frozen for Western blot analysis.

Western Blot Analyses of PrP^{res}

Frozen mouse brain samples were prepared as 10% (wt/vol) homogenates in 5% glucose in distilled water in grinding tubes (Bio-Rad, Hercules, CA, USA) by using a TeSeE Precess 48 homogenizer (Bio-Rad) following the manufacturer's instructions. Samples were analyzed by Western blotting as described (*24*). For immunoblotting experiments, mAbs Sha31 (*25*), 9A2 (*26*), 12B2 (*26*), Saf84 (*25*), and R145 (Vetyerinary Laboratories Agency, Weybridge, UK) were used at concentrations of 1 μg/mL. Sha31 recognizes $_{156}$YEDRYYRE$_{163}$ epitope, 9A2 recognizes $_{110}$WNK$_{112}$ epitope, 12B2 recognizes $_{101}$WGQGG$_{105}$ epitope, Saf84 recognizes $_{175}$RPVDQY$_{180}$ epitope, and R145 recognizes $_{231}$RESQUA$_{235}$ epitope of the bovine PrP sequence.

Histopathologic Analysis

All procedures involving brains from infected mice were performed as described (*27*). Samples were fixed in neutral-buffered 10% formalin (4% formaldehyde) before being embedded in paraffin. Once deparaffinated, 2-μm tissue sections were stained with hematoxylin and eosin. Lesion profiles of brains were established according to the standard method described by Fraser and Dickinson (*28*). For paraffin-embedded tissue blots, the protocol described by Andréoletti et al. (*29*) was used.

Results

Expression of *113L*BoPrP in Transgenic Mice

Seven lines (founders) of *113L*$BoPrP^C$ heterozygous transgenic mice carrying the endogenous murine *Prnp* gene (*Prnp* $mu^{+/-}$ *113L*$bo^{+/-}$) were obtained. Lines *113L*BoPrP-Tg037 and *113L*BoPrP-Tg009 were selected on the basis of their expression levels, and bred to homozygosity in a murine *Prnp* null background. To achieve this expression, selected lines were crossed with *Prnp* null mice (*Prnp* $mu^{-/-}$) to

achieve transgene-hemizygous lines (*Prnp* mu$^{-/-}$ *113L*bo$^{+/-}$). Absence of the murine *Prnp* gene was determined by using PCR with specific primers. Transgene expression levels were then determined in brain homogenates by serial dilution and compared with PrP^{C} levels found in bovine brain homogenates. Transgene expression levels for the two 1-month-old mice with hemizygous Tg lines *113L*BoPrP-Tg037 and *113L*BoPrP-Tg009 were found to be ≈3× and 0.5×, respectively. Mutant *113L*BoPrP expressed in 009 and 037 transgenic lines showed an electrophoretic profile similar to that of wild-type bovine PrP^{C} from BoPrP-Tg110 mice or cow brain, although only small differences in glycoform ratios were observed (Figure 1). Next, by crossing hemizygous animals, we obtained transgene-homozygous animals (*Prnp* mu$^{-/-}$ *113L*bo$^{+/+}$) (*30*).

Spontaneous Neurologic Disease in Transgenic Mice Expressing Mutant *113L*BoPrP

Spontaneous neurologic disease developed in *113L*BoPrP-Tg037 mice expressing mutant *113L*BoPrP. These mice had reduced lifespans compared with either non-Tg (*Prnp*$^{-/-}$) mice or transgenic mice expressing similar or higher levels of wild-type BoPrP (Table 1). However, disease did not develop in *113L*BoPrP-Tg009 mice expressing low levels of mutant protein, and these mice had survival times similar to non-Tg (*Prnp*$^{-/-}$) mice or BoPrP-Tg110 mice. Onset of clinical signs and survival times were dependent on the expression level of *113L*BoPrP (Table 1) (i.e., transgene-homozygous *113L*BoPrP-Tg037 mice showed an earlier onset of clinical signs and reduced survival times than hemizygous mice of the same line). Neurologic alterations generally involved motor impairment with ataxia affecting mainly the hind limbs. Mice showed a rough coat and prominent hunch at the early stages of clinical signs. Most mice had a wobbling gait and slight paralysis in the back limbs. Some mice had conjunctivitis and showed compulsive scratching in the head area. At the end stage of the disease, mice had highly restricted movement and lethargy. No signs of hyperactivity were detected in these mice.

Neuropathologic Alterations in Transgenic Mice Expressing Mutant *113L*BoPrP

All *113L*BoPrP-Tg037 mice at the terminal disease stage showed spongiosis in the cerebral cortex, thalamus, and hilus of the dentate gyrus, but not in the CA1 region of the hippocampus and granule cell layer of the dentate gyrus, compared with age-matched control BoPrP-Tg110 mice (Figure 2). Marked granule cell loss, spongiosis in the molecular layer, granule cell layer, subcortical white matter, and Bergmann glia hypertrophy and hyperplasia were also observed in these mice at the terminal disease stage. However, at the early stages of clinical signs, no spongiform changes were found, although neuronal loss was observed when *113L*BoPrP-Tg037 mice were compared with age-matched control BoPrP-Tg110 mice. These findings were particularly evident in the hippocampus proper (including hilus) and granular cell layer of the cerebellum.

Changes were more pronounced in animals with severe clinical manifestations. Neurons with a shrunken cytoplasm and nucleus were observed in all *113L*BoPrP-Tg037 mice, and these appeared in the molecular layer of the cerebellum, hippocampus (mainly plexiform layers and hilus), thalamus, and pons. Morphologic PrP aggregates, congophilic materials, or thioflavin-positive deposits were not detected in *113L*BoPrP-Tg037 mice. Astrocyte gliosis was observed throughout the brain in *113L*BoPrP-Tg037 mice, even at the early stage of the disease. The number and size of reactive astrocytes increased, as shown by immunolabeling with antibody against GFAP, in the cerebral cortex, hippocampus, striatum, thalamus, cerebellum, and brain stem. Microglial proliferation, as visualized with *Lycopericum esculentum*

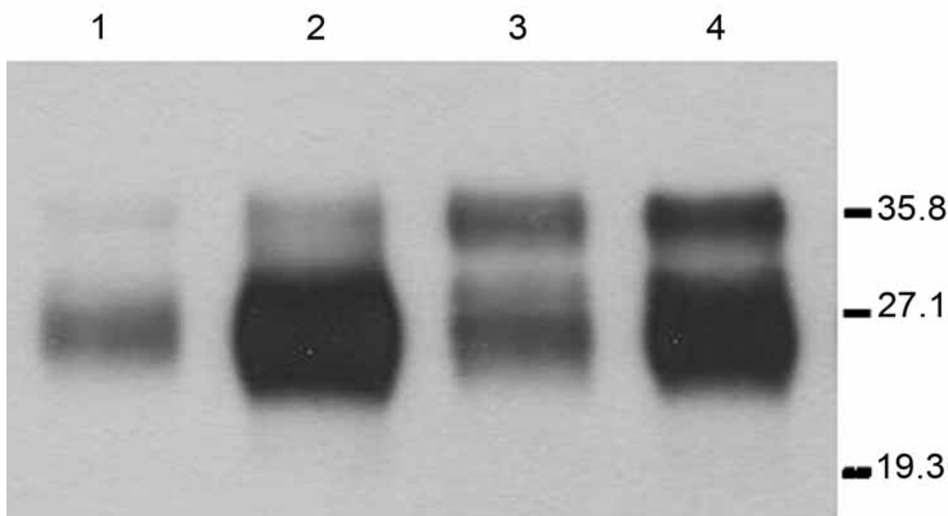

Figure 1. Immunoblots of brain extracts from hemizygous *113L*BoPrP-Tg037$^{+/-}$ *113L*BoPrP-Tg009$^{+/-}$ mouse lines compared with those of cow brain extract and BoPrP-Tg110 mouse brain extract. Brain homogenates were analyzed by Western blotting with monoclonal antibody 2A11 (*30*). Lane 1, *113L*BoPrPTg-009; lane 2, *113L*BoPrPTg-037; lane 3, Cow; lane 4, BoPrPTg-110. Equivalent amounts of total protein were loaded into each lane. *113L*, leucine substitution at codon 113; BoPrP, bovine prion protein. Values on the right are molecular masses in kilodaltons.

Table 1. Onset of clinical signs and survival times for transgenic mice expressing different levels of mutant *113L*BoPrP or wild-type BoPrP*

Transgenic mouse line	Transgene expression level†	Onset of clinical signs, days ± SEM (no. diseased/no. tested)	Death, days ± SEM
*113L*BoPrP-Tg009$^{+/–}$	0.5×	>550 (0/9)	>550
*113L*BoPrP-Tg009$^{+/+}$	1×	>550 (0/10)	>550
*113L*BoPrP-Tg037$^{+/–}$	3×	272 ± 33 (10/10)	345 ± 49
*113L*BoPrP-Tg037$^{+/+}$	6×	187 ± 18 (6/6)	223 ± 47
BoPrP-Tg110$^{+/–}$‡	4×	>550 (0/6)	>550
BoPrP-Tg110$^{+/+}$‡	8×	>550 (0/10)	>550
Non-Tg (*Prnp*$^{–/–}$)	0×	>550 (0/9)	>550

*BoPrP, bovine prion protein; *113L*, leucine substitution at codon 113; $^{+/–}$, hemizygous for bovine prion protein (*Prnp*) gene; $^{+/+}$, homozygous for bovine *Prnp* gene. All transgenic animals were murine *Prnp*$^{–/–}$.
†Relative to cattle PrP expression.
‡This BoPrP transgenic mouse line has been described (*20,22*).

lectin, was evident in brains of *113L*BoPrP-Tg037 mice but not in age-matched control mice.

Modification of Biochemical Properties of Bovine-PrP^C by Mutation *113L*

To explore changes in biochemical properties of mutant *113L*BoPrP, we solubilized brain homogenates from control BoPrP-Tg110, *113L*BoPrP-Tg037, and *113L*BoPrP-Tg009 mouse lines in extraction buffer and ultracentrifuged them at 100,000 × *g* for 1 h. Western blotting of soluble and insoluble fractions indicated differential biochemical properties of mutant *113L*BoPrP compared with wild-type BoPrP (Figure 3); the *113L* mutation resulted in a more insoluble protein. This insolubility was detected in *113L*BoPrP-Tg037 and *113L*BoPrP-Tg009 mouse lines. However, when the *113L*BoPrP-Tg009 mouse line was compared with the other 2 mouse lines, the expression level was lower and insolubility was detectable only when an 8-fold equivalent brain tissue mass was used (Figure 3, panel B) to obtain equivalent PrP signal.

Insolubility was detected early in the lifespan (30 days after birth) of mice (Figure 3, panel C), which suggested that quantification of *113L*BoPrP would reflect a cumulative effect. PK resistance was not found in mutant *113L*BoPrP or in wild-type BoPrP, which were digested at the PK concentration used (Figure 3).

Spontaneous Generation of Infectious Prions by *113L*BoPrP-Tg037 Mice

To test potential infectivity of brains of mutant *113L*BoPrP-Tg037 mice, we intracerebrally inoculated brain homogenates from sick animals into transgenic mice expressing wild-type bovine PrP (BoPrP-Tg110). In the first experiment (Table 2), we used a brain homogenate from a unique terminally sick animal. In this instance, neurologic signs developed in only 1 of 5 (attack rate 20%)

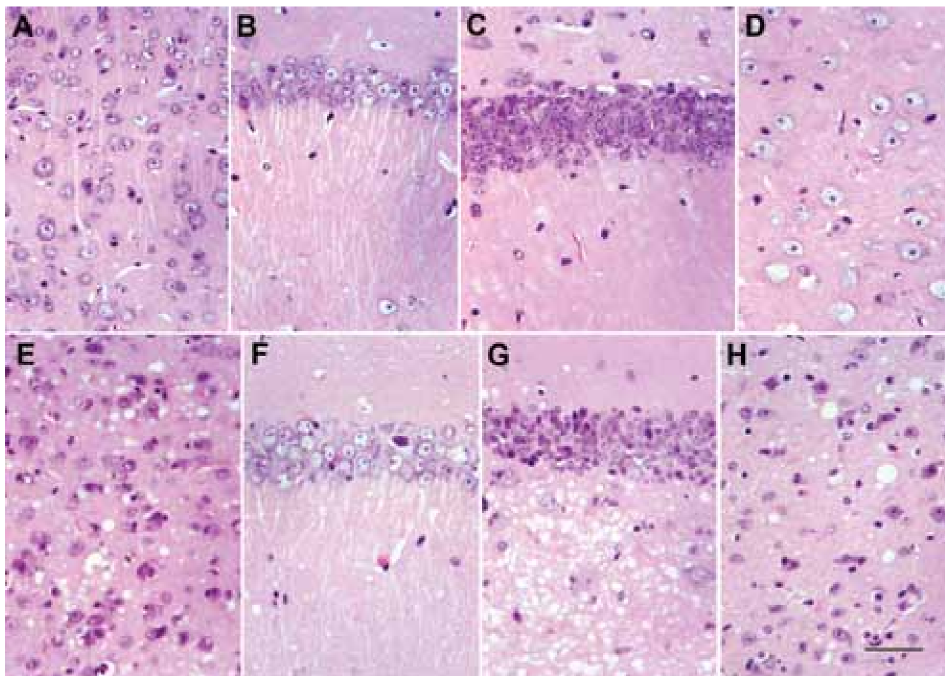

Figure 2. Comparison between homozygous bovine prion protein (BoPrP)-Tg110$^{+/+}$ control mice (panels A–D) and hemizygous *113L*BoPrP-Tg037$^{+/–}$ mice with end-stage disease (panels E–H) in parietal cortex (panels A and E), CA1 region of the hipocampus (panels B and F), dentate gyrus (panels C and G), and medial thalamus (panels D and H). Severe spongiosis is seen in the cerebral cortex, hilus ofdentate gyrus, and medial thalamus, but not in the CA1 area of the hippocampus and granule cell layer of the dentate gyrus. *113L*, leucine substitution at codon 113. Paraffin-embedded sections were stained with hematoxylin and eosin. Scale bar in panel H = 25 μm.

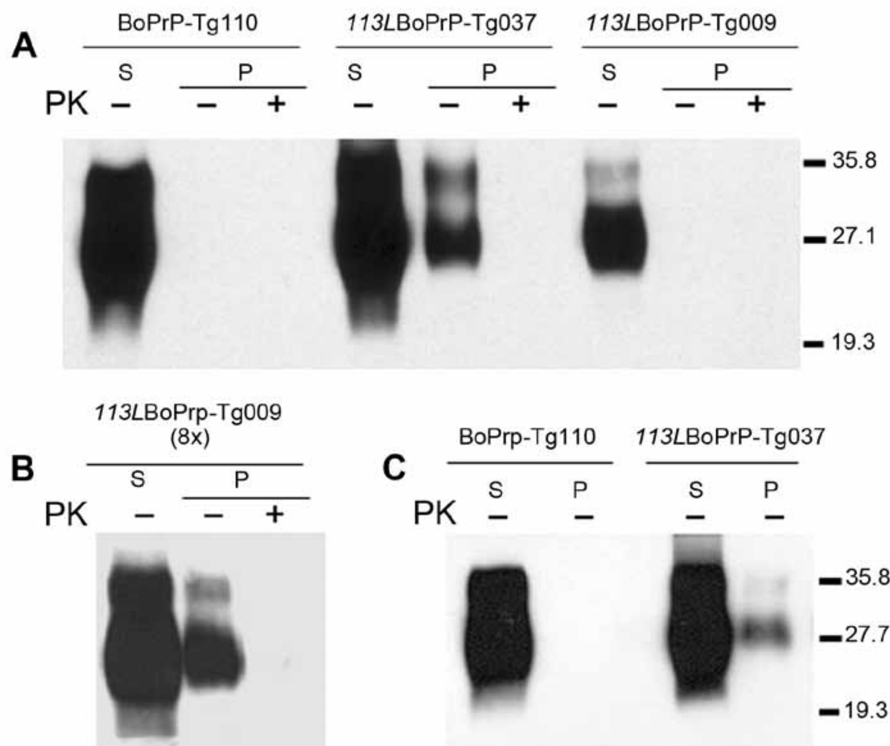

Figure 3. Host cellular prion protein (PrP^C^) solubility and proteinase K (PK) resistance studies in homozygous *113L*BoPrP-Tg037, *113L*BoPrP-Tg009, and control BoPrP-Tg110 mice. Western blot analysis with monoclonal antibody 2A11 of soluble (S) and insoluble (P) fractions obtained from mouse brain extracts (5% sarkosyl in phosphate-buffered saline, pH 7.4, previously cleared by centrifugation at 2,000 × *g*) after ultracentrifugation at 100,000 × *g* for 1 h. P fractions were treated with 5 µg/mL of PK (PK+) at 37°C for 60 min or left untreated (PK–). Panels A and B, show brain extracts from diseased *113L*BoPrP-Tg037 mice or from 500-day-old *113L*BoPrP-Tg009 and BoPrP-Tg110 mice. Panel C shows brain extracts from 30-day-old mice. In panels A and C, equivalent amounts of brain material were solubilized, centrifuged, and loaded onto the gel. In panel B, an 8-fold (8×) equivalent brain tissue mass was loaded to obtain equivalent PrP^C^ signals for the other 2 mouse lines. *113L*, leucine substitution at codon 113; BoPrP, bovine prion protein. Values on the right are molecular masses in kilodaltons.

inoculated *Tg110* mice. This mouse died at 333 dpi and contained a considerable amount of PrP^{res} in its brain, as shown by Western blot (Figure 4).

When brain homogenate from this mouse was reinoculated into 6 *Tg110* mice (second passage), neurologic signs developed in all recipients, and these mice showed a shorter mean ± SEM incubation period (272 ± 38 dpi), which was suggestive of an increased infectious titer that was maintained in subsequent passages (Table 2). These results were confirmed in a second independent experiment with a brain homogenate derived from a pool of 5 terminally sick *113L*BoPrP-Tg037 mice. In this instance, 2 of 6 (attack rate 33%) inoculated mice were infected (*Tg110* mice) (33% attack rate) and had incubation periods of 322 and 406 dpi, respectively. As in the first experiment, second passage (using a pool containing both mouse brains) produced an attack rate of 100% and a shorter incubation period (291 ± 23 dpi), which were maintained in subsequent passages (Table 2).

Brain homogenate from *Tg110* mice expressing comparable amounts of wild-type BoPrP, as well as brain homogenate from healthy mice, was also used to inoculate

Table 2. Infectivity of *113L*BoPrP-Tg037 mice brain homogenates in BoPrP-Tg110*

	Mean ± SEM survival time, days (no. diseased/no. tested)†		
Inoculum	First passage	Second passage	Third passage
*113L*BoPrP-Tg037 (179 d old)‡	330 (1/5)	272 ± 38 (6/6)	252 ± 23 (6/6)
*113L*BoPrP-Tg037-pool (150–250 d old)§	322, 406 (2/6)	291 ± 23 (6/6)	255 ± 14 (7/7)
wtBoPrP-Tg110-pool (500–600 d old)	>650 (0/12)	>650 (0/12)	NA
Normal mouse brain (500–600 d old)	>650 (0/12)	>650 (0/12)	NA
Classical BSE-C (VLAPG817/00) ¶	295 ± 12 (6/6)	265 ± 35 (6/6)	NA
Atypical BSE-H (CASE 03–2095)#	292 ± 5 (6/6)	296 ± 7 (6/6)	NA
Atypical BSE-L (02.2528)**	207 ± 7 (6/6)	199 ± 1 (6/6)	NA

*BoPrP, bovine prion protein; *113L*, leucine substitution at codon 113; wtBoPrP, wild-type bovine prion protein; NA, not available; BSE, bovine spongiform encephalopathy.
†Intracerebral inoculation with 2 mg brain tissue equivalent in BoPrP-$Tg110^{+/+}$ (8× expression level).
‡Brain homogenate from a unique terminally sick animal.
§Brain homogenate from a pool of 5 terminally sick mice that died at 150–250 d of age.
¶Brainstem homogenate of 1 cow naturally infected with classical BSE (RQ 225:PG817/00) supplied by the Veterinary Laboratories Agency (New Haw,
#Data from Torres et al. (*31*).
**Brainstem samples from naturally affected cows given a diagnosis of atypical BSE-L by the Agence Française de Sécurité Sanitaire des Aliments (Lyon, France).

uninfected mice, which served as negative controls. Neurologic signs did not develop in any of the inoculated mice (Table 2). These mice were euthanized at 650 dpi and did not show any PrP^{res} in their brains by Western blot.

For comparative studies, material from the brainstem of cows that contained classical bovine spongiform encephalopathy (BSE), atypical BSE-H, and atypical BSE-L prions was also inoculated into mice by the same procedure. These 3 inocula induced a typical neurologic disease after primary transmission and showed an attack rate of 100% (Table 2). Survivals times of mice inoculated with brainstem of *113L*BoPrP-Tg037 mice on second and third passages were similar to those produced by the classical BSE-C isolate (Table 2), as well as by other isolates reported for the same *Tg110* mouse line (*20,22,32*).

Properties of P*113L*-BSE Prion

Western blot analysis with mAb 9A2 against brain-PrP^{res} produced by BoPrP-Tg110 mice infected with the new *113L*-BSE prion (Figure 4, panel A) showed a typical BSE PrP banding pattern characterized by small fragments (19-kDa fragment for the aglycosyl band) and prominent diglycosylated species in all challenged PrP^{res}-positive mice. This result was indistinguishable from that produced by classical BSE-C prion in these mice but differed from that observed after inoculation with atypical BSE-H or BSE-L prions (Figure 4, panel A).

Further characterization of PrP^{res} with other mAbs showed that the new *113L*-BSE prion was not recognized by mAb 12B2 (Figure 4, panel B), whose epitope ($_{101}$WGQGG$_{105}$ according to the bovine PrP sequence) is known to be poorly protected from PK digestion (*26,32*) in the classical BSE-derived prion but well preserved in the atypical BSE-H prion (Figure 4, panel B) (*31*). Furthermore, PrP^{res} immunolabeling with mAbs Saf84 and R145 showed that mice infected with the new *113L*-BSE prion, in contrast to mice infected with the H-type prion, did not show the characteristic PrP^{res} band profile (4 bands) of cattle BSE-H, but showed a PrP^{res}-profile (3 bands) similar to that of the BSE-C prion (Figure 4, panels C, D).

Comparative study of PrP^{Sc} accumulation in spleen from *Tg110*-infected mice showed that mice infected with *113L*-BSE or BSE-C prions consistently showed positive results for presence of PrP^{res} by Western blot. In contrast, no PrP^{Sc} deposits were detected in mice infected with either BSE-L or BSE-H prions. Similar results were obtained in subsequent passages.

We next examined vacuolation and PrP^{Sc} distribution in the brain, which are known to vary by strains/TSE prions (*28,33*). In general, we observed that PrP^{Sc} deposition patterns in brains of *113L*-BSE–infected mice were different from mice infected with BSE-H or BSE-L prions, but these overlapped mostly with those infected with BSE-C

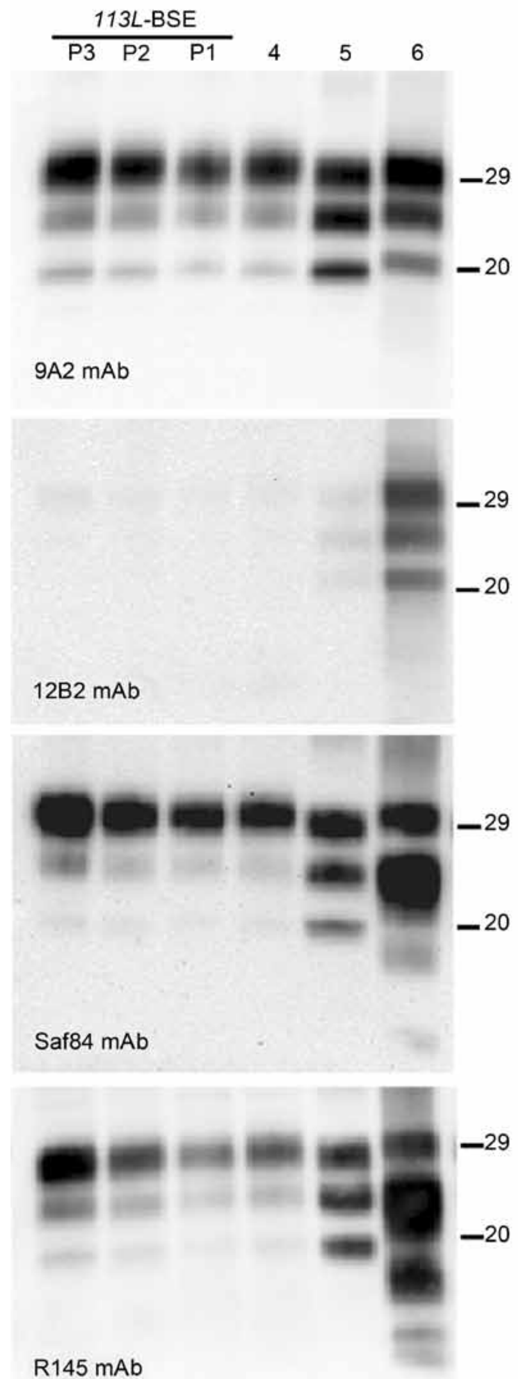

Figure 4. Comparative Western blot analyses of brain prion protein resistant to proteinase K digestion (PrP^{res}) from BoPrP-Tg110 mice infected with bovine spongiform encephalopathy (BSE)-C, *113L*-BSE, BSE-L, and BSE-H prions. Mice infected with newly generated *113L*-BSE prion at first (P1), second (P2), and third (P3) passages are compared with mice infected with BSE-C (P1) (lane 4); BSE-L (P1) (lane 5); and BSE-H (P1) (lane 6) prions. Each panel was identified by using the monoclonal antibody (mAb) listed at the bottom left. The same quantities of PrP^{res} were loaded in all lanes. BoPrP, bovine prion protein; *113L*, leucine substitution at codon 113. Values on the right are molecular masses in kilodaltons.

prion (Figure 5). However, *113L*-BSE–infected mice at the terminal stage of disease showed only spongiform changes that remained limited to the thalamus even after 3 passages (Figure 6). This finding was in contrast with lesion profiles observed in the mice infected with BSE-C, BSE-H or BSE-L prions (Figure 6) in which substantial vacuolar changes were observed in various brain areas. These results indicate that *113L*-BSE is an authentic infectious prion that phenotypically differs from BSE-H and BSE-L prions but has biochemical characteristic and histopathologic features similar to those of the classical BSE-C prion.

Discussion

We showed that the *113L* mutation in the bovine *Prnp* gene gives rise to a spontaneous neurodegenerative disease when expressed in transgenic mice. Neurologic symptoms of ataxia, rigidity, and lethargy accompanied by spongiform degeneration throughout the brain spontaneously develop in these mice. The rate at which illness progresses is related to expression levels of the mutant *113L*BoPrP (Table 1). Neurologic alterations did not develop in several mouse lines expressing similar or higher levels of wild-type bovine PrP^C during their lifespan, which is similar to observations in wild-type mice (Table 1) (*20*). Although the mechanism inducing the disease is unclear, we suggest that the *113L* mutation in bovine PrP^C could give rise to a different structure with respect to wild-type PrP^C, which shows reduced solubility (Figure 3). Enhanced aggregation of mutant PrP could affect the appearance of the disease. Other mutations in the *Prnp* gene have also been related to enhanced aggregation of the mutant PrP in transgenic mice (*12–14,34*). However, the mechanisms through which these mutations may influence the aggregation properties of PrP^C are unclear.

In previous studies, overexpression of murine PrP carrying the *101L* mutation (equivalent to human *102L* and bovine *113L* mutations) led to spontaneous neurodegenerative disease in mice (*15,35,36*). However, when this mutation was introduced into the murine *Prnp* gene by gene targeting, mice homozygous for the *101*L mutation showed no spontaneous spongiform encephalopathy (*37*). As proposed by Manson et al., the lifespan of a mouse carrying only 1 or 2 copies of the mutant gene is insufficiently long enough to enable the stochastic event that makes TSE occur (*37*). Transgenic mice expressing high levels of human PrP^C carrying the familial *101L* mutation were reported to be free of disease (*38*). These results suggest that an equivalent mutation in PrPs from different species might have different structural consequences. A possible explanation is that species-specific interaction sites for PrP cofactors or chaperones are required, and that in mice they are compatible for bovine PrP but not for human PrP.

We also show that spontaneous neurodegenerative disease induced by the single *113L* amino acid substitution is transmissible to mice expressing wild-type bovine PrP^C, indicating spontaneous generation of infectious prions. Transmissibility of this genetically initiated disease to mice not carrying *113L* mutations provides crucial support for a causal link between PrP misfolding and the spontaneous generation of a transmissible prion. Whether the small amount of insoluble PrP we detected in brain homogenates (Figure 3) constitutes the infectious prion in our mice, or some other as yet uncharacterized species, remains to be determined.

Several transgenic mouse models expressing PrP with various familial mutations have been reported (*12–14,34*). Most of these transgenic mouse models have confirmed that the presence of these mutations triggers spontaneous disease, but spontaneous generation of a transmissible prion

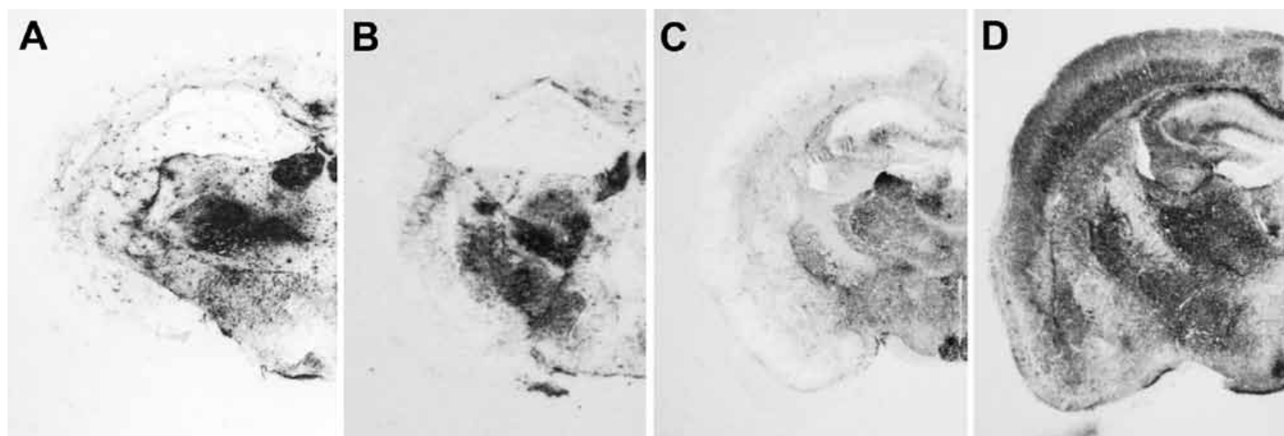

Figure 5. Immunochemical analysis of paraffin-embedded tissue blots of representative coronal sections of the hippocampus, showing deposition patterns of abnormal isoform of host-encoded prion protein in brains from BoPrP-Tg110 mice infected with bovine spongiform encephalopathy (BSE)-C (A), *113L*-BSE (B), BSE-H (C), and BSE-L (D) prions. BoPrP, bovine prion protein; *113L*, leucine substitution at codon 113. Monoclonal antibody Sha31 stained by using the procedure of Andréoletti et al. (*29*). Original magnifications ×20.

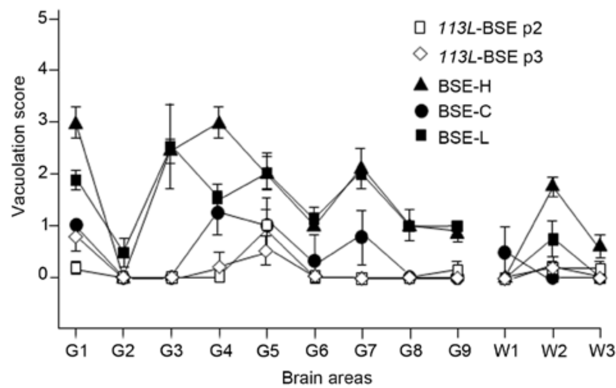

Figure 6. Vacuolar lesion profile in brains from BoPrP-Tg110 mice inoculated with bovine spongiform encephalopathy (BSE)-C (black circles, n = 6 animals), BSE-H (black triangles, n = 6 animals), BSE-L (black squares, n = 5 animals), *113L*-BSE second passage (black squares, n = 5 animals), and *113L*-BSE third passage (open diamonds, n = 5 animals) prions. Lesion scoring was conducted for 9 areas of gray matter (G) and 3 areas of white matter (W) in mouse brains. G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, medial thalamus; G6, hippocampus; G7, septum; G8, medial cerebral cortex at the level of the thalamus; G9, medial cerebral cortex at the level of the septum (G9); W1, cerebellum; W2, mesencephalic tegmentum; W3, pyramidel tract. BoPrP, bovine prion protein; *113L*, leucine substitution at codon 113. Error bars indicate SE.

has only been reported for mutation D178N, associated with human FFI (*16*). This study reported the spontaneous appearance of infectivity in knock-in mice carrying the mouse-equivalent D177N mutation. Spontaneous infectivity has also been reported in transgenic mice expressing a mouse PrP with 2 point mutations (170N and 174T), which subtly affect the structure of its globular domain (*17*).

The new *113L* BSE prion generated shares some phenotypic features with the classical BSE-C prion when inoculated in the same *Tg110* mouse line according to various criteria: 1) apparent molecular mass of PrP^{res}, 2) PrP^{res} glycosylation pattern, 3) lack of immunoreactivity with mAb 12B2, 4) pattern of labeling with mAbs Saf84 and R145, 5) detectable PrP^{res} in spleens of infected animals, and 6) spatial distribution of PrP^{res} in brain. However, the vacuolation profile in brain was distinct from those of all known bovine prion strains (classical BSE-C, atypical BSE-H, and atypical BSE-L prions). These differences were maintained after subsequent passages, indicating that the novel prion, spontaneously produced by transgenic mice expressing mutant *113L*BoPrP, is distinct from all known bovine prion strains, although it shares many phenotypic features with the classical BSE-C prion.

These observations demonstrate that mutations in bovine PrP can result in spontaneous generation of infectious prion diseases and support the hypothesis of a genetic origin for the epidemic BSE prion. Different features exhibited by the new *113L*-BSE prion, compared with those of the classical BSE prion, suggest that if the origin of BSE was genetic, it is unlikely that the causal mutation would be related to the *113L* mutation. However, slight phenotypic differences observed could be the results of evolution of the epidemic BSE prion in field conditions in cattle, which must be different from those of our transgenic mouse model. Although BoPrP with the *113L* mutation has not been found in nature, a potential pathogenic mutation (E211K) within PrP has been recently reported in a cow with an H-type BSE phenotype (*39*). This mutation is equivalent to a common mutation (E200K) in humans, which is associated with genetic TSEs.

Spontaneous appearance of infectivity reported in transgenic mice expressing a mutated BoPrP and in mice expressing mutated mouse PrP reported by Stöhr et al. (*40*) supports the hypothesis that infectious TSE prions, could originate by a random genetic mutation that can induce de novo generation of infectious prions, and that this mechanism could constitute a source of prion diversity. These considerations enable us to hypothesize that the BSE epidemic could have begun by a random genetic mutation that was able to generate de novo infectious prions, which were included in meat and bone meal fed to cattle and then broadly expanded in the cattle population. According to this hypothesis, a key strategy for controlling BSE would involve preventing cows from consuming products from cows with spontaneous cases of BSE.

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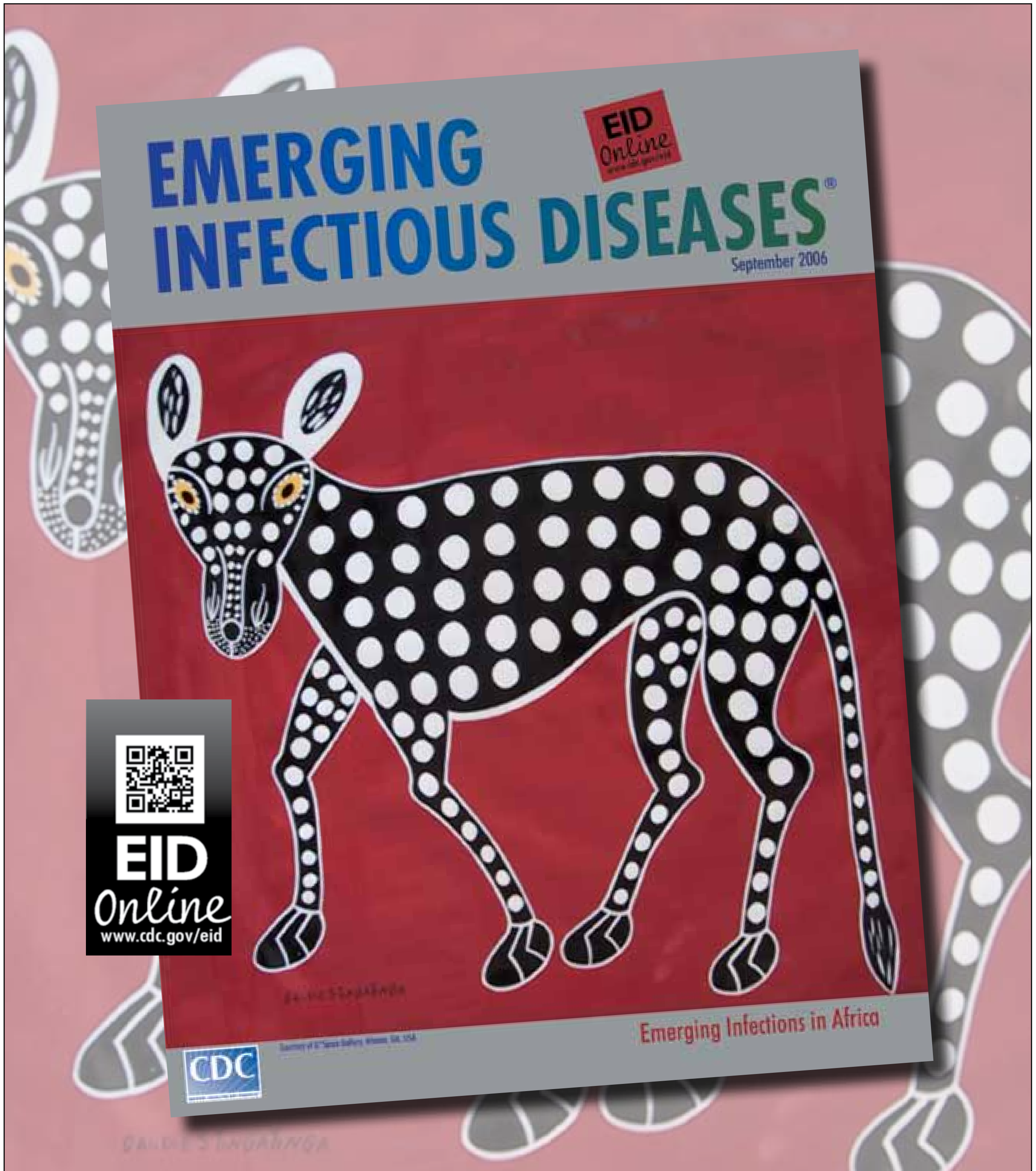

Zoonotic *Chlamydiaceae* Species Associated with Trachoma, Nepal

Deborah Dean, James Rothschild, Anke Ruettger, Ram Prasad Kandel, and Konrad Sachse

Trachoma is the leading cause of preventable blindness. Commercial assays do not discriminate among all *Chlamydiaceae* species that might be involved in trachoma. We investigated whether a commercial Micro-ArrayTube could discriminate *Chlamydiaceae* species in DNA extracted directly from conjunctival samples from 101 trachoma patients in Nepal. To evaluate organism viability, we extracted RNA, reverse transcribed it, and subjected it to quantitative real-time PCR. We found that 71 (70.3%) villagers were infected. ArrayTube sensitivity was 91.7% and specificity was 100% compared with that of real-time PCR. Concordance between genotypes detected by microarray and *ompA* genotyping was 100%. Species distribution included 54 (76%) single infections with *Chlamydia trachomatis*, *C. psittaci*, *C. suis,* or *C. pecorum,* and 17 (24%) mixed infections that includied *C. pneumoniae.* Ocular infections were caused by 5 *Chlamydiaceae* species. Additional studies of trachoma pathogenesis involving *Chlamydiaceae* species other than *C. trachomatis* and their zoonotic origins are needed.

Trachoma was first recognized as an ocular disease in the 27th century BC in China (*1*). Subsequent reports documented the disease among the Egyptians and Greeks in the 19th and 1st centuries BC, respectively. The word trachoma derives from the Greek word for rough swelling, referring to the follicles that appear on the tarsal conjunctiva. Epidemic trachoma was spread from the Middle East to Europe during the Crusades and was a major cause of blindness during the Napoleonic era (*1*). The disease was eliminated from most industrialized countries after the industrial revolution, which heralded the institution of improved sanitation, hygiene, and nutrition. Currently, trachoma prevalence is hypoendemic, mesoendemic, and hyperendemic among populations residing in tropical developing countries.

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During the past few decades, rates of trachoma have increased; in response, at the end of the 1990s, the World Health Organization developed the SAFE program with the goal of eliminating blinding trachoma by the year 2020. SAFE refers to Surgery, Antibiotics, Facial cleanliness, and Environmental improvements, specifically, surgery to correct trichiasis (in-turned eyelashes), oral antimicrobial drugs to treat *Chlamydia trachomatis* infections, facial cleanliness to decrease ocular infections, and environmental improvements such as latrines and wells to provide clean water. Unfortunately, most efforts have focused on the surgery and antimicrobial drug components and had disappointing results. Trichiasis often recurs months to years after surgery for 25%–75% of patients (*2*,*3*) and can be a result of reinfection (*3*). Infection often returns to pretreatment levels 6–24 months after termination of treatment (*4*,*5*). The recurrence of infection and disease is probably multifactorial. There is evidence that oral treatment of *C. trachomatis* infection blunts the immune response, increasing the patient's susceptibility to reinfection (*4*). Furthermore, additional species of *Chlamydiaceae*, namely *Chlamydia pneumoniae* and *C. psittaci*, have been implicated in trachomatous disease by our group (*6*) and by another independent research group from Paris working in Guinea, Africa (*7*). To eliminate infections with species other than *C. trachomatis,* longer treatment intervals might be required (*8*).

Although some *Chlamydiaceae* screening tests and strain-typing methods exist, they are expensive, are time-consuming, require trained personnel, and are available only in specialized laboratories; most do not discriminate among species of *Chlamydiaceae*. The tests or methods include serotyping of the major outer membrane protein by using monoclonal or polyclonal antibodies that are species or genus specific; commercial nucleic acid amplification tests for *C. trachomatis* only (*9*); conventional species-specific and genus-specific PCRs (*10*); direct sequence analysis of *ompA*, 16S rRNS, or 23S rRNA genes (*11*,*12*); multilocus sequence typing for *C. trachomatis (13, 14)* and other species (*14*); real-time (RT)-PCR (*6*,*15*), multilocus variable number tandem repeat analysis (*16*); and

the commercial micro ArrayTube or ArrayStrip (Alere Technologies, Jena, Germany) (*17*). Serotyping requires a cultured isolate, and techniques that involve sequencing might not be able to detect mixed-strain or mixed-species infections unless multiple strain–specific or species-specific primers are used, which require sufficient quantities of DNA. The advantage of the ArrayTube or ArrayStrip is that minimal DNA is required for amplification, and the hybridization patterns indicate species-specific nucleotide polymorphisms in regions of high sequence similarity.

The commercial ArrayTube assay has been successfully used to identify mixed infections among animals infected with multiple species of *Chlamydiaceae (18,19)*. Because of these benefits, we investigated whether the ArrayTube could discriminate among *Chlamydiaceae* species in DNA that was extracted directly from conjunctival samples from trachoma patients residing in a trachoma-endemic region of Nepal. We also evaluated the correlation of the ArrayTube test with *ompA* genotypes. As an independent test for viability of *Chlamydiaceae* organisms, RNA was isolated from the same samples and tested by quantitative RT-PCR (qRT-PCR).

Methods

Study Population and Samples

We used a table of random numbers to randomly select 101 villagers, 1–65 years of age, who had follicular trachomatous inflammation and/or intense trachomatous inflammation and who resided in a trachoma-endemic region of the Lumbini Zone of southwestern Nepal. Patients were enrolled after they provided informed consent. For trachoma grading, we used the modified World Health Organization scale. Upper tarsal conjunctival samples were obtained by using dacron swabs (Hycor Biomedical, Portland, ME, USA), which were immediately placed in M4 transport media (Remel, Lenexa, KS, USA) and stored in liquid nitrogen as described (*6*). To avoid contamination, study personnel changed gloves between participants.

Ethics Statement

The study was approved by institutional review boards of the Nepal Netra Jhoti Shang (Kathmandu, Nepal) and the Children's Hospital Oakland Research Institute (Oakland, CA, USA). Informed consent was obtained from each study participant. Oral consent was approved by both institutional review boards because of the high rate of illiteracy among the population. Consent was documented on the form by the team member who obtained the consent; the team member obtaining the consent signed the form stating that consent had been obtained. Since some study participants were minors, parents provided consent for their child to participate.

RNA and DNA Purification

Genomic DNA was extracted from the conjunctival swab samples by using the Roche High Pure kit (Roche, Pleasanton, CA, USA), and RNA was extracted by using the RNeasy kit (QIAGEN, Valencia, CA, USA) as described (*6*,*20*). RNA was reverse transcribed to cDNA by using a TaqMan reverse transcription kit (Applied Biosystems, Foster City, CA, USA) as described (*6*,*20*). DNA and RNA were stored at -80°C until use.

DNA Microarray Assay

To examine samples for the presence of any of the 9 *Chlamydiaceae* species and *Waddlia chondrophila* and *Simkania negevensis*, we performed the ArrayTube assay as described (*17*,*19*). Briefly, DNA from each sample was amplified and biotin labeled in 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s by using primers U23F-19 and 23R-22 (Table 1). Hybridization was conducted in the ArrayTube vessel at 58°C for 1 h. After 3 wash steps, hybridization signals were visualized by using streptavidin-conjugated peroxidase-catalyzed precipitation. The resulting patterns were processed by using the ATR-01 ArrayTube reader (Alere Technologies) and the Iconoclust 2.3 program (Alere).

Genus-Specific and Species-Specific qRT-PCR and *ompA* Genotyping

qRT-PCR was conducted by using genus-specific and species-specific primers (Table 1) along with appropriate controls including β-actin as described (*6*,*20*). Briefly, each reaction contained 1X SYBR Green PCR MasterMix (Applied Biosystems), 300 nmol/L of each primer, and 5 μL of sample DNA in a volume of 25 μL in duplicate in a 96-well plate. The thermocycling consisted of 10 min at 94°C followed by 40 cycles of 15 s at 94°C and 1 min at 60°C. Samples that were positive by qRT-PCR were subjected to PCR with 16S rRNA genus-specific primers in addition to species-specific *ompA* primers (Table 1). The PCR reagents, controls, thermocycling, and sequencing by BigDye Terminator (Applied Biosystems) automated capillary sequencing were used or performed as described (*6*,*20*). In addition, *ompA* genotyping of samples showing monoinfection with *C. trachomatis* was conducted by using the ArrayStrip microarray assay as described (*21*).

Data Analysis

Outcome variables included single or mixed infection, *Chlamydiaceae* species causing the infection, and *ompA* genotype. The association between discrete variables was analyzed by using the Fisher exact test or the Pearson χ^2 test by Stata 10 (College Station, TX, USA). A p value of <0.05 was used as the cutoff for determining statistical significance.

Table 1. Oligonucleotide primers used for the ArrayTube, quantitative real-time –PCR, and PCR for subsequent sequencing

Gene	Primers	Primer sequence, 5′→ 3′	Gene location	Base pair	Reference
23S rRNA*	U23F-19	ATTGAMAGGCGAWGAAGGA			(*17*)
	23R-22	biotin-GCYTACTAAGATGTTTCAGTTC			
Chlamydiaceae 16S rRNA†	16SrRNA-9	GCGAAGGCGCTTTTCTAATTTAT	734–756‡	76	(*6*)
	16SrRNA-10	CCAGGGTATCTAATCCTGTTTGCT	809–786‡		
Chlamydia trachomatis ompA†	OmpA-9	TGCCGCTTTGAGTTCTGCTT	33–52§	75	(*6*)
	OmpA-10	GTCGATCATAAGGCTTGGTTCAG	108–86§		
C. pneumoniae ompA†	Cpn ompAF1	ATAGACCTAACCCGGCCTACAATAAG	301–330	108	(*6*)
	Cpn ompAR1	GTGAACCACTCTGCATCGTGTAA	353–333	53	
C. psittaci ompA†	CpsF	GCAACTCCTACGGAGTCTTAA	260–279	93	(*6*)
	CpsB	GGCATCTTGTAAATGTTTCCCTAT	331–354		
C. pecorum ompA†	Cp-F	GTTTTCGACAGAGTCCTCAA	208–227	118	This study
	CpRT-R	ATTCTAATTTGCTCTTCTGG	325–305		
C. abortus ompA†	CpaOMP1-F	GCAACTGACACTAAGTCGGCTACA	763–786	82	(*15*)
	CpaOMP1-R	ACAAGCATGTTCAATCGATAAGAGA	845–821		
C. suis ompA†	Cs-F	GGAGATTATGTTTTCGATCGC	195–216	122	This study
	Cs-R	TAAGCTGCATTACTCGTTGTTTCA	338–292		
β-actin†	β-actin-3	GGTGCATCTCTGCCTTACAGATC	412–434¶	73	(*6*)
	β-actin-4	ACAGCCTGGATAGCAACGTACAT	52–30#		
*C. trachomatis ompA***	ompAF-1	GTGCCGCCAGAAAAAGAT	60–40§	1542	(*6*)
	ompAR-2	CCAGAAACACGGATAGTGTTATTA	55–31††		
*C. pneumoniae ompA***	CPF1	TTACAAGCCTTGCCTGTAGGGA	70–91‡‡	1098	(*8*)
	CPB4	AGAATCTGGACTGACCAGATACGTGAG	1169–1142‡‡		
*C. psittaci ompA***	Cps-1	GTATTAAAAGTTGATGTGAATAA	217–239§§	1022	(*8*)
	Cps-B4	TTGATTAAGCGTGCTTCACCAGTGATT	1169–1143§§		
*C. suis ompA***	Cs-F	GGAGATTATGTTTTCGATCGC	195–216	959	This study
	Cs-R	TAGAATCTGAATTGAGCGTTTACGTGA	1154–1128		
*C. pecorum ompA***	Cp-F	GTTTTCGACAGAGTCCTCAA	208–227	966	This study
	Cp-R	GAATCTGAACTGACCAGATACGTGAG	1173–1148		
16Sr RNA**	16SrRNA-F	CAGTCGAGAATCTTTCGCAAT	362–382c	904	(*6*)
	16SrRNA-R	TACTGCCCATTGTAGCACGTGTGT	1265–1232c		

*Primers used in ArrayTube (Alere Technologies, Jena, Germany).
†Primer pairs used for real-time PCR of *ompA* DNA and of cDNA from RNA for *16SrRNA* for detecting *Chlamydiaceae.*
‡Primer location based on reference strain L_2/434 *16SrRNA* sequence.
§Primer location based on reference strain L_2/434 *ompA* sequence.
¶Primer location based on position within intron 3 of the human β-*actin* sequence.
#Primer location based on position within exon 3 of the human β-*actin* sequence.
**Primer pairs used for PCR.
‡‡Primer location based on intergenic region of reference strain L_2/434 downstream of *ompA* sequence.
‡‡Primer location based on *C. pneumoniae* strain TW183 *ompA.*
§§Primer location based on *C. psittaci* avian type C strain *ompA.*

Results

The distribution of villagers by age and sex is shown in Table 2; none lived in the same household. The rate of mixed infections was significantly higher for female than for male participants (p = 0.0011), although the overall rates of infection did not differ. Single or mixed infections did not differ by age group, although the rate of single and mixed infections among those >10 years of age was significantly higher (p = 0.0472). The mean age was 26 years for female and 28 years for male participants.

For purposes of cross-comparison, the ArrayTube and qRT-PCR analyses were run independently. For the 101 samples, the ArrayTube had a sensitivity of 91.7% and a specificity of 100% compared with sensitivity and specificity of qRT-PCR (online Technical Appendix Table, wwwnc.cdc.gov/EID/article/19/12/13-0656-Techapp1.pdf). None of the samples yielded positive results for *C. abortus* by ArrayTube or qRT-PCR. Six samples were not detected by the ArrayTube but were *C. trachomatis* positive by qRT-PCR. All samples positive by qRT-PCR were

Table 2. Correlation of sex and age with single and mixed *Chlamydiaceae* species infections

Patient variable	Total infected, no. (%); n = 71	p value	Single infections, no. (%); n = 54	p value	Mixed infections, no. (%); n = 17	p value
Sex						
M (n = 33)	22 (66.7)	0.6448	21 (63.6)	0.2025	1 (3.0)	0.0098
F (n = 68)	49 (75.1)		33 (48.5)		16 (23.5)	
Age, y						
1–10 (n = 44)	26 (59.1)	0.0472	19 (43.2)	0.0748	7 (15.9)	1.000
>10 (n = 57)	45 (78.9)		35 (61.4)		10 (17.5)	

genotyped; the *ompA* genotypes matched 100% of those identified by ArrayTube.

Of the 101 participating villagers, 71 (70.3%) were infected (Figure 1); 26 (37%) of the infections involved a single or mixed infection with a species other than *C. trachomatis* or in combination with *C. trachomatis*. The 54 (76%) single infections were 48 (88.9%) *C. trachomatis,* 2 (3.7%) *C. psittaci*, 2 (3.7%) *C. suis,* and 2 (3.7%) *C. pecorum.* Most infections were caused by *C. trachomatis* strains C, C1, and C3, although urogenital strains B, D, E, F, and L_2 were also represented. The 17 (24%) mixed infections were 15 (88.2%) *C. trachomatis* plus another species and 2 (11.8%) other species. Specifically, they were 7 (41.2%) *C. trachomatis* plus *C. psittaci*, 5 (29.4%) *C. trachomatis* plus *C. suis*, 2 (11.7%) *C. psittaci* plus *C. suis*, 1 (5.9%) *C. trachomatis* plus *C. pneumoniae*, 1 (5.9%) *C. trachomatis* plus *C. pecorum*, and 1 (5.9%) *C. psittaci* plus *C. suis* and *C. trachomatis.* There were no statistically significant differences by patient age or sex for infections caused by *C. trachomatis* or other species. Figure 2, panel A, shows the ArrayTube assay for the sample from the patient infected with the latter 3 species. Figure 2, panel B, shows an example of microarray-based *omp*A genotyping.

Discussion

Although *C. trachomatis* is well established as a cause of trachoma (*1*), the high percentage of infections with other species (37%) found in this study suggests a role for these pathogens in trachoma pathogenesis. In addition, the use of 2 independent methods to detect *Chlamydiaceae*, one of which used RNA to demonstrate the presence of viable *Chlamydiaceae* species in trachomatous eyes, lends further support to this theory. Our findings also support 2 previous studies; 1 conducted by our group and 1 conducted by an independent research group in Paris. Our group detected *C. trachomatis*, *C. psittaci*, and *C. pneumoniae* DNA and RNA among Nepali villagers in all age groups (*6*). Just under 60% of the cases were caused by single or mixed infections with species other than *C. trachomatis*. In addition, infection with each species was significantly associated with antibodies to chlamydial heat shock protein 60, a known virulence factor for the organism associated with inflammation and trachomatous scarring. The Paris group conducted their study in a trachoma-endemic region of Guinea, Africa, and detected *C. trachomatis*, *C. psittaci*, and *C. pneumoniae* among children 1–10 years of age by using RT-PCR that targeted a conserved sequence of 16S rRNA (*7*).

These findings are not surprising for several reasons. Historically, swab samples from persons in a trachoma-endemic area were cultured and then serologically typed by using microimmunofluorescence, a technique that requires technical expertise, and only 1 strain each of *C. pneumoniae* and *C. psittaci* was included. However, cross-reactivity among species has been observed *(22)*. Immunoreactivity to other species, therefore, might not be considered as evidence for infection with these pathogens. However, in a study in Sudan where trachoma is hyperendemic, immunoreactivity to all 3 species was found among 3% of persons with clinical trachoma *(23)*. Their findings suggest either cross-reactivity or infection with these species. Currently, conjunctival swab samples from patients in trachoma-endemic areas are usually only tested by *C. trachomatis*–specific commercial nucleic acid amplification tests or by *C. trachomatis*–specific in-house PCR. Although a pan-Chlamydiales PCR is available, it might not detect mixed infections because an additional cloning step is required *(24)*. Furthermore, this test has not been applied to samples from persons in a trachoma-endemic area. Consequently, unless investigators think to use specific tests to look for other species, they will not be detected.

Our study identified ocular infections with 5 *Chlamydiaceae* species among trachoma patients. Over the past 5 years, improved technology has enabled increasing identification of single and mixed infections with *Chlamydiaceae* species among mammals and birds (*10*,*19*,*25*). Co-infections with *C. psittaci* and *C. abortus* have been found in cow milk, and co-infections with *C. abortus* and *C. pecorum* have been identified in conjunctival and nasal swab specimens from calves (*19*). Mixed infections among bovine abortion cases have included *C. abortus* and *C. suis (25)*. When cloacal swab samples and fecal samples from pigeons were tested, mixed infections with *C. psittaci,* combined with *C. pecorum*, *C. abortus,* or *C. trachomatis,* along with unclassified *Chlamydiaceae* spp., were discovered (*18*). Most studies used the commercial ArrayTube or ArrayStrip.

Prevalence of ocular infections with *Chlamydiaceae* species in different mammals is high, which supports the

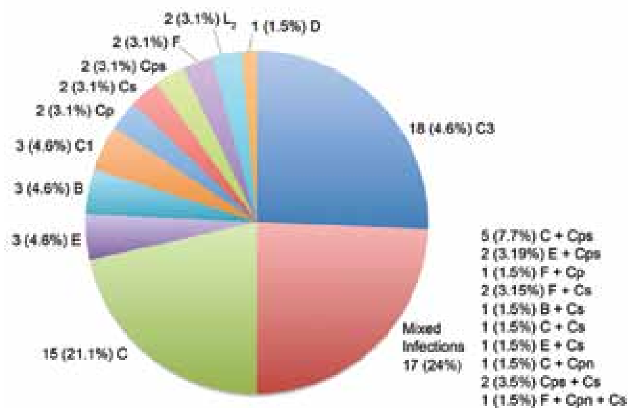

Figure 1. *Chlamydiaceae* infections among 101 villagers residing in a trachoma-endemic region of southwestern Nepal identified by the ArrayTube (Alere Technologies, Jena, Germany), real-time PCR, and *ompA* genotyping. The number and percentage for each infection are shown. Single infections included each species and the designated *ompA* genotypes (n = 71). *C. trachomatis* (Ct) trachoma strain C predominated, but single infections with *C. psittaci* (Cps), *C. pecorum* (Cp), and *C. suis* (Cs) also occurred). Mixed infections included those with Ct, Cps, *C. pneumoniae* (Cpn), Cp, and Cs.

notion that humans also are probably susceptible to ocular infection and disease with zoonotic species. A recent study identified several *Chlamydiaceae* species infecting the diseased and healthy eyes of sheep; these species were *C. abortus*, *C. pecorum*, *C. suis*, and uncultured *Chlamydia*-like organisms (*26*). *C. psittaci* has been associated with ocular diseases in sheep and koalas (*27*). *C. suis* is also well known as a cause of conjunctivitis among pigs (*28*), and *C. pecorum* is associated with keratoconjunctivitis among sheep and goats (*26*). One study tracked *Chlamydiaceae* infections among humans and their domesticated animals and found *C. psittaci* on ocular swabs from humans, cattle, buffaloes, sheep, and goats inhabiting the same compounds (*29*).

The plethora of mixed infections, along with single infections with various *Chlamydiaceae* species, among domesticated animals (such as ducks, pigs, cows, sheep, goats, horses, and cattle) suggests multiple opportunities for transmission to humans and development of disease. *C. pneumoniae (30)* and *C. psittaci (31,32)* are known to be transmissible from human to human. Alternatively, infection and reinfection from animals could be another mechanism for transmission in which human-to-human transmission, which is necessary for the human-confined pathogen *C. trachomatis*, would not be required. In addition, clinical outcomes could be worse when caused by mixed chlamydial infections rather than monoinfection, as was suggested in a study of ovine abortion (*33*).

A major question is whether *C. trachomatis* strains that cause sexually transmitted diseases (STDs) or *Chlamydiaceae* species that cause zoonotic infection are capable of causing chronic infection and the cycles of reinfection that are characteristic of trachoma caused by *C. trachomatis*. STD-strain infections among children causing trachoma-like disease have been reported. In a study by Harrison et al. (*34*), urogenital strain J was isolated from the conjunctiva of a Navajo child who had trachoma. This strain was also noted to have been isolated from the urogenital tract of Native American women in the same area. Mordhorst et al. (*35*) isolated strains Ba, D, E, G, and H from 14 patients in Denmark for whom onset of infection and trachoma occurred during childhood. Of these 14 patients, trachomatous disease was severe for 5. We have also documented trachoma-like disease caused by *C. psittaci* and *C. pneumoniae* in the United States (*8*). Another report notes the isolation of *C. pneumoniae* from a laboratory technician with acute follicular conjunctivitis who had been working with the agent (*36*). Incidents of ocular infection with *C. suis* have occurred among pig farm and slaughterhouse workers (D. Vanrompay, pers. comm.); however, these infections were asymptomatic.

Animal models of trachoma also lend support to the pathogenic role of STD strains and other zoonotic *Chlamydiaceae* species in trachoma. STD strains E and G have been shown to produce severe ocular disease similar to trachoma in macaque or baboon models of trachoma (*37–39*). There is also a guinea pig model of trachoma in which *C. caviae* has been shown to produce pathologic changes similar to those of trachoma (*40*). These collective findings indicate that multiple zoonotic species can probably infect the eyes of humans and might contribute to trachomatous disease pathogenesis.

In our study, we randomly selected villagers with follicular or intense trachomatous inflammation to screen for *Chlamydiaceae* infections. None of the selected villagers lived in the same household. Most infections were with trachoma C strains, although urogenital strains B, D, E, F, and L_2 were also detected. We have documented urogenital infections in trachoma patients in Nepal but not with L_2 (*6*), perhaps because of the small sample size. We identified 4 other zoonotic species as etiologic agents in single or mixed infections. These species are common among domesticated animals (such as pigeons, pigs, cows, and buffaloes), which in trachoma-endemic communities are commonly kept for consumption or agricultural purposes. In our previous study (*6*), we tested for only *C. psittaci* and *C. pneumoniae* in addition to *C. trachomatis*.

The mixed infection rate of 24% found in our study was comparable to the rate of 35% found in our previous study, although the higher rate might have reflected the testing of multiple family members who would have had the same exposure to infected animals (*6*). The significantly

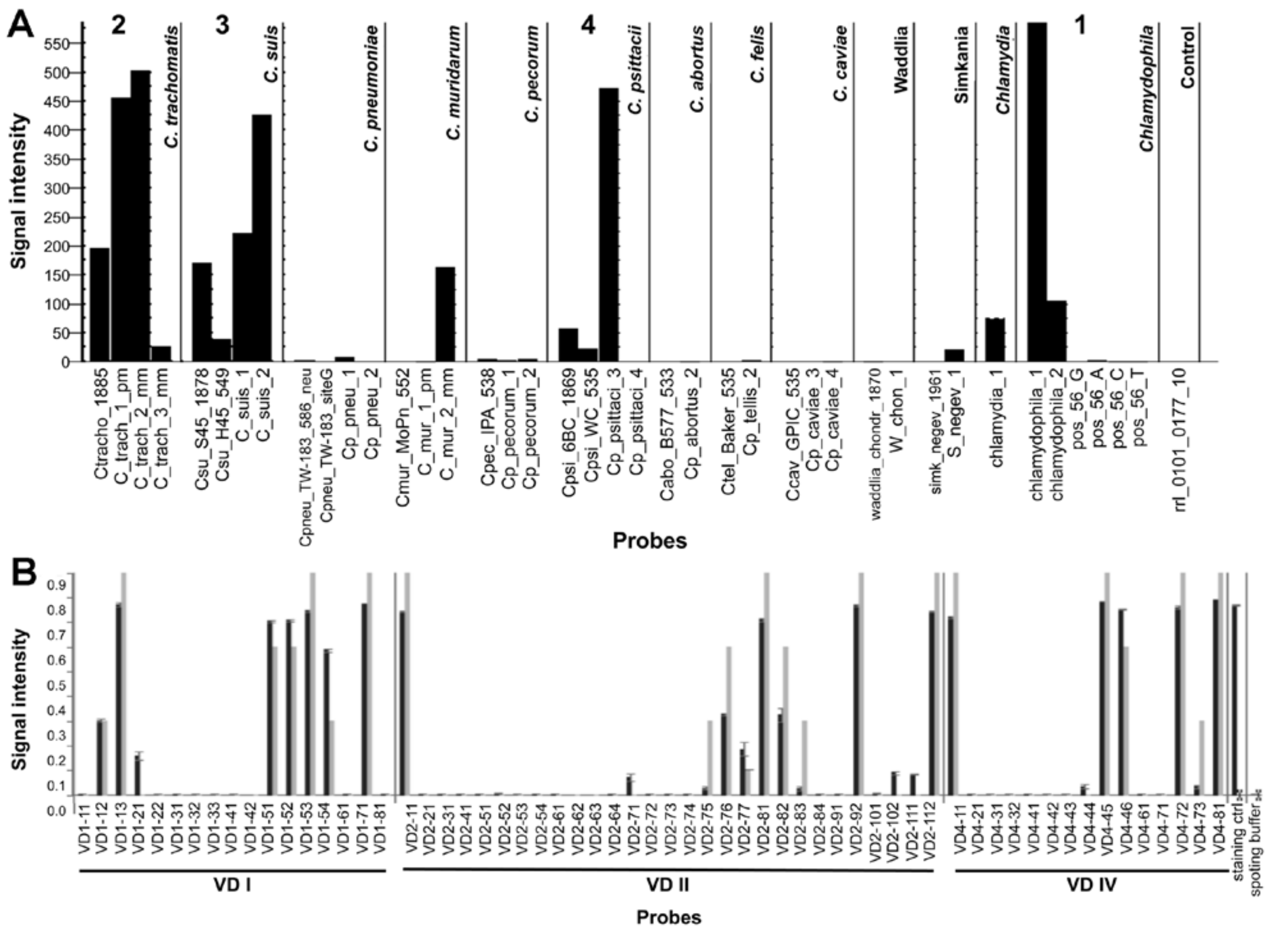

Figure 2. Identification of *Chlamydiaceae* triple infection by using the ArrayTube (Alere Technologies, Jena, Germany) assay. A) Biotinylated PCR product from a DNA extract was hybridized to a DNA microarray carrying species-specific probes from the 23S rRNA gene locus (*17*). Bar graph shows specific hybridization signals for genus *Chlamydia* (1), *C. trachomatis* (2), *C. suis* (3), and *C. psittaci* (4) in sample 67. Other signals represent nonspecific cross-hybridization. B) *omp*A genotyping of the *C. trachomatis* strain from sample 64 conducted by using the ArrayStrip platform that is specific for *C. trachomatis*. The best match of this sample was genotype C. The genotype has been determined by automatic comparison of experimentally obtained (black bars) and theoretically constructed (gray bars) hybridization patterns with use of the software's PatternMatch algorithm. The numerical values of matching score MS (measure of similarity between sample and reference strain) and Delta MS (numerical difference between best and second best match) indicate that the identification is highly accurate (*21*). The rightmost bars represent internal staining control (biotinylated oligonucleotide probe) and spotting buffer (background).

higher rate of mixed infections among female participants is consistent with their societal role of caring for domesticated animals and the enhanced opportunity for contact with potentially infected animals. No significant associations were found between age or sex and infecting species. This finding is similar to that of the previous study (*6*), indicating that these species are probably prevalent in the communities and that all villagers are susceptible to zoonotic infection.

The ArrayTube was 91.7% sensitive and 100% specific compared with qRT-PCR. That 5 samples were negative by the ArrayTube might reflect the higher sensitivity of amplifying a single target in qRT-PCR compared with amplifying multiple targets in a single ArrayTube assay. Nonetheless, the ArrayTube assay is a relatively quick assay for screening populations in trachoma-endemic areas for *Chlamydiaceae* species. It is ideally suited for detecting mixed infections that might be missed by tests that target a single species, that amplify only the most abundant species in the sample, or that require additional DNA for multiple strain-specific or species-specific amplifications.

Identifying *Chlamydiaceae* species distribution among persons in a trachoma-endemic area is critical for understanding disease prevalence and instituting appropriate therapeutic regimens for the specific species (*8*). For assessment of the prevalence of infections caused by all *Chlamydiaceae* species and for a better understanding of

their zoonotic origins, additional studies using the ArrayTube are warranted in other trachoma-endemic countries worldwide. For prevention of transmission from animal to human hosts, interventions will probably need to be instituted. Our results, then, represent findings that could help guide the World Health Organization program for eliminating blinding trachoma by the year 2020. Finally, understanding the full effects of multiple *Chlamydiaceae* species on the epidemiology, immunopathology, and disease outcome of trachoma will be a major research challenge. Although additional studies are needed, on the basis of our findings, vaccine design will probably need to consider the potential diversity of the host immune response to different *Chlamydiaceae* pathogens.

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Guillain-Barré Syndrome Surveillance during National Influenza Vaccination Campaign, New York, USA, 2009

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The New York State Department of Health (NYSDOH) collected information about hospitalized patients with Guillain-Barré syndrome (GBS) during October 2009–May 2010, statewide (excluding New York City), to examine a possible relationship with influenza A(H1N1)pdm09 vaccination. NYSDOH established a Clinical Network of neurologists and 150 hospital neurology units. Hospital discharge data from the Statewide Planning and Research Cooperative System (SPARCS) were used to evaluate completeness of reporting from the Clinical Network. A total of 140 confirmed or probable GBS cases were identified: 81 (58%) from both systems, 10 (7%) from Clinical Network only, and 49 (35%) from SPARCS-only. Capture–recapture methods estimated that 6 cases might have been missed by both systems. Clinical Network median reporting time was 12 days versus 131 days for SPARCS. In public health emergencies in New York State, a Clinical Network may provide timely data, but in our study such data were less complete than traditional hospital discharge data.

In the fall of 1976, the outbreak of a swine-origin influenza virus prompted a mass vaccination campaign in the United States. Although an influenza epidemic did not occur, epidemiologic investigations demonstrated a small but significant risk for Guillain-Barré syndrome (GBS) among adult vaccine recipients within 6 weeks after vaccination (*1–4*). Some studies found that a relatively small risk extended slightly beyond the 6 weeks after vaccination (*1*,*2*). The estimated attributable risk for GBS after swine influenza vaccination was slightly less than 1 case per 100,000 persons vaccinated (*1*,*3*,*4*). Because of this association, GBS surveillance was established for the 3 subsequent influenza seasons; however, no increased risk for GBS was identified after influenza vaccination (*5*,*6*). The underlying reason for the association with the 1976 vaccination remains unknown.

In April 2009, influenza A(H1N1)pdm09 virus was first identified (*7–9*). Its emergence and rapid global spread prompted swift development of a new vaccine. The previous association of GBS with the 1976 vaccine raised concerns about the potential for a similar association with the new A(H1N1)pdm09 monovalent vaccines.

In June 2009, the Centers for Disease Control and Prevention (CDC) engaged the 10 CDC-funded Emerging Infection Program (EIP) sites (*10*,*11*), including New York State (NYS), to rapidly collect and report information about hospitalized persons with GBS during October 1, 2009–May 31, 2010, to examine a possible relationship with A(H1N1)pdm09 vaccines. Some participating sites had the capability to collect hospital discharge data in real time and used this method as a primary reporting source. However, NYS has inherently long delays in hospital discharge data reporting, so to conduct real-time surveillance, NYS established a network of practicing neurologists as primary reporters. Hospital discharge data were used to supplement and retrospectively evaluate the completeness of the active physician-based reporting system.

Results of the overall national EIP GBS surveillance system during the A(H1N1)pdm09 vaccination campaign, which includes NYS data from hospital discharge data and the physician-based reporting system, have been described

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(*10*,*11*). Because of the rarity of GBS and the small excess risk identified by multistate efforts associated with A(H1N1)pdm09 vaccines (*10*,*11*), the NYS Department of Health's (NYSDOH) EIP did not attempt to study the association between vaccination and GBS. Presented here is a comprehensive evaluation of the NYSDOH EIP's use of a neurologist-based reporting surveillance system. Capture–recapture was used to compare hospital discharge data with neurologist reports to evaluate the completeness of the overall NYSDOH surveillance system.

Materials and Methods

Data Sources

NYSDOH conducted surveillance for hospitalized persons with GBS who were admitted during October 1, 2009–May 31, 2010, among NYS residents, excluding New York City. The total population under surveillance was ≈11.1 million persons.

Neurologist-based GBS Surveillance

Under the authority of NYS Public Health Law 206(1)(j), the NYSDOH Commissioner approved a time-limited request for physician reporting of GBS, not usually a notifiable condition. This request enabled NYSDOH to establish real-time surveillance by asking neurologists to report all suspected GBS cases. The NYSDOH Institutional Review Board approved the surveillance protocols.

Neurologists

Licensed neurologists practicing in NYS were identified in 2 ways: from a list from the NYSDOH Physician Profile System, in which all licensed physicians are required to maintain updated information (e.g., their specialty, practice location), and from a list provided by the American Academy of Neurology of NYS members. These lists were combined to create a singular deduplicated dataset with current address information for >2,600 neurologists.

Hospital-based Neurology Units

To focus surveillance efforts, study staff analyzed hospital discharge data to identify high-volume hospitals (i.e., hospitals that treated ≥5 GBS cases during 2003–2008) and lower-volume hospitals (i.e., hospitals that treated <5 GBS cases during 2003–2008). Of 183 NYS hospitals, 101 high-volume hospitals treated 95% of GBS cases diagnosed during 2003–2008. Study staff contacted the 101 high-volume hospitals to identify whether a neurology inpatient clinical unit was present or whether a private practice provided the hospital's neurology inpatient services. These inquiries produced 150 neurology clinical units/neurology practices recruited as active reporters. A second group of passive reporters comprised the remaining neurologists.

Therefore, 2 groups were created: 1) 150 active reporting sites based in hospital clinical neurology units or neurology practices and 2) 2,494 passive reporting neurologists identified through the deduplicated physician list. We refer to this combined group of active and passive reporters as the Clinical Network. Active reporters were mailed an information packet in mid-October 2009 that included a letter emphasizing the importance of reporting and the authority under which surveillance was conducted and a standardized case report form. Contact information was requested for a person at the practice or hospital who could serve as liaison. To facilitate reporting of suspected GBS, the liaison at the 150 active reporting sites received a biweekly email or phone call in accordance with the facility's preference. Some hospitals identified infection preventionists as the primary reporters. The 2,494 neurologists in the passive reporting group received the initial informational packet but no biweekly follow-up. In early March 2010, both groups received a second letter emphasizing the importance of continued reporting and providing a summary of preliminary results.

Hospital Discharge Data GBS Surveillance

New York's Statewide Planning and Research Cooperative System (SPARCS) collects administrative data on all hospital discharges in NYS. Reporting facilities are required to submit 95% of hospital discharge data within 60 days after the discharge month and 100% within 180 days after the facility's fiscal year ends. Because these delays render SPARCS ineffective as a primary real-time reporting system, we used SPARCS as a secondary system to retrospectively evaluate completeness of reporting from the Clinical Network. Staff reviewed SPARCS data monthly beginning January 1, 2010, for a primary or secondary GBS discharge diagnosis (International Classification of Diseases, Ninth Revision, Clinical Modification, code 357.0 [acute infective polyneuritis]) in NYS residents who were admitted during the study period. Because of SPARCS reporting delays, review continued through November 2010 to capture at least 95% of all admissions during the study period that had a GBS discharge code.

GBS Case Definition

Case status was determined by using the Brighton Collaboration Case Criteria for GBS, which incorporates 7 clinical and 2 diagnostic study criteria in hospitalized patients (*12*). Before staff carefully reviewed medical records, all patients reported as having GBS and/or having a primary or secondary GBS diagnosis code in SPARCS during the study period were considered to have suspected cases.

After medical record review, cases were assigned to 1 of 3 case definitions: confirmed, probable, and noncase.

Confirmed case-patients met all clinical criteria and at least 1 diagnostic study criteria; probable cases met all clinical criteria but did not meet diagnostic study criteria; and noncases did not fulfill clinical criteria or an alternative diagnosis was provided. Methods used for case classification have been described (*10*,*11*).

Follow-up of Reported Cases

NYSDOH staff followed up on all reported GBS cases, regardless of reporting source. For patients transferred between facilities, medical records were reviewed at the hospital where most of the diagnostic work-up and treatment was provided. Medical record reviews were conducted by using a standardized CDC Medical Record Review Form to assess case status, patient vaccination history (for both A[H1N1]pdm09 and seasonal influenza), and additional variables of interest in accordance with CDC protocol (*10*). The study coordinator discussed any unclear or missing information after medical record review with the case-patients' consulting neurologists and CDC. To ensure accurate and timely results, NYSDOH contracted with 11 public health and hospital-based nurses throughout NYS trained to conduct onsite medical record reviews. Staff conducted voluntary patient interviews with all confirmed and probable case-patients or their family members by using a standardized interview form. Vaccine histories were collected and verified by using 4 sources: medical record review, patient interview, information from primary care providers, and the NYS Immunization Information System.

Data Analysis

We comprehensively evaluated data obtained through the Clinical Network and SPARCS. Using medical record reviews as a standard, we calculated positive predictive values (PPVs) and compared them for the Clinical Network and SPARCS. Cohen's κ coefficient was used to assess overall agreement between the 2 systems and data reliability.

Capture–recapture methods have been applied to epidemiologic data (*13*–*18*). Therefore, to evaluate the completeness of the overall GBS surveillance system, we used Chapman capture–recapture methods using 2 data sources (*19*). By matching case-patients identified through the Clinical Network and SPARCS on sex, birth, and admission and discharge dates, we calculated an estimate of the total number of GBS cases and its 95% CI (*19*,*20*). The total number of GBS cases was estimated by $n = [(b+1)(c+1)/(a+1)] - 1$, where b and c are the numbers of persons in the first and second capture, respectively, and a is the number identified in both captures (*19*).

We used standard definitions to compare timeliness of reporting and timeliness to review of the Clinical Network and SPARCS. Time to report was defined as the difference, in days, between patient's hospital admission date and date a report was received by NYSDOH. Median time to report was compared between the 2 reporting systems. Time to review was defined as the difference, in days, between the date a report was received by NYSDOH and date of the medical record review. Median time to review was compared between the 2 reporting systems.

We evaluated reporting completeness by comparing Clinical Network cases with cases identified through SPARCS-only. To assess for biases in Clinical Network case reporting, SPARCS-only cases were reviewed to identify reporting differences by admission date, average age, sex, antecedent events, active/passive reporters, and A(H1N1)pdm09 vaccination status. Variables were compared by reporting source (Clinical Network vs. SPARCS) using Fisher exact test with $p \leq 0.05$ considered statistically significant.

Data were stored in Microsoft Access 2007 (Microsoft, Redmond, WA, USA). Data were cleaned and analyzed by using Microsoft Excel 2007 (Microsoft) and SAS Software version 9.1 (SAS Institute, Cary, NC, USA).

Results

Case Reports

NYSDOH received 576 suspected GBS cases from the combined Clinical Network and SPARCS surveillance system among residents with hospital admission dates during October 1, 2009–May 31, 2010 (Figure). All 240 reported patients who met study eligibility requirements were reviewed and assigned a case status, 140 were classified as having confirmed/probable GBS (Figure). When the Clinical Network data were compared with SPARCS data, 81 cases were identified in both systems, 10 cases were identified by the Clinical Network only, and 49 cases were identified by SPARCS-only (Figure). SPARCS detected 130 of the confirmed cases (sensitivity 92.9%); the Clinical Network detected 91 confirmed cases (sensitivity 65.0%). PPV was higher for the Clinical Network (82%) than for SPARCS (59%). Cohen's κ coefficient was 0.52, indicating moderate agreement between the 2 reporting sources (*21*).

Capture–Recapture Analysis

Capture–recapture analysis indicated that the entire NYSDOH surveillance system missed only 6 cases, yielding 146 (95% CI 140–152) GBS cases. Thus, NYSDOH surveillance identified 96% of the estimated cases.

Timeliness of Case Reporting and Medical Record Review

For SPARCS, median time to report was 131 days after hospital admission (Table 1). In contrast, the Clinical Network had a median time to report of 12 days after hospital

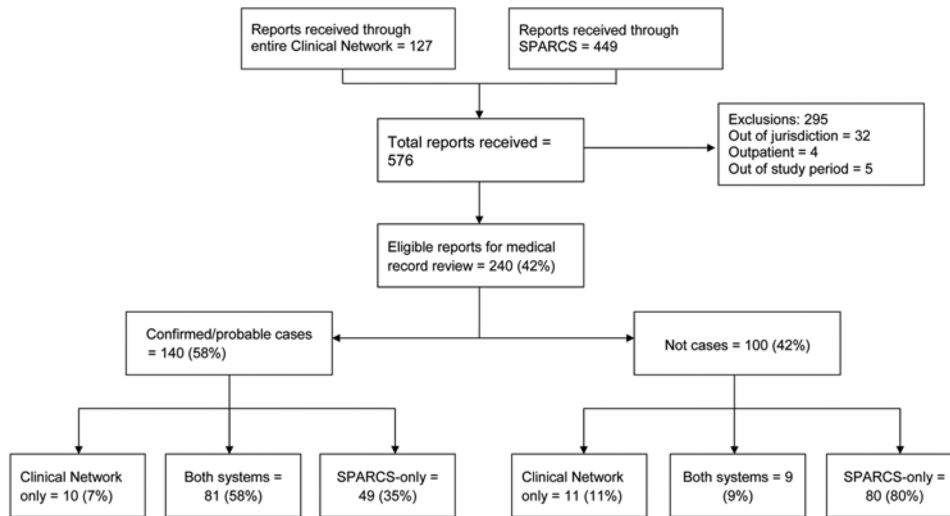

Figure. Surveillance for Guillain-Barré syndrome during the A(H1N1)pdm09 National Influenza Vaccination Campaign, New York, USA, October 1, 2009–May 31, 2010. SPARCS, Statewide Planning and Research Cooperative System.

admission. Medical records for reports identified through the Clinical Network were reviewed within a median of 7 days, compared with 14 days for SPARCS reports.

Analysis of Hospital Discharge Data as a Primary Reporting System

When analyzing SPARCS as the sole reporting source, 219 GBS patient reports were identified, of which 130 (59%) met confirmed/probable case definition (Table 2) and 89 (41%) were determined upon review to be false positive. However, when data were stratified by primary and secondary diagnosis, using only a primary GBS diagnosis code, 116 (89%) of the total 130 cases were identified, and PPV increased from 59% to 78% (Table 2).

Assessment of Bias in Clinical Network Reporting

The 49 confirmed/probable cases missed by the Clinical Network were reviewed further to identify possible biases in reporting. For 17 (35%) cases, admission date was either early or late in the surveillance period, with 8 cases missed in October 2009 and 9 missed in May 2010. No differences were found between case-patients in SPARCS-only and in the Clinical Network on the basis of sex (male 59% vs. 52%, respectively), age (mean 54.5 years vs. 53.3 years), or antecedent event 1–6 weeks before GBS symptom onset (65% vs. 56%). Among 91 Clinical Network–reported cases, 82 (90%) were received from active reporters and 9 (10%) from passive reporters. Among 49 SPARCS-only cases missed by the Clinical Network, 42 (86%) patients were under the care of active reporters and 7 (14%) were under the care of passive reporters.

Exposure to A(H1N1)pdm09 Vaccines

Nineteen (14%) of 140 patients with confirmed/probable GBS received A(H1N1)pdm09 vaccine (Table 3); GBS developed in 8 (42%) of persons within 1–6 weeks after vaccination and in 11 (58%) >6 weeks after vaccination. Six (75%) of the 8 patients with confirmed/probable GBS that developed within 1–6 weeks after vaccination were identified by the Clinical Network, and 2 (25%) were identified by SPARCS-only. Of the 91 confirmed/probable Clinical Network–reported cases, 14% received A(H1N1)pdm09 vaccine before GBS diagnosis. This proportion is similar to that found by the total surveillance system (14%) and that found if SPARCS was the stand-alone system (15%) (Table 3). Although not statistically significant, a difference was noted between the 2 surveillance systems related to recording vaccination history in the medical record. Of 19 confirmed/probable GBS case-patients who received A(H1N1)pdm09 vaccine, 9 (69%) of 13 Clinical Network–reported cases had vaccination status noted in the medical record, compared with 2 (33%) of 6 SPARCS–identified cases.

Discussion

National active GBS surveillance was implemented at EIP sites to assess whether a statistically significant

Table 1. Timeliness of reporting data to NYSDOH Guillain-Barré syndrome surveillance system, 2009–2010*

Reporting source, time, d	Mean	Median	Range
Clinical Network†			
To report‡	18	12	0–127
To review§	9	7	0–42
SPARCS¶			
To report	130	131	58–196
To review	28	14	4–184

*NYSDOH, New York State Department of Health; SPARCS, Statewide Planning and Research Cooperative System.
†Network of >2,494 reporting clinical neurologists.
‡Time lapse between patient's hospital admission date and NYSDOH receipt of report.
§Time between date NYSDOH received report and medical record review.
¶SPARCS collects administrative data on all hospital discharges in the state.

Table 2. Use of primary and secondary diagnosis codes for GBS identified by SPARCS, New York State Department of Health GBS surveillance system, 2009–2010*

Diagnosis	Total cases reported	Confirmed/probable cases, no. (%)
Primary	149	116 (78)
Secondary	70	14 (20)
Total	219	130 (59)

*GBS, Guillain-Barré syndrome; SPARCS, Statewide Planning and Research Cooperative System. SPARCS collects administrative data on all hospital discharges in state facilities.

association existed between GBS and A(H1N1)pdm09 vaccines. NYSDOH contributed a quarter of the total population under surveillance to national EIP efforts, identifying suspected cases by using physician-based reporting and SPARCS. NYSDOH's use of a Clinical Network as primary reporters and SPARCS as a supplementary source was an effective and complete method of GBS surveillance, identifying ≈96% of all GBS cases in the study population. The Clinical Network had a high PPV (82%) and its data were timely but lacked completeness, identifying 65% of total cases. However, the Clinical Network and the overall surveillance system had an equal proportion (14%) of case-patients vaccinated for A(H1N1)pdm09 virus, suggesting that using only the Clinical Network would not have biased results related to vaccination. During this emergency situation, timeliness and a high PPV for the primary source of reporting (i.e., the Clinical Network) were vital to ensure timely review and transmission of data to CDC for further analysis of vaccine safety. If the Clinical Network had a low PPV, as was seen in SPARCS (59%), our capacity to rapidly review medical records would have been diminished. An influx of false-positive reports would have delayed the time to review and delayed transmission of complete data to CDC.

The rapid time to report and the quantity of reports received from the Clinical Network demonstrated a strong collaboration among the clinical neurology community, infection preventionists, and public health authorities. The Clinical Network provided timely reporting with a median time to report of 12 days. An evaluation of US public health infectious disease reporting systems that used public health and biomedical literature found a median of 12 days (1–54 days) for meningococcal disease from diagnosis to initiation of investigation by the state public health agency and a median of 21 days (2–41 days) for *Escherichia coli* O157:H7 (*22*) infection. Therefore, the Clinical Network's and NYSDOH's time to report and investigate cases (median 19 days [12 days to report, 7 days to review]) was comparable to that for infectious disease surveillance systems. This finding suggests that during a potential public health crisis, neurologists, who may be unaccustomed to reporting to public health authorities, may be timely and competent reporters if the reasons to report are compelling and clear and the Commissioner of Health has officially requested reporting.

Adequate staffing is necessary for successful surveillance, and contracting public health/hospital-based nursing staff enabled timely review of medical records. Staff reviewed cases identified through the Clinical Network in a median of 7 days versus 14 days for SPARCS. SPARCS cases had a longer time to record review because many of these patients had been discharged and medical records departments required additional time to locate records. Many case-patients reported through the Clinical Network were hospitalized at the time of review, enabling easier access to medical records and thus a quicker review process.

If SPARCS had been the sole reporting source, using a primary diagnosis code of 357.0 would have identified 83% of total cases, including all cases in vaccinated persons. A routine GBS surveillance system based solely on hospital discharge data would be substantially less resource dependent (because of its ability to batch medical record reviews and its need for fewer staff) and have high sensitivity. The Clinical Network required a full-time staff member, 2 research assistants to maintain data and conduct biweekly follow-up calls to the neurology practices, and 11 study nurses to review medical records. Time invested by the neurology practices, infection preventionists, and medical records departments also must be considered in the overall cost of establishing and maintaining such a robust surveillance system. This evaluation has shown that for future surveillance efforts involving GBS, using a primary diagnosis code of 357.0, requires fewer resources

Table 3. Vaccination status of patients with confirmed or probable GBS, New York State Department of Health GBS surveillance system, 2009–2010*-

Reporting source	Total confirmed/probable GBS case-patientss	A(H1N1)pdm09 monovalent vaccine status	
		Received, no. (%)	Did not receive, no. (%)
Clinical Network† and SPARCS‡	140	19 (14)	121 (86)
SPARCS	130	19 (15)	111 (85)
Clinical Network	91	13 (14)	78 (86)
SPARCS-only¶	49	6 (12)	43 (88)

*GBS, Guillain-Barré syndrome; SPARCS, Statewide Planning and Research Cooperative System.
†Network of >2,494 reporting clinical neurologists.
‡SPARCS refers to all suspected cases identified through hospital discharge data (some of these cases might have been identified by the Clinical Network as well).
¶Cases missed by the Clinical Network and identified only through SPARCS.

than establishing a Clinical Network, has a high PPV, and can identify a high proportion of all GBS cases. However, in NYS, these costs were necessary because of the lack of timeliness of SPARCS. For this surveillance system, even if SPARCS were timely, it would still carry a substantial cost because of the intensive follow-up required. Receipt of reports took a median of 11 times longer from diagnosis date through SPARCS and double the time to review than through the Clinical Network. Reporting timeliness is a key surveillance system metric, and its importance is specific to the health-related event under surveillance (*23*). For GBS surveillance related to vaccination, the long delay in SPARCS reporting was unacceptable. The mass vaccination campaign necessitated rapid collection and analysis of data to determine vaccine safety and reassure providers and the public.

The Clinical Network may have failed to identify 49 cases found through SPARCS-only for several reasons. Because of logistical issues, information packets notifying the Clinical Network of the surveillance system were not sent until mid-October 2009, and NYSDOH staff did not receive a contact person for most of active reporters for biweekly follow-up until early November 2009. Many neurologists did not review their records retrospectively to October 1, 2009, and instead reported prospectively from mid-October 2009, causing underreporting in October. Reporter fatigue was noted late in the surveillance period, with only 4 confirmed/probable Clinical Network reported cases in May 2010. Supporting the suspicion that underreporting was largely a logistical issue, no differences based on demographic or vaccination status were identified between cases identified by the Clinical Network and SPARCS-only cases. Although a statistically significant difference was not identified, some reporters expressed confusion about whether to report GBS in nonvaccinated patients throughout the surveillance period.

Our conclusions are subject to 2 possible limitations. First, some GBS cases might have been misidentified; however, this circumstance was minimized by use of standardized case definitions and standardized training of the surveillance officer and contracted nursing staff. In addition, all reported GBS cases received equal follow-up regardless of reporting source, including discussing the case with the patient's consulting neurologist when information was unclear or missing. Second, specificity values could not be calculated because there was no standard for comparison; therefore, Cohen's κ coefficient was used to assess the reliability of the data. Sensitivities of 65% and 93% were found for the Clinical Network and SPARCS, respectively, but these sensitivities may be overestimated because of the possible cases missed by both systems. However, capture–recapture analysis suggests that few cases were missed.

When a credible public health emergency arises, physicians unaccustomed to reporting noninfectious diseases might be asked for assistance to protect public health and safety. For emergency GBS reporting, the Clinical Network reported quickly and well with a high PPV, and record reviews were conducted rapidly by using contracted nursing staff. These efforts led to the prompt transmission of data to CDC for the timely analysis of vaccine safety. The Clinical Network did not achieve complete case ascertainment in comparison with hospital discharge data, but no systematic bias with regard to A(H1N1)pdm09 vaccination status was evident, so data were judged valid for inclusion in the national multistate study of the risk for GBS in persons receiving A(H1N1)pdm09 vaccine. However, because of the resources needed to develop and maintain this system, it is not recommended for routine surveillance or research studies. Hospital discharge data can be used for nonemergent situations, routine surveillance, and research, but users should be aware of the built-in eporting delays.

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Antiviral Susceptibility of Highly Pathogenic Avian Influenza A(H5N1) Viruses Isolated from Poultry, Vietnam, 2009–2011

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We assessed drug susceptibilities of 125 avian influenza A(H5N1) viruses isolated from poultry in Vietnam during 2009–2011. Of 25 clade 1.1 viruses, all possessed a marker of resistance to M2 blockers amantadine and rimantadine; 24 were inhibited by neuraminidase inhibitors. One clade 1.1 virus contained the R430W neuraminidase gene and reduced inhibition by oseltamivir, zanamivir, and laninamivir 12-, 73-, and 29-fold, respectively. Three of 30 clade 2.3.4 viruses contained a I223T mutation and showed 7-fold reduced inhibition by oseltamivir. One of 70 clade 2.3.2.1 viruses had the H275Y marker of oseltamivir resistance and exhibited highly reduced inhibition by oseltamivir and peramivir; antiviral agents DAS181 and favipiravir inhibited H275Y mutant virus replication in MDCK-SIAT1 cells. Replicative fitness of the H275Y mutant virus was comparable to that of wildtype virus. These findings highlight the role of drug susceptibility monitoring of H5N1 subtype viruses circulating among birds to inform antiviral stockpiling decisions for pandemic preparedness.

Sporadic transmission of highly pathogenic avian influenza (HPAI) A(H5N1) viruses from birds to humans has been documented since 1997 (*1*), and these viruses continue to cause severe illness and death in humans. Their wide geographic spread and rapid evolution have raised concerns over emergence of a novel, virulent virus that could efficiently transmit among humans, leading to a pandemic. Vietnam is among the countries experiencing the highest number of human fatalities caused by zoonotic H5N1 subtype infections. Since the introduction of HPAI (H5N1) viruses into poultry in Vietnam during 2003 (*1*,*2*), there have been dynamic changes in their genetic and antigenic properties. Clade 1 viruses predominated in Vietnam before 2007, and were the most commonly detected H5N1 subtype group in the Mekong Delta region through 2010 (*3*). However, in northern Vietnam provinces, clade 2.3.4 viruses became the predominant group during 2007–2010. Since 2010, viruses of clade 2.3.2.1 have been detected in poultry from both regions (*3*). Since 2009, multiple subgroups of 2.3.2.1 rapidly emerged and have circulated among domestic poultry in Asia, including several provinces of Vietnam (*4*).

Genetic and antigenic divergence of HPAI (H5N1) viruses among poultry challenges development of effective vaccines for poultry and to pandemic preparedness and development of antiviral drugs for humans. Assessment of drug susceptibility has become an integral part of subtype H5N1 virus surveillance. To assist laboratories worldwide in their surveillance and pandemic preparedness efforts, the Influenza Division of the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA, along with other partners, developed the H5N1 Genetic Changes Inventory that includes established and potential markers of drug resistance (*5*). Resistance to matrix 2 (M2) protein blockers amantadine and rimantadine, caused by mutations in the M2 protein, is detected commonly in clade 1.1 (S31N) and clade 2.1.3 (V27A) H5N1 virus subtypes and sporadically in other groups (*6*,*7*). Oseltamivir, an orally administered neuraminidase (NA) inhibitor, is the most prescribed medication for the treatment of persons with influenza virus infections. Emergence of resistance to NA inhibitors among H5N1 virus subtypes, especially oseltamivir resistance among H5N1 subtypes caused by the H275Y mutation,

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is a constant threat (*8*). Assessment of susceptibility to NA inhibitors is hampered by several factors: insufficient knowledge of molecular markers of resistance, lack of harmonized approaches for testing and data analysis and, most critically, lack of established laboratory correlates of clinically relevant resistance. Taking into account these and other limitations, the current method for monitoring susceptibility to NA inhibitors is a critical element needed to evaluate pandemic risk.

In this study, we assessed drug susceptibility profiles of HPAI A(H5N1) viruses isolated from poultry specimens collected in Vietnam during 2009–2011. The antiviral drugs tested included FDA-approved medications and investigational antiviral agents. We report the detection of an oseltamivir-resistant virus with H275Y mutation from the expanding clade 2.3.2.1.

Materials and Methods

Viruses

Viruses collected from poultry on farms, in backyard flocks, and in live-poultry markets in Vietnam during 2009–2011 were identified as HPAI A(H5N1) at the National Center for Veterinary Diagnostics (NCVD), Vietnam, by using the World Health Organization (WHO) protocol (*9*). Viruses were then sent to the WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza at CDC, where they were isolated from eggs and further propagated according to WHO protocol (*9*). Virus handling was conducted under enhanced Biosafety Level 3 containment according to institutional guidelines.

Sequencing and Phylogenetic Analysis

Full-length gene sequences were generated by the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and assembled by using Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA). Phylogenetic trees were generated by using MEGA version 5.0 (www.megasoftware.net) neighbor-joining methods implemented with 1,000 bootstrap replicates. Phylogenetic data for the strain A/goose/Guangdong/1/1996 (clade 0) were used as a reference for tree rooting and numbering, and trees were annotated according to the WHO/World Organisation for Animal Health/Food and Agriculture Organization of the United Nations criteria (*10*). Sequences were deposited into the Global Initiative on Sharing All Influenza Data database. Accession numbers are listed in online Technical Appendix 1 (wwwnc.cdc.gov/EID/article/19/12/13-0705-Techapp1.xlsx). For NA sequences, N1 aa numbering is used throughout the text (*11*). The pyrosequencing method was used to detect NA residue 275 in inoculated ferret nasal wash samples (*12*).

NA Inhibitors, Neuraminidase Inhibition Assay, and 50% Inhibitory Concentration Analysis

Susceptibility to the drugs zanamivir (GlaxoSmithKline, Uxbridge, UK), oseltamivir (Roche Diagnostics GmbH, Mannheim, Germany), peramivir (BioCryst Pharmaceuticals, Birmingham, AL, USA), and laninamivir (compound R-125489; Biota, Begbroke, UK) was assessed by fluorescent neuraminidase inhibition (NI) assay, by using inhibitor concentrations ranging from 0.03 nmol/L to 1,000 nmol/L (*13*). The 50% inhibitory concentration (IC_{50}) values, the drug concentration needed to inhibit virus NA activity by 50%, were determined by using a CDC in-house program, the JASPR v1.2 curve-fitting software (*14*). Statistical analysis of IC_{50} values was performed by using SAS 9.2 software (SAS Institute, Cary, NC, USA) to identify outliers, using a statistical cutoff value U = Q3+3.0*(interquartile range). The interquartile range was determined as Q3–Q1; Q1 or Q3 denoted 25th or 75th percentile, respectively. The resulting value was applied for clade and drug. Mild outliers were defined as viruses that had IC_{50} values >U and <10 times the median, and extreme outliers as having IC_{50}values >U and ≥10 times the median. SigmaPlot 12 (Systat Software, Chicago, IL, USA) was used to generate box-and-whisker plots to visualize outliers. The median/mean IC_{50} values among virus clades were analyzed by using the Kruskal-Wallis 1-way analysis of variance and the Dunn's multiple comparison test, respectively.

Susceptibility to Antiviral Agents in Cell Culture

Susceptibilities to the M2 blocker amantadine and to investigational agents DAS181 and favipiravir (T705) were assessed in a virus yield reduction assay on Madin-Darby canine kidney (MDCK) SIAT1 cells (*15*,*16*). In brief, the confluent cell monolayers seeded on 96-well plates were treated before inoculation with amantadine or favipiravir for 30 minutes or with DAS181 for 2 hours (online Technical Appendix 2 Figure 1, wwwnc.cdc.gov/EID/article/19/12/13-0705-Techapp2.pdf). After the drug was removed, cells were inoculated with either wildtype (WT) virus, which did not contain the H275Y mutation in NA, or oseltamivir-resistant virus with the H275Y mutation (as positive control) at a low multiplicity of infection of 0.0001 PFU/cell and incubated for 1 hour at 4°C. Cells were washed and added to fresh media containing drug dilutions, and incubated at 37°C for 24 hours. Supernatants were harvested to determine infectious virus yield 50% tissue culture infectious dose per mL ($TCID_{50}$/mL) in MDCK-SIAT1 cells. The 90% effective concentration (EC_{90}) of drug (drug concentration that reduces the infectious virus yield by 90%) was determined by using the 4-parameter logistic nonlinear regression model equation in GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA).

Replicative Capacity in Cell Culture and Ferret Upper Respiratory Tract

Comparison of replicative capacity of WT and oseltamivir-resistant H275Y mutant virus was achieved by inoculating MDCK-SIAT1 cells at a multiplicity of infection of 0.0001 PFU/cell in 24-well plates. Cells were incubated at 37°C, and supernatants were collected every 12 hours until 72 hours postinoculation. At each time point, the infectious virus yields ($TCID_{50}$/mL) were determined by titrating the supernatants on MDCK-SIAT1 cells (*17*). In vivo replicative capacity was assessed in ferrets by inoculating naïve, anesthetized animals, 3–5 months of age, with 10^6 $TCID_{50}$ of either WT or H275Y virus. Virus titers ($TCID_{50}$/mL) were measured in the nasal washes collected postinoculation on days 1–3, 5, 7, and 9.

Results

Phylogenetic Analysis and Resistance Markers

Knowledge of subtype H5N1 virus genomics, especially that of hemagglutinin (HA), NA, and M2 genes, is required for interpretation of drug susceptibility data and for uncovering new trends. According to the HA gene phylogeny, viruses isolated from poultry in Vietnam during 2009–2011 were assigned to 3 clades: 1.1 (n = 25), 2.3.2.1 (n = 70), and 2.3.4 (n = 30) (Figure 1; online Technical Appendix 2 Figure 2, panel A; online Technical Appendix 2 Table). The viruses of clade 2.3.4 were categorized as 3 subclades, termed 2.3.4.1, 2.3.4.2, and 2.3.4.3. The NA phylogenetic tree topology (online Technical Appendix 2 Figure 2, panel B) was comparable to the HA tree for clades 1.1 and 2.3.2.1, but not for clade 2.3.4 viruses, suggesting NA gene reassortment among these viruses. Two reassortant viruses of subclade 2.3.4.1 contained NA genes similar to those of subclade 2.3.2.1 viruses.

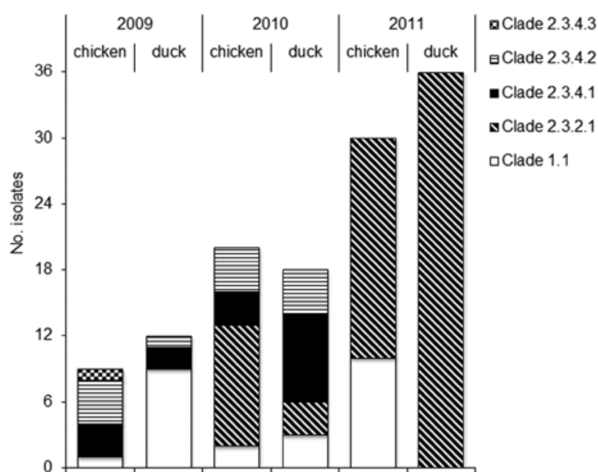

Figure 1. Description of 125 highly pathogenic avian influenza A(H5N1) viruses collected from poultry in Vietnam during 2009–2011 and tested during this study.

The M tree was similar to the HA tree and showed evidence of reassortment for a single clade 2.3.4.2 virus that clustered with M genes from subclade 2.3.4.1 (online Technical Appendix 2 Figure 2, panel C). The H5N1 Genetic Changes Inventory (*5*) was used to screen NA and M gene alignments for molecular markers associated with potential drug resistance. Clade 1.1 viruses contained S31N in M2 protein, the most common marker of M2 blocker resistance. This mutation was present in combination with L26I, a typical feature of clade 1 and 1.1 viruses. The remaining viruses had no known markers of M2 blocker resistance. In the NA gene, the H275Y mutation, a marker of oseltamivir-resistance, was detected in the virus A/duck/VN/NCVD-664/2010 (clade 2.3.2.1). The virus was isolated in Ninh Binh Province in northern Vietnam. In subclade 2.3.4.2 (n = 13), 3 viruses contained the mutation I223T, which may affect susceptibility to NA inhibitors. One clade 1.1 virus carried the mutation V149A, which was previously linked to slightly reduced susceptibility to zanamivir (*18*).

Drug Susceptibility in NI Assay

To identify viruses with potential resistance to NA inhibitor(s), we performed the NI assay using oseltamivir, zanamivir, and peramivir. IC_{50} values were calculated for each virus and drug. The median IC_{50} values were similar for oseltamivir and zanamivir (0.44 nmol/L and 0.36 nmol/L, respectively) and ≈2-fold lower for peramivir (online Technical Appendix 2 Table). The influence of the NA sequence diversity on IC_{50} values was most noticeable from the wide-ranging oseltamivir IC_{50} values (>13,000-fold difference between minimum and maximum), whereas the range was much narrower for zanamivir (145-fold) and intermediate for peramivir (1,300-fold). Analysis of IC_{50} values was further achieved by individual clade to enable better correlation with NA sequences (Table 1; Figure 2). The 2 reassortants were excluded from clade 2.3.4 analysis because the associated NA gene from each was related to those from clade 2.3.2.1.

The median oseltamivir IC_{50} value for clade 1.1 was lower than that of clades 2.3.2.1 and 2.3.4, by 7- to 27-fold, respectively. In clade 1.1, virus A/chicken/Vietnam/NCVD-780/2011 was identified as an extreme outlier and showed a 12-fold increased oseltamivir IC_{50} value (Table 2; Figure 2). This virus contained a previously unreported change: the presence of NA mutation R430W (online Technical Appendix 2 Figure 3). Virus A/chicken/Vietnam/NCVD-776/2011 was identified as a mild outlier: it had the H253Y mutation and showed a 9-fold increase in oseltamivir IC_{50} values; virus A/chicken/Vietnam/NCVD-878/2011, which carried V149A, exhibited a 3-fold increase. Notably, the oseltamivir

IC_{50} values of all 3 outliers described were similar to, or less than, the median oseltamivir IC_{50} value of clade 2 viruses (Tables 1, 2; Figure 2).

Influenza virus strain H5N1 A/duck/Vietnam/NCVD-664/2010 was identified as an extreme outlier for oseltamivir susceptibility in clade 2.3.2.1; it contained the marker H275Y and exhibited a 1,353-fold elevation in IC_{50}. Two mild outliers (3–5-fold increase) that carried the V424I change were identified within the same clade. In clade 2.3.4 viruses, 4 outliers for oseltamivir were detected, 3 of which possessed I223T, which conferred a 6–7-fold increase in IC_{50} values. The fourth virus had a V147R substitution and exhibited a 4-fold increase in IC_{50} (Table 2). As anticipated from the results of phylogenetic analysis, oseltamivir IC_{50} values of the 2 reassortant viruses (HA of clade 2.3.4 but NA from clade 2.3.2.1) matched those of clade 2.3.2.1 viruses (Table 1).

When tested for zanamivir susceptibility, an extreme outlier that had a 73-fold increase in IC_{50} was detected in clade 1.1 (Table 2): this was the same virus, A/chicken/Vietnam/NCVD-780/2011, that showed a previously unknown R430W change and was identified as an extreme outlier for oseltamivir susceptibility. Three mild outliers were identified from clades 1.1, 2.3.2.1, and 2.3.4 and had amino acid changes at the V149A, H275Y, and G147R substitutions, respectively.

The virus A/duck/Vietnam/NCVD-664/2010 that carried the H275Y mutation was predictably identified as an extreme outlier for peramivir with a 415-fold increase in IC_{50} values; the remaining viruses showed no increase. Among a subset of viruses (n = 38) tested with laninamivir, the virus that carried the R430W mutation showed a 29-fold increase, and the virus that had the H275Y mutation showed a 6-fold increase in IC_{50} values.

The WHO criteria for reporting NI assay data for influenza viruses (*19*) are based on fold difference between IC_{50} values of the test virus and a reference IC_{50} value (such as median IC_{50}); different criteria are set for seasonal type A and type B viruses. The reporting for H5N-1subtypes is not specified; therefore, we followed the criteria as outlined for seasonal type A viruses, but grouped the IC_{50} values by clade (Table 1). For clade 1.1, the virus that had the R430W mutation showed reduced inhibition by oseltamivir, zanamivir, and laninamivir; in clade 2.3.2.1, the virus that had the H275Y mutation showed highly reduced inhibition by oseltamivir and peramivir.

Characterization of the Oseltamivir-Resistant H275Y Virus

The oseltamivir-resistant virus was also tested with antiviral agents with mechanisms of action other than NA inhibition. The infectious virus yields of WT and the oseltamivir-resistant virus were reduced by >2 logs at 1 µg/mL of amantadine (data not shown), which is consistent with the M2 blocker–sensitive genotype. Inoculation of cells with DAS181 before incubation was equally effective in inhibiting replication of the virus with H275Y mutation and the WT virus (Table 3; online Technical Appendix 2 Figure 1). Both viruses were equally susceptible to favipiravir, expressing EC_{90} values of 3 µmol/L–6 µmol/L (Table 4; online Technical Appendix 2 Figure 1). For risk assessment, it was essential to investigate whether the H275Y mutation had a detrimental effect on virus replication. In MDCK-SIAT1 cells, the H275Y-mutated virus replicated at a similar rate as the WT virus and reached infectious titers as high as 10^9 $TCID_{50}$/mL at 72 hours post infection (Figure 3, panel A). In the ferret

Table 1. Clade-specific analysis of drug susceptibility of highly pathogenic avian Influenza A(H5N1) viruses in the neuraminidase inhibition assay*

Neuraminidase inhibitor	Clade	No.	IC_{50} (nmol/L) Min-Max	Median	Mean±SD†	Baseline‡ No.	Min-Max	Mild outliers§ No.	IC_{50}	Extreme outliers¶ No.	IC_{50}
Oseltamivir	1.1	25	0.04–0.69	0.06	0.07 ± 0.02	23	0.04–0.16	1	0.52	1	0.69
	2.3.2.1	70	0.13–527.26	0.41	0.43 ± 0.17	67	0.13–0.87	2	1.72–2.01	1	527.26
	2.3.4	28	0.51–11.36	1.62	1.48 ± 0.74	24	0.21–2.79	4	6.76–11.36	0	NA
	2.3.4/R**	2	0.48–0.52	NA ‡‡	0.50 ± 0.10	2	0.48–0.52	0	NA	0	NA
Zanamivir	1.1	25	0.13–18.89	0.26	0.28 ± 0.10	23	0.13–0.54	1	1.33	1	18.89
	2.3.2.1	70	0.14–1.11	0.32	0.34 ± 0.12	68	0.14–0.68	1	1.11	0	NA
	2.3.4	28	0.21–1.71	0.51	0.52 ± 0.18	27	0.21–0.99	1	1.71	0	NA
	2.3.4/R	2	0.48–0.62	NA	0.55 ± 0.07	2	0.48–0.62	0	NA	0	NA
Peramivir	1.1	25	0.07–0.31	0.13	0.14 ± 0.03	25	0.07–0.31	0	NA	0	NA
	2.3.2.1	70	0.09–91.22	0.20	0.21 ± 0.09	69	0.09–0.47	0	NA	1	91.22
	2.3.4	28	0.16–0.52	0.29	0.31 ± 0.10	30	0.16–0.52	0	NA	0	NA
	2.3.4/R	2	0.28–0.39	NA	0.33 ± 0.05	2	0.28–0.39	0	NA	0	NA

*IC 50, 50% inhibitory concentration; No., number of viruses analyzed; Min-Max, minimum to maximum of IC_{50} values; NA, not applicable; IQR, interquartile range.
†Mean and SD of IC_{50} values after exclusion of outliers.
‡ Viruses with IC_{50} values <U = Q3+3.0*(IQRIQR= Q3-Q1; Q1 = 25th percentile; Q3= 75th percentile.
§Mild outliers, with IC_{50} >U but <10-fold difference compared with the median IC_{50}.
¶Extreme outliers, with IC_{50} >U and ≥10-fold difference compared with the median IC_{50}.
**2.3.4/R,reassortants; the viruses belong to clade 2.3.4 but the neuraminidase genes are grouped with clade 2.3.2.1 (also see online Technical Appendix 2 Table, wwwnc.cdc.gov/EID/article/19/12/13-0705-Techapp2.pdf).

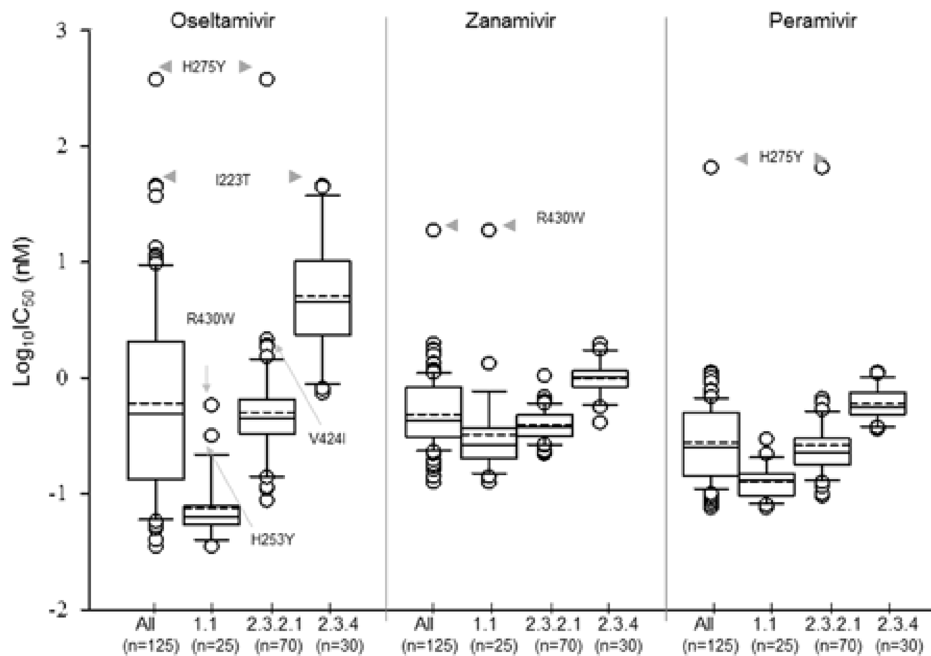

Figure 2. Distribution of log-transformed 50% inhibitory concentration (IC_{50}) values for oseltamivir, zanamivir, and peramivir: Box-and-whisker plot analysis of all tested highly pathogenic avian influenza A(H5N1) viruses (n = 125) and individual clade for each virus. The boxes represent the 25th (quartile 1) to 75th (quartile 3) percentiles; horizontal and dash lines within the box represent median and mean values, respectively; n, number of viruses tested.

model, the H275Y virus titers in the nasal washes collected at several points after inoculation were similar to those of the WT virus (Figure 3, panel B) and no differences in symptoms were noted. The stability of the H275Y mutation was demonstrated by analyzing pyrosequencing data of the viruses shed by the infected ferrets.

Discussion

In this study, we identified a single oseltamivir-resistant virus among 125 HPAI A(H5N1) viruses isolated from poultry in Vietnam during 2009–2011. It was recovered from a domestic duck in Ninh Binh Province, northern Vietnam. This virus belonged to the rapidly expanding clade 2.3.2.1 and contained the H275Y mutation, which is the principal marker of oseltamivir resistance in N1-subtype viruses (*11*). We observed no impairment in its replicative fitness in either cell culture or a ferret model. Our findings are in agreement with previous studies in which the reverse genetically engineered H275Y mutant from clade 1 retained its in vitro replicative efficiency and high pathogenicity in animals (*20*). Emergence of clade 1 viruses carrying the H275Y mutation was reported in oseltamivir-treated patients (*21*,*22*). When tested in ferrets, replication of the H275Y mutant virus was not inhibited by oseltamivir (*23*), confirming its oseltamivir-resistant phenotype. Of the 3,215 subtype H5N1 virus NA sequences available in GenBank, 6 contain H275Y; these viruses include a virus isolated from a patient treated with oseltamivir in Vietnam during 2005 (*21*), a patient in Indonesia (GenBank accession no. EU146786; unknown treatment history), and 4 viruses isolated from birds in Hong Kong (GenBank accession no. DQ250158) and Russia (GenBank accession no. DQ840522, DQ320136, and CY063862) (*7*). Although detection of H275Y mutations in H5N1 subtypes is rare, the global spread of subtype H1N1 viruses carrying the same mutation in the absence of drug exposure serves as a sobering reminder of the unpredictable nature of influenza virus evolution. As indicated in this and other studies, the HPAI (H5N1) NA gene is subject to reassortment between different HA clade-bearing viruses, which could accelerate this process (*3*).

Apart from the H275Y marker, it is difficult to predict the effect of natural genetic variation in HPAI (H5N1) viruses on susceptibility to NA inhibitors in humans. The range of H5N1 subtype oseltamivir IC_{50} values was wide (0.04–527.26 nmol/L), similar to previous findings (*24*). The WHO criteria for reporting NI assay data are based on a fold increase in the IC_{50} value of a test virus compared with that of a control or reference virus (or median) IC_{50} value (*18*). For influenza A, the result is interpreted as normal (<10-fold), reduced (10- to100-fold), or highly reduced inhibition (>100-fold).

In this report, the subtype H5N1 influenza virus with the H275Y NA mutation exhibited highly reduced inhibition by oseltamivir and peramivir, and normal inhibition by zanamivir and laninamivir. The current criteria need further clarification when applied to highly diverse H5N1 subtypes. For instance, oseltamivir IC_{50} values of clade 1.1 viruses were 7–27-fold lower than those of clade 2 viruses; this finding is in accord with previous reports (*25*). This difference stems from the presence of either histidine (clade 2) or tyrosine (clade 1) at position 253 in the NA

Table 2. Characterization of statistical outliers identified in neuraminidase inhibition assay*

Clade	Virus name	Neuraminidase gene change†	IC_{50}, nmol/L; mean ± SD (fold)‡			
			Oseltamivir	Zanamivir	Peramivir	Laninamivir
1.1	A/ck/VN/NCVD-780/2011	R430W	0.69 ± 0.25 (12)	18.89 ± 2.18 (73)	0.29 ± 0.03 (2)	2.62 ± 0.02 (29)
	A/ck/VN/NCVD-878/2011	V149A	0.16 (3)	1.33 (5)	0.13 (1)	NT
	A/ck/VN/NCVD-776/2011	H253Y	0.52 ± 0.24 (9)	0.20 ± 0.03 (1)	0.10 ± 0.03 (1)	0.14 ± 0.01 (1)
	Median	NA	0.06	0.26	0.13	0.09 ± 0.01§
2.3.2.1	A/dk/VN/NCVD-664/2010	H275Y	527.26 ± 201.10 (1,353)	1.11 ± 0.67 (3)	91.22 ± 44.34 (415)	1.36 ± 0.72 (6)
	A/dk/VN/NCVD-712/2011	V424I	2.01 (5)	0.52 (2)	0.26 (1)	NT
	A/dk/VN/NCVD-714/2011	V424I	1.72 (4)	0.53 (2)	0.31(1)	NT
	Median	NA	0.41	0.32	0.2	0.23 ± 0.01§
2.3.4	A/ck/VN/NCVD-296/2009	I223T	10.99 ± 2.38 (7)	0.86 ± 0.33 (2)	0.52 ± 0.17 (2)	0.49 ± 0.24
	A/ck/VN/NCVD-295/2009	I223T	10.37 ± 1.78 (6)	0.93 ± 0.19 (2)	0.50 ± 0.13 (2)	0.53 ± 0.18
	A/ck/VN/NCVD-283/2009	I223T	11.36 ± 3.43 (7)	0.77 ± 0.28 (2)	0.49 ± 0.24 (2)	0.42 ± 0.16
	A/dk/VN/NCVD-462/2010	G147R	6.76 ± 0.44 (4)	1.71 ± 0.48 (3)	0.45 ± 0.06 (2)	0.40 ± 0.17
	Median	NA	1.62	0.51	0.31	0.12 ± 0.02§
1	*H5N1* Reference viruses					
	A/VN/HN30408/2005, clone	H275Y	155.18 ± 5.77 (1,552)	0.63 ± 0.12 (1)	10.88 (64)	1.13 (6)
	A/VN/HN30408/2005, clone	N295S	2.99 ± 0.21 (30)	0.73 (2)	0.13 (1)	0.52 (3)
	A/Vietnam/1203/2004	NA	0.10 ± 0.02 (1)	0.46 ± 0.06	0.17 ± 0.03	0.18 ± 0.03
NA	*H1N1pdm09* Reference viruses					
	A/North Carolina/39/2009	H275Y	138.06 ± 26.02 (727)	0.19 ± 0.03 (1)	16.77 ± 4.47 (335)	0.26 ± 0.05 (1)
	A/California/07/2009		0.19 ± 0.05 (1)	0.18 ± 0.02	0.05 ± 0.01	0.17 ± 0.04

*IC_{50}, 50% inhibitory concentration; NT, not tested; NA, not applicable.
†Compared with the neuraminidase gene sequence of the closest match. (See Table 1 for median IC_{50} for each clade.)
‡Fold increase compared with the median IC_{50} of the same clade virus.
§Fold increase compared with the IC_{50} of the closest matching virus in the same clade. Global Initiative on Sharing All Influenza Data NA accession no. shown in online Technical Appendix 1 (wwwnc.cdc.gov/EID/article/19/12/13-0705-Techapp1.pdf).
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protein (*26*,*27*). Predictably, the identified clade 1.1 virus carrying the revertant H253Y mutation exhibited ≈9-fold higher IC_{50} values compared with the median IC_{50} value for this clade and ≈10-fold increase compared with A/ck/VN/NCVD-777/2011, a matching virus in which the only difference in the NA gene was an H253Y substitution. Such a virus would be reported as exhibiting reduced inhibition, yet its oseltamivir IC_{50} value was below the median IC_{50} value of typical clade 2.3.4 viruses tested in this study. In view of these findings, it may be more appropriate to consider all clade 1 viruses possessing histidine at position 253 as hypersensitive and use a median IC_{50} value of clade 2 viruses in fold difference calculations. If this rule were applied, 1 of the reference H5N1 subtype viruses (clade 1), possessing N295S (*23*), would increase by ≈2-fold in oseltamivir IC_{50} values (2.99 nmol/L) compared with values of clade 2.3.4 viruses (1.62 nmol/L) and, thus, would be reported as exhibiting normal inhibition. Like the H275Y-mutated viruses previously identified, this variant has also been isolated from an oseltamivir-treated patient (*22*) and its replication was not affected by oseltamivir treatment in ferrets (*23*). Therefore, it is essential to be able to detect and report these NA variants (*28*), despite an overall low IC_{50} value.

Because of lack of established laboratory correlates of clinically relevant resistance, analysis and interpretation of IC_{50} values generated in this study were completed according to the WHO criteria with a stipulation that fold change comparison was performed by using median IC_{50} values for individual clades. This approach facillitated identification of variants, such as the R430W-mutated virus from clade 1.1, which should be further studied. Certain discrepancies among reports are to be expected in the absence of standardized assays and criteria. For example, variant V149A of clade 1.1 was reported here as normally inhibited by zanamivir; such a variant was previously reported as showing mildly decreased susceptibility to zanamivir (*18*,*29*). Similarly, substitutions at variant I223 have previously been associated with reduced susceptibility to oseltamivir, zanamivir, or both (*5*). In this study, 3 clade 2.3.4 viruses carrying variant I223T were reported to show normal inhibition by NA inhibitors because of the relatively high

Table 3. Reduction of influenza virus yield in MDCK-SIAT1 cells in the presence of antiviral agent DAS181*

H5N1 subtype	NA	Mean ± SD virus yield (log_{10} $TCID_{50}$/mL), DAS181 (µmol/L)						EC_{90} (µmol/L), mean ± SD
		0	0.04	0.16	0.63	2.5	10	
A/dk/VN/NCVD-680/2011	Wildtype	6.6 ± 0.7	5.3 ± 0.7	3.3 ± 0.5	3 ± 0	†	†	0.02 ± 0.01
A/dk/VN/NCVD-664/2010	H275Y	7.0 ± 0.9	4.6 ± 0.8	4.3 ± 0.9	†	†	‡	0.01 ± 0.01

*Multiplicity of infection 0.0001/cell in 24-well plate. NA, neuraminidase; $TCID_{50}$/mL, 50% tissue culture infectious dose; EC_{90}, 90% effective concentration.
†Below the limit of detection, 1.3 × log_{10}/mL.

Table 4. Reduction of influenza virus yield in MDCK-SIAT1 cells in the presence of favipiravir*

H5N1 subtype	NA	Mean ± SD virus yield (log_{10} $TCID_{50}$/mL;), favipiravir (µmol/L)						EC_{90} (µmol/L), mean ± SD
		0	0.4	1.6	6.3	25	100	
A/dk/VN/NCVD-680/2011	Wildtype	6.9 ± 0.6	6.9 ± 0.5	5.7 ± 0.7	6.3 ± 0.4	3.8 ± 0.9	†	3.2 ± 2.4
A/dk/VN/NCVD-664/2010	H275Y	7.6 ± 0.5	6.9 ± 0.8	7.5 ± 0.5	6.9 ± 0.7	5.4 ± 0.8	†	5.9 ± 3.2

*Multiplicity of infection 0.0001/cell in 24-well plate. $TCID_{50}$/mL, 50% tissue culture infectious dose; EC_{90}, 90% effective concentration; NA, neuraminidase.
†Below the limit of detection, .3 × log_{10}/mL.

median IC_{50} values of this clade. Nevertheless, the oseltamivir IC_{50} values in this clade were ≈55-fold greater than the reference WT A(H1N1)pdm09 virus. Acquired additional changes in the NA (e.g., H275Y) may confer a higher level of resistance to the NA-inhibitor class of drugs among these viruses (*30*,*31*).

HPAI (H5N1) viruses resistant to M2 blockers are prevalent among poultry throughout Asia, including Vietnam (*7*,*32*). Most of these M2-resistant viruses belong to clade 1.1 (*32*,*33*), but they have also been found in other clades, including clade 2.3.4 (*34*). The oseltamivir-resistant H275Y-mutant virus detected in this study was sensitive to M2 blockers; however, the ease with which viruses can acquire resistance to this class of drugs emphasizes the need for alternative therapeutic options. The NA activity of the oseltamivir-resistant H275Y-mutant virus was inhibited by zanamivir and laninamivir in the NI assay. These NA inhibitors are delivered by inhalation, which limits their use for treatment of severely ill patients; an intravenous formulation of zanamivir is in clinical trial and is available on a compassionate use basis for treatment of hospitalized influenza patients (*35*,*36*). The replication of the H275Y-mutant virus and its WT counterpart were equally inhibited by investigational drugs DAS181 and favipiravir in cell culture.

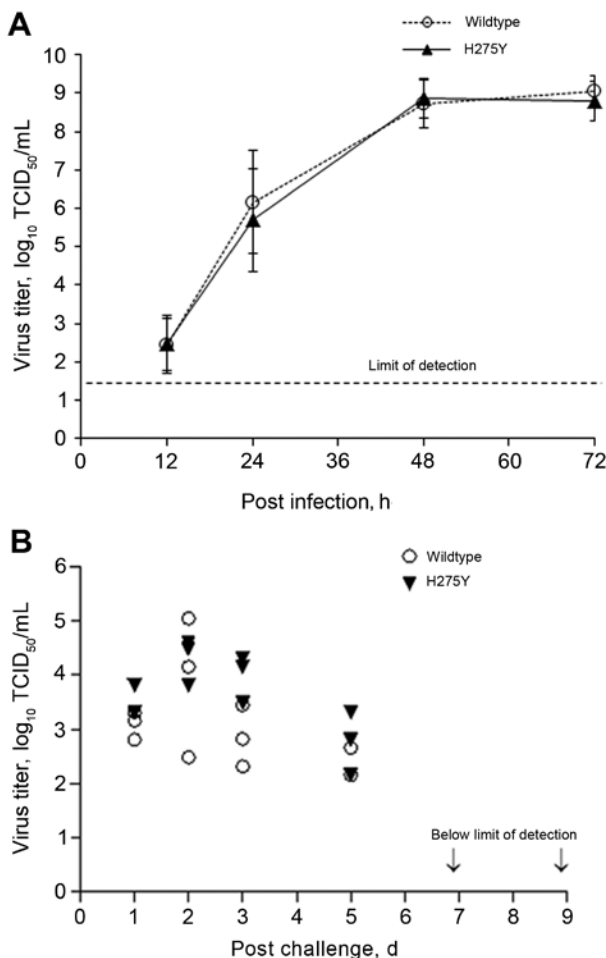

Figure 3. Replicative capacity of the oseltamivir-resistant highly pathogenic avian influenza A(H5N1) virus possessing the H275Y substitution and the wild type virus in (A) MDCK and MDCK-SIAT1 cell lines and (B) in the ferret upper respiratory tract; nasal washes were collected on days 1, 2, 3, 5, 7, and 9 post challenge. Of note, the limit of detection for virus titer was set at 1.3 x $log_{10.}$

Conclusions

Our findings demonstrate the critical role of ongoing monitoring of antiviral drug susceptibility in HPAI (H5N1) viruses sampled from poultry on informing antiviral stockpiling decisions for pandemic preparedness. Because 15 countries have reported human cases of HPAI (H5N1) virus infection to date, these findings also emphasize the need to enhance the armamentarium of available anti-influenza drugs worldwide for treatment of subtype H5N1-infected patients, including agents with diverse mechanisms of action, which could enable combination treatment (*37*), and host-directed antiviral therapy, and which may be less vulnerable to resistance.

Acknowledgments

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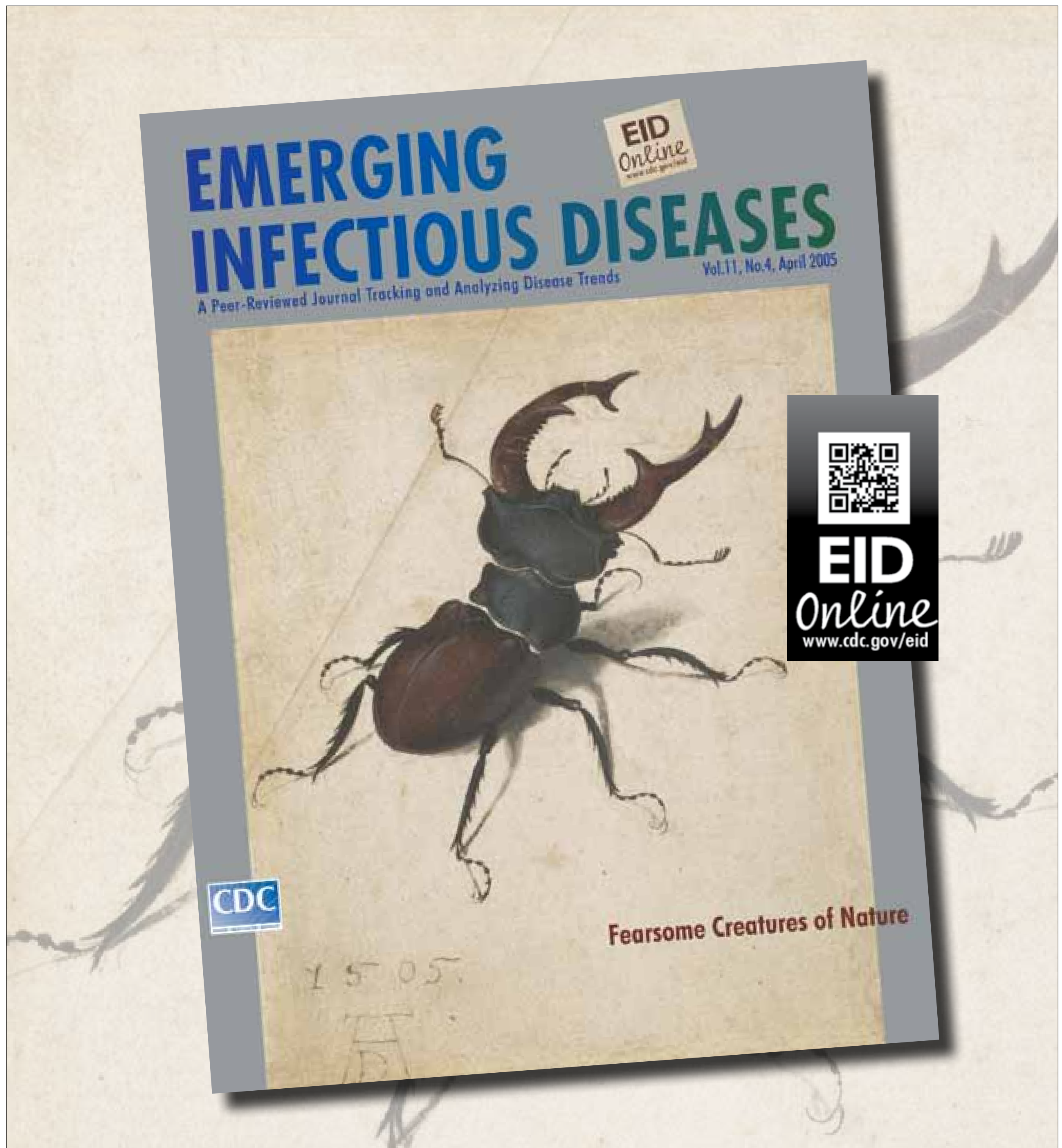

Novel Reassortant Influenza A(H1N2) Virus Derived from A(H1N1)pdm09 Virus Isolated from Swine, Japan, 2012

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We isolated a novel influenza virus A(H1N2) strain from a pig on January 13, 2012, in Gunma Prefecture, Japan. Phylogenetic analysis showed that the strain was a novel type of double-reassortant virus derived from the swine influenza virus strains H1N1pdm09 and H1N2, which were prevalent in Gunma at that time.

Influenza A viruses can be transmitted between humans, swine, and birds; virus subtypes have the potential to reassort and generate new viruses by cross-breeding in the various hosts (*1*). For example, influenza A subtype H1N1 viruses reassorted in swine, and the resulting swine influenza viruses (SIVs) were transmitted to humans. The reassorted combinations have resulted in pandemic viruses as well as low-pathogenicity viruses with low transmissibility among humans. Similarly, seasonal human subtypes of influenza are transmissible to swine (*2*). In 2009, a novel strain of the H1N1 SIV subtype emerged and was associated with a pandemic (*3*,*4*). The virus, later termed influenza A(H1N1)pdm09, hereafter referred to as pH1N1, was confirmed as a reassortant virus resulting from cross-breeding of a European avian subtype H1N1 virus and a North American triple reassortant virus (*5*). Subsequently, other strains reassorted from the pH1N1 virus (*6*–*8*). We report on an isolated new reassortant H1N2 SIV derived from the pH1N1 virus and SIVs originating in Japan.

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The Study

We collected 109 nasal swab samples from pigs for swine influenza surveillance during November 2011–February 2012. Nasal swab samples were collected from healthy pigs, 6 months of age, at an abattoir in Gunma Prefecture, Japan. All samples were inoculated onto MDCK cells (*9*). All cell culture supernatants were tested by using a hemagglutination assay of a 0.7% solution of guinea pig erythrocytes (*9*). To determine the subtype of the isolate, a hemagglutination inhibition assay was performed by using ferret antiserum for A/California/07/2009 [A(H1N1)pdm09], A/Victoria/210/2009 [A(H3N2)], B/Bangladesh/3333/2007 [B/Yamagata-lineage], and B/Brisbane/60/2008 [B/Victoria-lineage] (*9*). One strain of influenza A virus, designated A/swine/Gunma/1/2012, was isolated from the samples.

For full genome sequencing of the influenza A/swine/Gunma/1/2012 strain, we conducted reverse transcription PCR (*10*). Segment-specific primers used for amplification and sequencing are shown in online Technical Appendix Figure, panel A (wwwnc.cdc.gov/EID/article/19/12/12-0944-Techapp1.pdf). Phylogenetic analysis of the nucleotide sequences was conducted by using MEGA version 5 software (www.megasoftware.net) and Tree Explorer version 2.12 (http://en.bio-soft.net/tree/TreeExplorer.html) (*11*). Evolutionary distances were estimated according to the Kimura 2-parameter method (*12*). The phylogenetic trees of hemagglutinin (HA) and neuraminidase (NA) genes were constructed by using the neighbor-joining method (*13*). In addition, phylogenetic trees based on the matrix protein, nucleoprotein genes, nonstructural protein, polymerase acid, polymerase basic 1, and polymerase basic 2 were constructed by using the neighbor-joining method. The reliability of the trees was estimated with 1,000 bootstrap replications. GenBank accession numbers assigned to the gene sequences of the analyzed strain are the following: polymerase basic 2 (AB731582), polymerase basic 1 (AB731583), polymerase acid (AB731584), HA (AB731585), nucleoprotein (AB731586), NA (AB731587), matrix protein (AB731588), and nonstructural protein (AB731589).

Phylogenetic trees based on HA and NA gene sequences are shown in the Figure, panels A and B. The identities of the nucleotide sequences of each gene are shown in the Table. The A/swine/Gunma/1/2012 strain was confirmed as a strain of pH1N1 virus Figure, panel A). NA gene sequences showed that the virus was located within clusters of swine-type viruses documented in Japan as the representative strains, such as A/swine/Ehime/1/1980 (Figure, panel B). The sequence identity of the NA gene between the A/swine/Gunma/1/2012 strain and other Japanese H1N2 SIV strains ranged from 85.0 to 97.5%. The identities of other genes between the A/swine/Gunma/1/2012 strain and pH1N1 virus vaccine strain (A/California/07/2009) were highly

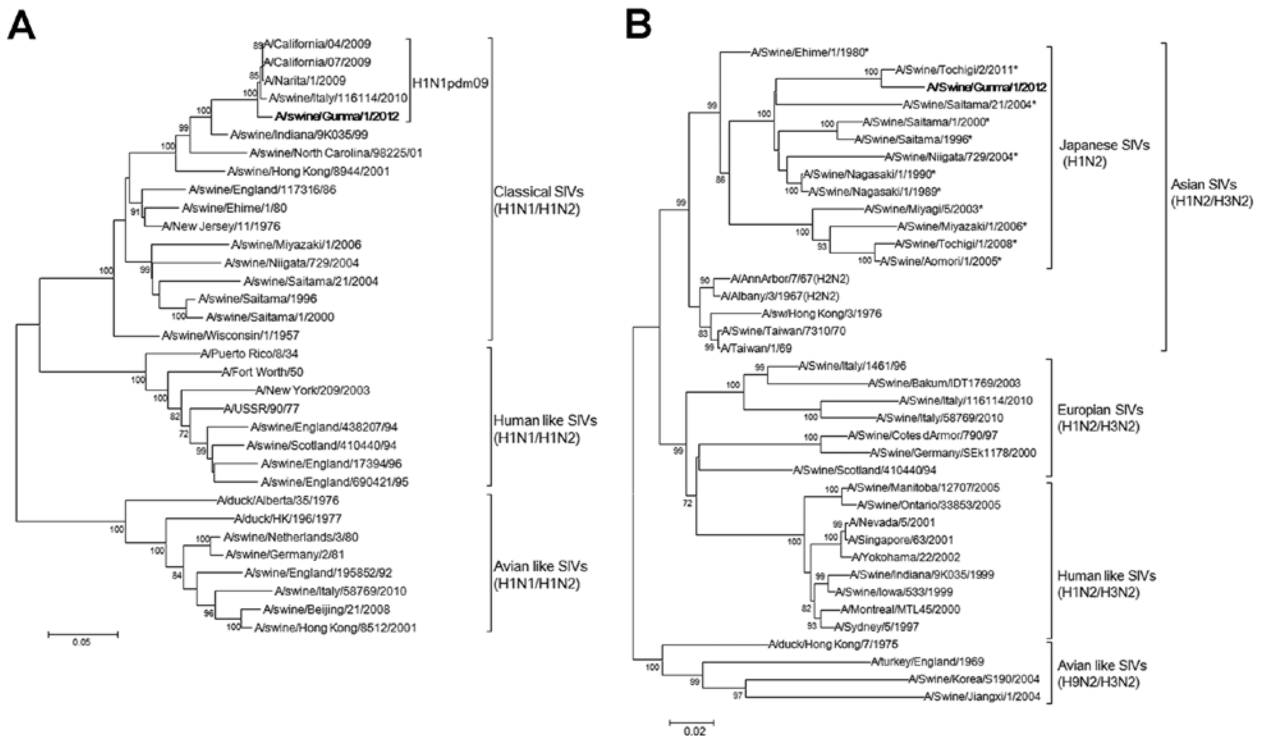

Figure. Phylogenetic tree based on the nucleotide sequences of hemagglutinin (A) and neuraminidase (B) genes of A/swine/Gunma/1/2012, a novel H1N2 swine influenza virus (SIV) strain. Distance was calculated according to the Kimura 2-parameter method; the trees were constructed by using the neighbor-joining method with labeling of the branches showing at least 70% bootstrap support. Boldface text indicates the novel strain reassorted from strains of the SIV H1N2 subtype. Asterisks indicate reference strains compared with A/Swine/Gunma/1/2012 used to calculate the identity of neuraminidase gene. Scale bars indicate nucleotide substitutions per site.

homologous (>90%; Table). These results suggest that the A/swine/Gunma/1/2012 strain was a new reassortant of the H1N2 SIV subtype derived from the pH1N1 virus.

We isolated 1 strain in this study. The samples (109 nasal swabs) were collected from different pig farms ≈60 km apart. The epidemiologic association may be low among the samples, because the quarantine inspection system is well established in Japan. All samples were collected from pigs 6 months of age; therefore, the potential for infection with the virus could have been low. Additional and larger studies investigating the emergence of the parent virus of the strain may be needed.

Table. Sequence identity of each gene of influenza strain A/swine/Gunma/1/2012, reassorted from influenza A(H1N1)pdm09 and A/California/07/2009*

Gene	Identity (%)
PB2	98.9
PB1	98.7
rPA	98.7
HA	98.4
NP	98.7
MP	99.3
NS	99.3

*PB, polymerase basic; PA, polymerase acid; HA, hemagglutinin; NP, nucleoprotein; MP, matrix protein; NS, nonstructural protein.

Conclusions

Vijaykrisna et al. found a new reassortant virus among avian-type, swine-type, and pH1N1 viruses (*6*). In addition, Monero et al. reported a new reassortant virus between SIV, identified in Italy, and pH1N1 viruses (*7*). Thus, pH1N1 virus and other types of influenza viruses can be reassorted. However, to our knowledge, reassortant H1N2 SIV strains derived from pH1N1 virus in Japan have not been identified before this report. Although the transmission of SIVs to humans has been reported sporadically, the infectious nature of this reassortant H1N2 strain among humans is unknown. The emergence of a novel H1N2 SIV strain raises further concerns about whether the virus will generate further genetic reassortments and gain virulence. Systematic influenza virus surveillance in pigs and humans should be considered.

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etymologia

Sarcocystis nesbitti

[sahr″ko-sis′tis nez-bit′ē]

In 1843, Swiss scientist Friedrich Miescher found “milky white threads” in the muscles of a mouse, which for years were known as “Miescher’s tubules.” In 1882, Lankester named the parasite *Sarcocystis*, from the Greek *sarx* (flesh) and *kystis* (bladder). Scientists were unsure whether to classify the species as protozoa or as fungi because only the sarcocyst stage had been identified. In 1967, crescent-shaped structures typically found in protozoa were seen in sarcocyst cultures, and it was determined to be a protozoan, a close relative of *Toxoplasma* spp. In 1969, A. M. Mandour described a new species of Sarcocystis in rhesus macaques, which he named *Sarcocystis nesbitti*, after Mr. P. Nesbitt, who saw the trophozoites in stained smears. Snakes are now known to be the definitive hosts of *S. nesbitti*, and several primates, including humans, can be intermediate hosts.

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Myocarditis after Trimethoprim/ Sulfamethoxazole Treatment for Ehrlichiosis

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The manifestations of human monocytic ehrlichiosis range from a mild febrile syndrome to a severe multisystem illness. Myocardial involvement is uncommon. We report a woman, 78 years of age, who was treated with trimethoprim/sulfamethoxazole after a tick bite, in whom myocarditis was subsequently diagnosed. She recovered completely after doxycycline therapy.

Human monocytic ehrlichiosis (HME) is caused by an obligate intracellular gram-negative bacteria of the family *Anaplasmataceae*. The first reported case of ehrlichiosis occurred in a man, 51 years of age, in Arkansas, United States. The man experienced a prolonged febrile illness after being bitten by ticks. Four years later, *Ehrlichia chaffeensis*, the causative agent of this syndrome, was recognized (*1*). Ticks acquire *E. chaffeensis* from a reservoir host, the white-tailed deer, and transmit the organism to humans during blood meals (*2*).

The clinical manifestations of HME range from a mild febrile syndrome to severe multisystemic illness. Although ehrlichiosis has sometimes been referred to as Rocky mountain spotted fever without the rash, a rash is not uncommon (*2*). Gastrointestinal, pulmonary, and central nervous system symptoms are well described, but cardiovascular disease is rare (*2*). Including the patient described herein, 4 persons in whom HME was diagnosed have had myocardial involvement.

The Study

A woman, 78 years of age, who had a 4-day history of fevers, chills, fatigue, and myalgias sought medical treatment after a syncopal episode. She denied dyspnea or chest pain. Ten days earlier, while in southern Virginia, she had noticed a tick on her ankle. After tick removal, the area became inflamed, and the patient received trimethoprim/sulfamethoxazole (TMP/SMX) from a local urgent care center.

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Her vital signs were as follows: oral temperature 39.3°C, blood pressure 120/60 mm Hg, heart rate 90 bpm, respiratory rate 16 breaths per min, and oxygen saturation 98% on room air. Except for a 2–cm erythematous patch on the medial aspect of her right ankle, results of her physical examination were normal. Her leukocyte count was 1.4×10^3 cells/µL (34% non-segmented neutrophils) and platelet count was 66×10^3/µL. Blood cultures were negative. The serum aspartate aminotransferase level was 173 U/L (reference <50 U/L), and the serum alanine aminotransferase level was 109 U/L (reference <50 U/L). Urinary sediment was unremarkable and a chest radiograph was normal.

The following day, the patient became dyspneic. A room air arterial blood gas revealed a pH of 7.43 (reference range 7.38–7.42), PO_2 of 70 mm Hg (reference range 75–100 mm Hg), and PCO_2 of 20 mm Hg (reference range 38-42 mm Hg). Pulmonary congestion without focal infiltrates was evident on a chest radiograph; an electrocardiogram indicated normal voltage, and saddle-shaped S-T elevations in 6 leads did not indicate ischemic distribution. On hospital day 3, her cardiac enzyme levels were markedly elevated: creatine kinase was 2,524 U/L (reference <325 U/L), creatine kinase-MB 27.3 ng/mL (reference <2.3 ng/mL), and troponin 27.3 ng/mL (reference 0–0.03 ng/mLl). A transthoracic echocardiogram revealed left ventricular systolic dysfunction with global hypokinesis and an ejection fraction of 30% (reference range 55–70%). Cardiac magnetic resonance imaging showed mild atherosclerosis.

Review of the patient's peripheral blood smear from the day of admission showed several monocytes with characteristic morulae. She was empirically treated with doxycycline, and experienced prompt defervescence. Her cardiac decompensation resolved over several days and an ejection fraction of 70% was noted on echocardiogram 10 weeks after her admission.

Serologic results for *Borrelia bergdorferi*, *Rickettsia rickettsii*, *Anaplasma phagocytophilum*, and *Francisella tularensis* were negative. Initial serologic analysis for *E. chaffeensis* demonstrated a positive result (IgM titer 320) and a negative IgG result . Convalescent-phase serologic analysis 1 month after discharge showed an IgG titer for *E. chaffeensis* of 8,192, indicating recent infection; markers of cardiac injury had returned to reference levels.

Conclusions

The Dallas criteria for a diagnosis of myocarditis are applied based on histopathologic findings of an inflammatory cellular infiltrate with or without myocyte necrosis. Unfortunately, these criteria have low sensitivity, lack prognostic value, and necessitate an invasive procedure. Autopsy studies have shown that myocardial inflammation

is not homogeneous and that sampling issues can contribute to a high rate of false negative endomyocardial biopsies. In clinical practice, the diagnosis is made based on the clinical syndrome, cardiac biomarkers, and electrocardiographic and echocardiographic findings (*3*).

Although a wide variety of pathogenic organisms have been associated with myocarditis, the etiology of this disorder in most patients remains idiopathic. In those cases in which an etiologic agent has been identified, viruses, in particular coxsackie B viruses, have been most frequently implicated (*4*). Endomyocardial biopsies during the late 1990s revealed other viral agents such as adenovirus, influenza A and B, cytomegalovirus, parvovirus B-19, and Epstein-Barr virus, among others (*3*,*4*). Myocardial involvement and cardiac dysfunction are frequently recognized in patients with HIV infection.

Trichinella spiralis and *Toxoplasma gondii* have also been recognized as causes of myocarditis, and, outside the United States, trypanosomes are commonly recognized etiologic agents (*4*). Bacteria can cause myocarditis through the effects of toxins, as is the case with diphtheria. Endotoxins have a direct suppressant effect on myocardial contractility, and myocardial dysfunction can occur in patients infected with gram-negative bacteremia. Alternatively, the presence of other organismsa, such as *Mycoplasma pneumoniae*, *Chlamydophilia* spp., or *Borrelia bergdorferi* within the myocardium has been demonstrated, indicating a more direct effect (*3*,*4*).

Myocardial involvement is a rare complication of HME. Studies in which dog models were used have shown that acute infection with *E. canis* is a risk factor for myocardial injury (*5*). In 2 earlier reported cases of HME with myocardial involvement, previously healthy men had clinical symptoms of HME confirmed by serologic analysis and left ventricular dysfunction and electrocardiographic abnormalities developed, similar to those of the patient in this study. Myocarditis resolved in both patients after doxycycline therapy (*6*,*7*). A third case of HME-related myocarditis occurred in a patient with Wegener granulomatosis (*8*). The patient was immunosuppressed by use of a tumor necrosis factor-α inhibitor, which may have contributed to the more severe manifestation of HME. This patient also appeared to recover after initiation of doxycyline. Jahangir et al. reported the sudden death of an otherwise healthy outdoor worker 19 days after the worker was bitten by a tick. An autopsy revealed transmural myocarditis and pulmonary congestion and serology consistent with human granulocytic anaplasmosis (*9*).

There is a demonstrated association between use of trimethoprim/sulfamethoxazole and fulminant manifestations of rickettsial diseases; the mechanism is unknown. Severe cases of HME have been reported in otherwise healthy adolescents taking short courses of trimethoprim/sulfamethoxazole (*10*,*11*), and in transplant patients receiving long-term regimens of sulfa drugs as prophylaxis (*12*,*13*). Our patient's disease severity may have been exacerbated by her recently prescribed regimen of trimethoprim/sulfamethoxazole.

Myocarditis associated with human monocytic ehrlichiosis is distinctly uncommon. The possibility that this condition is caused by simultaneous infection with another microorganism cannot be excluded, although such dual infection is rare (*14*). Whether subclinical myocarditis may occur more frequently is unknown, but increased evaluation of cardiac function may reveal that this is a more common phenomenon.

The mechanism whereby this organism produces transient myocardial dysfunction is unknown. Rickettsial infection is characterized by direct endothelial cell infection and inflammation mediated by cytokine and chemokine activity, which leads to increased microvascular permeability (*15*). Occasionally, small vessel occlusion and local ischemia may occur. In patients with ehrlichiosis, perivascular lymphohistiocytic infiltrates may be seen on histopathologic examination (*16*).

The observed improvement in myocardial function following doxycycline therapy in the patient in this study and in those previously described suggests that myocardial dysfunction may be caused by direct infection of the myocyte or a toxic effect of sulfa drugs, or may be secondary to an innate inflammatory response mediated by *E. chaffeensis*. It would be less likely to be a result of the host's adaptive immune response, e.g., rheumatic fever. Considering the spread of this organism throughout the south central and southeastern United States, and the frequent use of trimethoprim/sulfamethoxazole to treat localized soft-tissue infections, it is necessary to recognize this pathogen as a treatable cause of myocarditis.

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All work was completed at George Washington University Medical Center, Washington, DC.

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Distinct Lineage of Vesiculovirus from Big Brown Bats, United States

Terry Fei Fan Ng, Cindy Driscoll, Maria Paz Carlos, Algernon Prioleau, Robert Schmieder, Bhakti Dwivedi, Jakk Wong, Yunhee Cha, Steven Head, Mya Breitbart, and Eric Delwart

We identified a novel rhabdovirus, American bat vesiculovirus, from postmortem tissue samples from 120 rabies-negative big brown bats with a history of human contact. Five percent of the tested bats were infected with this virus. The extent of zoonotic exposure and possible health effects in humans from this virus are unknown.

Bats are reservoirs for many emerging viral pathogens, including Ebola viruses, Marburg viruses, henipaviruses, and severe acute respiratory syndrome coronaviruses; >80 bat virus species have been characterized (*1*,*2*). The diversity of these viruses and their high infection rates in bats may be attributed to multiple factors might that facilitate virus transmission and maintenance, including bats' large social group size, high species diversity, long life, long-distance migration, roost sharing by multiple species, and social habits such as mutual grooming and biting (*1*,*2*).

Rabies virus (family *Rhabdoviridae*, genus *Lyssavirus*) is commonly detected in bats from the United States. Analyses of several cases of human rabies infections have reported insectivorous bats as the source (*3*). The *Rhabdoviridae* family contains 6 formally approved genera, but most bat rhabdoviruses belong to the *Lyssavirus* genus (Figure). Nonrabies lyssaviruses have been characterized from bats in other parts of the world, including Australia, Europe, Africa, and Asia (*4*–*9*). In contrast to the known diversity in bats of the extensively analyzed *Lyssavirus* genus, the diversity of other *Rhabdoviridae* genera in bats remains largely undetermined. Vesiculoviruses (genus *Vesiculovirus*), such as vesicular stomatitis virus, cause fever and vesicular diseases in animals such as cattle, horses, and pigs. Some vesiculoviruses, including Chandipura virus and vesicular stomatitis virus, are also zoonotic and cause acute diseases in humans.

The bat virome has not been fully characterized. Most bat virome studies have been conducted by analyzing fecal, anal swab, or pharyngeal swab specimens from healthy bats (*10*–*13*). These studies have revealed a variety of viruses but no new rhabdoviruses. However, viruses in fecal and pharyngeal samples could include ingested and inhaled viruses that originated from insects and plants (*10*–*13*). To focus specifically on viruses infecting the bats themselves, we performed unbiased metagenomic sequencing of RNA viruses purified from the lungs and livers of 120 rabies-negative big brown bats (*Eptesicus fuscus*) collected in Maryland, USA.

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The Study

During 2008, more than 500 bats associated with possible human exposure were submitted to the Maryland Department of Health and Mental Hygiene State Laboratory for postmortem diagnosis of rabies by direct fluorescent antibody assay (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/12/12-1506-Techapp1.pdf). For this study, virus particles were purified from the lungs and livers of 120 rabies-negative bats with good carcass condition, and viral nucleic acids were extracted, randomly amplified, and sequenced by using 454 pyrosequencing (Roche, Mannheim, Germany) and Solexa Illumina sequencing (Illumina, San Diego, CA, USA). A total of ≈100,000 pyrosequences and 13.5 million Solexa Illumina sequences were generated and then assembled to form contigs. More than 30 contigs showed low protein identities to known vesiculoviruses (BLASTx; http://blast.ncbi.nlm.nih.gov/Blast.cgi), indicating a possible novel virus. PCR and rapid amplification of cDNA ends were performed to obtain the complete genome of this virus (primers shown in online Technical Appendix Figures 1, 2). We proposed the name American bat vesiculovirus (ABVV) for this virus.

The full-length ABVV genome (GenBank accession no. JX569193) consists of 10,692 nt of negative-sense, single-stranded RNA beginning with a 103-nt 5′ untranslated region, followed by open reading frames encoding for the nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and polymerase protein genes (Figure, panel A). Phylogenetic analyses showed that ABVV is related to vesiculoviruses including Chandipura virus and Isfahan virus, both of which are associated with encephalitic illness in humans. ABVV is located close to the root of vesiculoviruses in the Bayesian analysis of the nucleoprotein gene

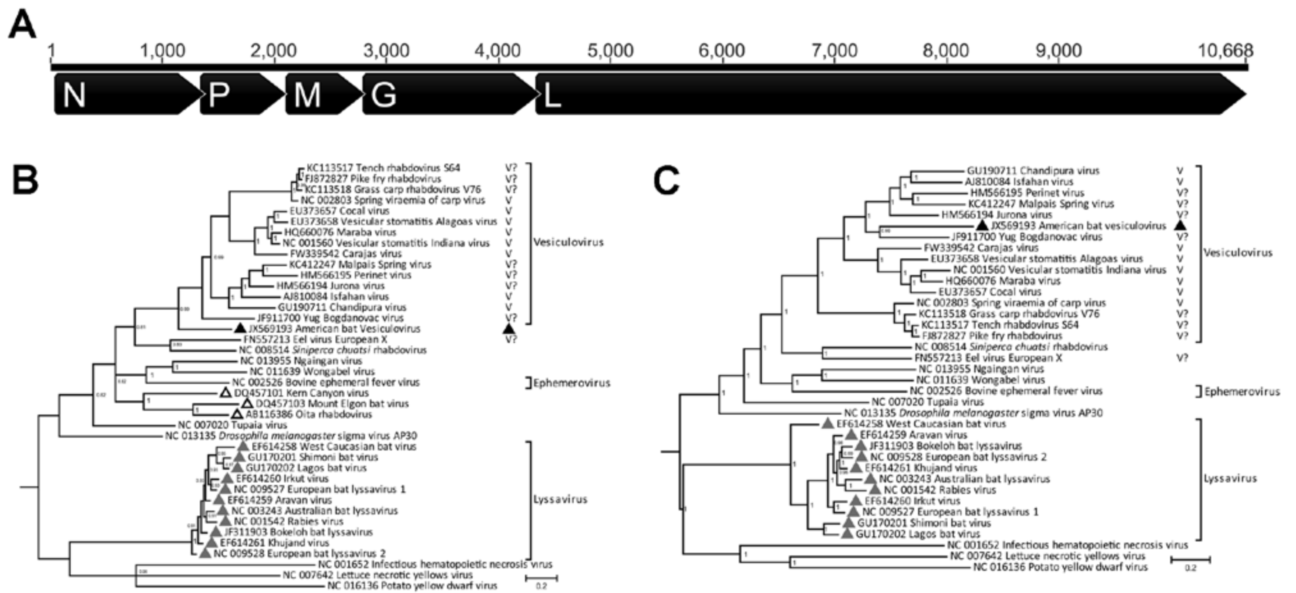

Figure. Analyses of American bat vesiculovirus (ABVV) compared with other members of the family *Rhabdoviridae*. A) Genome organization of ABVV; B) Bayesian inference tree of the ABVV N gene; C) Bayesian inference tree of the 5 concatenated ABVV genes (N, P, M, G, L). For the Bayesian analyses, sequences from the entire gene were used, except for a few partially sequenced genomes for which only ≈100 aa were publicly available. Posterior probabilities (>75%) of the Bayesian analysis are shown next to each node. Formally classified vesiculoviruses are labeled with "V," whereas potential vesiculoviruses not formally recognized by the International Committee on Taxonomy of Viruses are labeled with "V?." Distinct clades of bat rhabdoviruses are labeled with triangles of different colors: black, vesiculovirus; gray, lyssavirus; white, unclassified. N, nucleoprotein; P, phosphoprotein; M, matrix protein; G, glycoprotein; L, polymerase protein. Scale bar indicates amino acid substitutions per site.

(Figure, panel B) and shares 41%–49% aa identity with known vesiculoviruses, similar to the vesiculovirus interspecies identities reported (47.9%–72.5%) and higher than the intergenera identities between vesiculoviruses, lyssaviruses, and ephemeroviruses (17.0%–33.1%) (*14*). Analyses of the polymerase gene alone (online Technical Appendix Figure 3) and of a concatenation of all 5 genes (Figure, panel C) suggested that ABVV lies within the vesiculovirus clade, more closely related to mammalian than fish vesiculoviruses. Combined, these analyses indicate that ABVV is likely to belong to the *Vesiculovirus* genus, rather than representing a novel genus. The basal phylogenetic position of ABVV suggests early divergence from other mammalian vesiculovirus species.

Lung and liver tissues from 60 of the bats used for the pooled metagenomic analyses were screened individually for ABVV by using reverse transcription PCR targeting the polymerase gene (online Technical Appendix). Three (5%) bats tested positive for ABVV: 1 adult female, 1 adult male, and 1 juvenile male. Viral RNA was found in liver tissue from the 2 male bats and in lung and liver tissues from the female adult bat.

Considering the extensive lyssavirus diversity in bats, we hypothesize that bat vesiculoviruses are similarly diverse. To facilitate characterization of diverse vesiculoviruses in bats, we designed 2 pairs of degenerate PCR primers (VesiConAF-KCDGAYAARAGYCAYTCVATGA; VesiConAR-TGNGCNACDGTNARDGCATT; VesiConBF-GGNMGRTTYTTYTCHYTDATGTC; VesiConBR-TCHGCNGAYTGCATNGTYTCA) on the basis of a sequence alignment of the polymerase gene of ABVV and the formally classified mammalian vesiculoviruses. When the ABVV-positive bat liver cDNA was used as a control, the nested PCR yielded an amplicon of 704 bases, and its sequence was confirmed by cloning and Sanger sequencing. Future studies may use these pan-vesiculovirus PCR primers to investigate vesiculovirus diversity in other bat species and in other regions.

Conclusions

Big brown bats are prevalent in North America, where their geographic range overlaps extensively with that of humans, and considerable interactions occur between big brown bats and humans and their pets. Big brown bats from this region are a known reservoir of rabies virus; our analysis shows that these bats also constitute a sylvatic mammalian reservoir of vesiculoviruses.

The characterization of ABVV sheds light on vesiculovirus diversity in bats. The other bat rhabdoviruses—Mount Elgon bat virus, Oita rhabdovirus, and Kern Canyon

virus—do not belong to the vesiculovirus clade but cluster together in a separate clade (*14*) (Figure, panel B). A recent report described several rhabdoviruses in oropharyngeal swab specimens from Spanish bats, but the short reads (100 bases) precluded a detailed phylogenetic analysis (*15*). Of the bats tested in our study, 5% were infected with ABVV, a finding that suggests vesiculoviruses are prevalent in bats. The characterization of a novel rhabdovirus in bats with a history of human contact raises questions for further research, including health effects on the virus' hosts, seroprevalence, possible transmission by insect vectors, and the extent of zoonotic exposure in humans. ABVV-specific and vesiculovirus-consensus PCRs, as well as future endeavors to culture this virus, will help address these questions.

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Acute *Toxoplasma gondii* Infection among Family Members in the United States

Despina G. Contopoulos-Ioannidis, Yvonne Maldonado, and Jose G. Montoya

We investigated 32 families of persons with acute toxoplasmosis in which ≥1 other family member was tested for *Toxoplasma gondii* infection; 18 (56%) families had ≥1 additional family member with acute infection. Family members of persons with acute toxoplasmosis should be screened for infection, especially pregnant women and immunocompromised persons.

Only isolated case reports and small case series have been published on acute *Toxoplasma gondii* infections among family members (*1–6*). When a case of acute toxoplasmosis is identified in a family, additional household members might have been infected around the same time period; family members frequently share common exposures to food or environmental sources potentially contaminated with *T. gondii*. Identification of additional infections could lead to earlier implementation of appropriate interventions for persons in certain high-risk groups, such as immunocompromised persons and pregnant women.

Large-scale evaluation of the prevalence of acute*T. gondii* infections among family members in the United States has not been performed (*4*). Therefore, we investigated the prevalence of acute toxoplasmosis among household and family members of patients who had acute toxoplasmosis.

The Study

We performed a retrospective cohort study using data collected by the Palo Alto Medical Foundation Toxoplasma Serology Laboratory (PAMF-TSL; www.pamf.org), Palo Alto, California, USA, during 1991–2010. Patient blood samples were sent from diverse laboratories from throughout the United States, and testing was conducted at the PAMF-TSL. The study was approved by the Institutional Research Board at the PAMF Research Institute.

From the PAMF-TSL database, we identified families that 1) had an index case-patient with a diagnosis of acute toxoplasmosis and 2) had ≥1 additional household/family member who had been tested for *T. gondii* infection at PAMF-TSL. Details of the process used to identify additional household/family members are described in the online Technical Appendix (wwwnc.cdc.gov/EID/article/19/12/12-1892-Techapp1.pdf). All identified family/household members were categorized as acutely infected (<6 months before sample collection time); recently infected (6–12 months before sample collection time); chronically infected (>12 months before sample collection time); or never infected. The criteria used for this categorization are described in the online Technical Appendix. These criteria are routinely used in the daily clinical practice at PAMF-TSL to estimate the most likely time of the *T. gondii* infection; the accuracy of these criteria has been previously validated (*7–11*).

All identified families were categorized in 3 family groups (online Technical Appendix). Group 1 consisted of families with an index case-patient who had acute toxoplasmosis and ≥1 additionally tested family/household member who had acute or recently acquired *T. gondii* infection. Group 2 consisted of families with an index case-patient who had acute toxoplasmosis; ≥1 additionally tested family/household member who had chronic *T. gondii* infection; and no other tested household members who had evidence of acute or recently acquired *T. gondii* infection. Group 3 consisted of families with an index case-patient who had acute toxoplasmosis and in which no additionally tested family/household members showed evidence of *T. gondii* infection.

We defined as prevalence of acute *T. gondii* infection in >1 family members (prevalence of group 1 families) the number of group 1 families divided by the total number of study families over the 20-year study period (primary endpoint). As secondary endpoint, we also calculated the prevalence of group 2 families. We also tested whether the IgG-Dye test titers and IgM-ELISA titers of the index case-patients were different across the 3 family groups by using the Kruskal-Wallis test. All analyses were done in Stata/SE version 12 (StataCorp LP, College Station, TX, USA).

Among 97,279 persons serologically tested for *T. gondii* in the PAMF-TSL over the 20 year study period, we identified 107 persons who had ≥1 person from their household with a diagnosis of acute toxoplasmosis and ≥1 additional household member serologically tested for *T. gondii* infection. Those 107 persons were grouped into 32 study families (Figure). Patient demographic and clinical characteristics are shown in Table 1; serologic test results

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for members of group 1 families are shown in Table 2, Appendix (wwwnc.cdc.gov/EID/article/19/12/12-1892-T2.htm), and for members of groups 2 and 3 families in the online Technical Appendix.

Table 1. Demographic and clinical information for persons in the 18 group 1 study families identified from data on acute toxoplasmosis cases collected during 1991–2010 by the Palo Alto Medical Foundation Toxoplasma Serology Laboratory, Palo Alto, California, USA*

IC patient no.	Clinical information for IC	No. additional household members tested	Infection status of additional household members	Clinical information for additional household members	Risk factors reported by ≥1 household member
IC-1	LN	2	Wife: acute infection	Pregnant, first trimester	Ate raw lamb
			Daughter: no infection	NA	
			(Baby girl: status not ascertained)		
IC-2	8 wks pregnant	1	Husband: acute infection	LN	NR
			(Fetus: AF PCR–)		
IC-3	8 wks pregnant	1	Husband: acute infection	Asymptomatic	Contact with cat feces, eating undercooked meat, gardening
			(Baby boy: could not R/O CT; no follow-up beyond 1 mo of age)		
IC-4	27 wks pregnant	2	Husband: acute infection	NA	NR
			Son: acute infection	NA	
			(Fetus: AF PCR–)		
IC-5	11 wks pregnant	1	Husband: acute infection	NA	None
			(Fetus: AF PCR–)		
IC-6	Infant with CT	2	(Mother: acute infection)	NA	NR
			Father: acute infection	NA	
			Brother: acute infection	NA	
IC-7	LN, fever, headache	3	Wife: acute infection	LN	Poor cleaning of cooking surfaces
			Daughter 1: acute infection	Posterior cervical LN	
			Household member: chronic infection	NA	
			Son/daughter 2: not tested		
IC-8	13 wks pregnant	1	Husband: acute infection	NA	Ate deer meat that had positive results for *T. gondii* by PCR
			(Baby Boys A and B: status not ascertained)		
IC-9	22 wks pregnant	1	Husband: acute infection	NA	NR
			(Fetus: NA)		
IC-10	Pregnant, third trimester	2	Daughter 1: Recent infection	Asymptomatic	Children played in uncovered sandbox
			Daughter 2: acute infection	Asymptomatic	
			(Baby girl A: asymptomatic; CSF PCR–, could not R/O CT; baby girl-B: CT, macular scar, ascites, AF PCR+, CSF PCR+)		
IC-11	Infant with CT†	2	(Mother: recent infection)	NA	NR
			Father: recent infection	NA	
			Sister: no infection	NA	
IC-12	LN, fever, hepatitis	3	Wife: acute infection	LN	Ate raw lamb
			Household member 1: acute infection	LN	
			Household member 2: acute infection	NA	
IC-13	21 wks pregnant	1	Husband: acute infection	LN	Ate venison tartare
			(Fetus: CT, ascites, hydrocephalus; abortion)		
IC-14	Infant with CT	1	(Mother: acute infection)	NA	Ate bear meat; ate deer meat that had positive results for *T. gondii* by PCR
			Father: acute infection	Fever, flu-like symptoms	
IC-15	9 wks pregnant	1	Husband: acute infection	NA	None
			(Baby boy: status not ascertained)		
IC-16	Febrile illness (fibromyalgia)‡	3	Daughter 1: Recent infection	NA	Ate deer meat that had positive results for *T. gondii* by PCR
			Daughter 2: no infection	NA	
			Grandson: no infection	NA	
IC-17	Eye disease	3	Son: acute infection	NA	NR
			Daughter 1: acute infection	Asymptomatic	
			Daughter 2: no infection	NA	
IC-18	LN	1	Wife: Recent infection	NA	NR

*Mother-infant pairs were counted as 1 unit/household member; infection status of these is shown in parenthesis. IC, index case-patient; LN, lymphadenopathy; NA, not available; NR, not reported; AF, amniotic fluid; R/O, rule out; CT, congenital toxoplasmosis; CSF, cerebrospinal fluid.
†Infant with CT with hydrocephalus, high bilirubin, abnormal liver function tests, low platelets, and positive PCR results on CSF.
‡Female patient taking chronic corticosteroids; patient died.

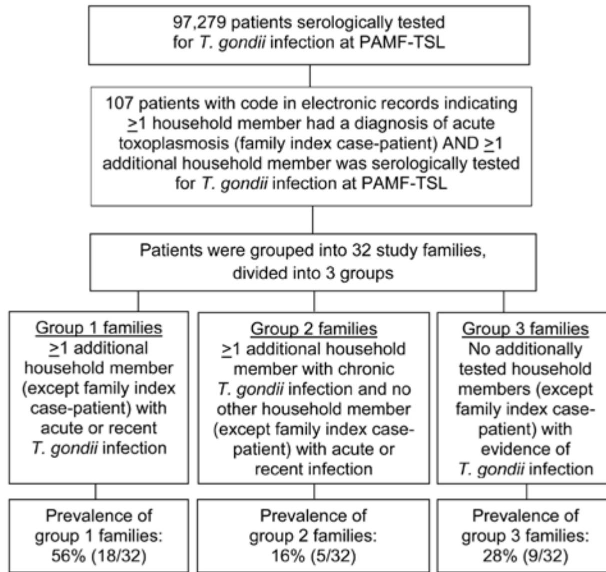

Figure. Flowchart for the identification of families with an index case-patient who had acute toxoplasmosis and ≥1 family member with acute or recent *Toxoplasma gondii* infection. Data were extracted from the database of the Palo Alto Medical Foundation Toxoplasma Serology Laboratory (PAMF-TSL; Palo Alto, CA, USA), from patient samples sent to PAMF-TSL during 1991–2010 from laboratories throughout the United States.

The prevalence of group 1 families in our study was 56% (18/32); group 2 families, 16% (5/32); and group 3 families, 28% (9/32) (Figure). The IgG-Dye test and the IgM-ELISA titers of the index case-patients were not significantly different across the 3 family groups ($p = 0.27$ for IgG and $p = 0.07$ for IgM) (Table 2, Appendix; online Technical Appendix). For group 1 families, all additional family members with acute/recently acquired infection had serologic profiles (titers of IgG, IgM, and/or IgA/IgE and avidity) that were similar to those of the index case-patients, indicating that they were infected at about the same time (Table 2, Appendix).

Conclusions

Our data provide preliminary evidence that multiple cases of acute *T. gondii* infection may occur among family/household members. These findings are particularly critical for persons at high risk from *T. gondii* infection, such as women who are or may become pregnant or immunocompromised persons. Interpretation of our study findings would have been clearer had the background prevalence of acute toxoplasmosis in the United States been known. Although no such population-level empirical data exist, we have identified at PAMF-TSL 889 patients with acute *T. gondii* infection over the 20-year study period (estimated prevalence ≈9/1,000 patients screened at PAMF-TSL; unpub. data).

A limitation of our study is that the families tested at PAMF-TSL over this study period might represent a group in whom the prevalence of acute *T. gondii* infection in ≥1 family member has been overestimated. Only 4% of persons who had acute toxoplasmosis diagnosed at PAMF-TSL during the 20-year study period had samples sent from additional household members for *T. gondii* testing (32 index case-patients with acute toxoplasmosis/889 acute infections). The collection of those additional samples depended solely on the response of the referring physicians to a 1-time written request for testing of additional family members. It is possible that the response of the primary care providers to this request would have been more likely if any of those additional family/household members had symptoms suggestive of acute toxoplasmosis. In addition, the IgG-Dye test and IgM-ELISA titers of the index case-patients did not predict which families would have additional household members with acute toxoplasmosis.

Further replication of the estimated prevalence of acute *T. gondii* infection in consecutive US families is needed. Future studies might also compare the *T. gondii* serotypes among index case-patients and family members (type II vs. non–type II) (*12*), which could help clarify whether certain serotypes are more likely to be associated with family outbreaks. Moreover, it would be useful to screen for antibodies to sporozoite-specific antigens (*13*), which can provide further insight regarding the source of *T. gondii* infection that is more likely to be associated with acute toxoplasmosis in ≥1 family member (e.g., sporozoite-specific, related to contact with cat feces, vs. bradyzoite-specific, related to ingestion of undercooked meat [*14*]).

When a case of acute toxoplasmosis is diagnosed, screening of additional family members should be considered, especially if pregnant women or immunocompromised patients live in those households, so that appropriate preventive strategies and/or therapeutic interventions are applied. These within-family clusters of cases are not easy to predict based solely on clinical or epidemiologic information, except for situations of sharing common meal (i.e., with undercooked meat), because it is unlikely that other risk factors would be different. Thus, only routine serologic screening of household members of acutely infected persons might identify such acute *T. gondii* infection infections.

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Novel Orthoreovirus from Mink, China, 2011

Hai Lian, Ye Liu, Shoufeng Zhang, Fei Zhang, and Rongliang Hu

We identified a novel mink orthoreovirus, MRV1HB-A, which seems to be closely related to human strain MRV-2tou05, which was isolated from 2 children with acute necrotizing encephalopathy in 2005. Evolution of this virus should be closely monitored so that prevention and control measures can be taken should it become more virulent.

The family *Reoviridae* is a diverse group of viruses with double-stranded RNA genomes contained within icosahedral, nonenveloped, double-layered, protein capsids (*1*). Members of the genus *Orthoreovirus* contain 10 genome segments and have been isolated from a wide variety of reptiles, birds, and mammals (including humans) (*1*,*2*). The mammalian orthoreoviruses (MRVs) have 4 major serotypes: type 1 Lang, type 2 Jones,, type 3 Dearing, and type 4 Ndelle (*3*,*4*), which can be differentiated by neutralization and hemagglutination inhibition assays (*5*,*6*). It is well known that reovirus genomes are prone to various types of genome alterations, including intragenic rearrangement and reassortment under laboratory and natural conditions (*7*,*8*). Reassortment events, involving exchange of genome segments between 2 viruses, which could lead to increased virulence, are major driving forces for reovirus genome molecular diversity and evolution (*9*,*10*). In 1975, natural reovirus infection in mink was first described in Germany (*11*). In 1992, Liu et al. also reported the isolation of a reovirus from the feces of mink with diarrhea in China (*12*). However, to our knowledge, no genetic evidence of MRV strains isolated from mink has been reported.

We isolated a novel MRV strain (named MRV1HB-A) from a mink with diarrhea in Hebei Province in northern China. To track virus evolution and look for evidence of genetic reassortment, we used PCR sequencing and phylogenetic analysis to compare genetic relatedness of MRV1HB-A and other orthoreoviruses.

The Study

In 2011, minks on a breeding farm in Raoyang County, in southeastern Hebei Province, became ill with an unidentified disease. The illness rate was almost 100% among farmed minks (*Mustela vison*), although the death rate was <5%, mainly in minks <3 months of age. Clinical signs included anorexia, emaciation, unkempt fur, and diarrhea. PCR excluded all classical endemic and emerging viruses, mink enteritis virus, canine distemper virus, Aleutian mink disease virus, and mink coronavirus as the causative agent. To identify the cause of the disease, we homogenized fecal samples from affected minks in phosphate-buffered saline and subsequently inoculated the homogenate into FK81, Vero, and BHK-21 cells. On day 7, a strong cytopathic effect was observed in FK81 cells, including rounded and detached cells; on day 8, a similar cytopathic effect was observed in Vero cells; and on day 10, the cytopathic effect was observed in BHK-21 cells. Electron microscopy of infected cells demonstrated icosahedral, nonenveloped, viral particles characteristic of MRVs (Figure 1). The mink reovirus was able to hemagglutinate type O human erythrocytes (1% vol/vol) but not chicken, mouse, goose, or rabbit erythrocytes (1% vol/vol), a finding characteristic of MRVs. Using MRV-specific reverse transcription PCR assays, we obtained products of the predicted size of 416 bp for the polymerase large (L)1 gene regions. After direct sequencing of the PCR products, a BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) search showed the sequences to be authentic reovirus sequences, with closest similarity to those of the recently identified human MRV2tou05 strain, which had been isolated from 2 children with acute necrotizing encephalopathy in 2005 (*10*). These initial findings provide the genetic evidence that an enteric reovirus is shed in the diarrheal feces of mink, confirming a previous report suggesting MRV as an etiologic agent of acute viral enteritis in mink (*12*). We tested the pathogenicity of MRV1-HB-A by orally infecting 3-month-old minks at a dose of 3 $\times 10^5$ 50% tissue culture infective dose. Mucoid diarrhea was seen on day 5 after infection. The clinical signs were similar to those of naturally infected mink.

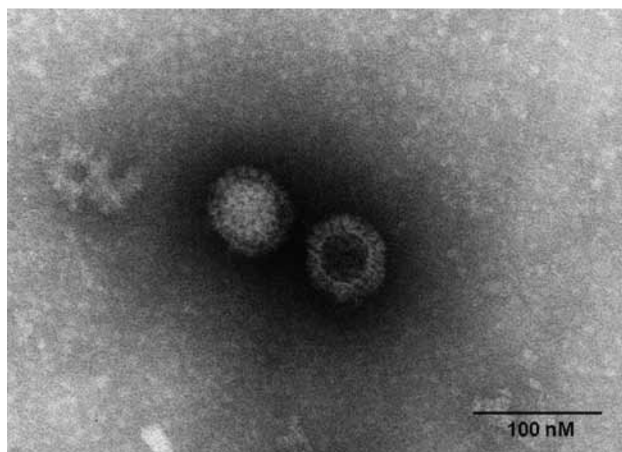

Figure 1. Electron micrograph of orthoreovirus MRV1-HB-A.

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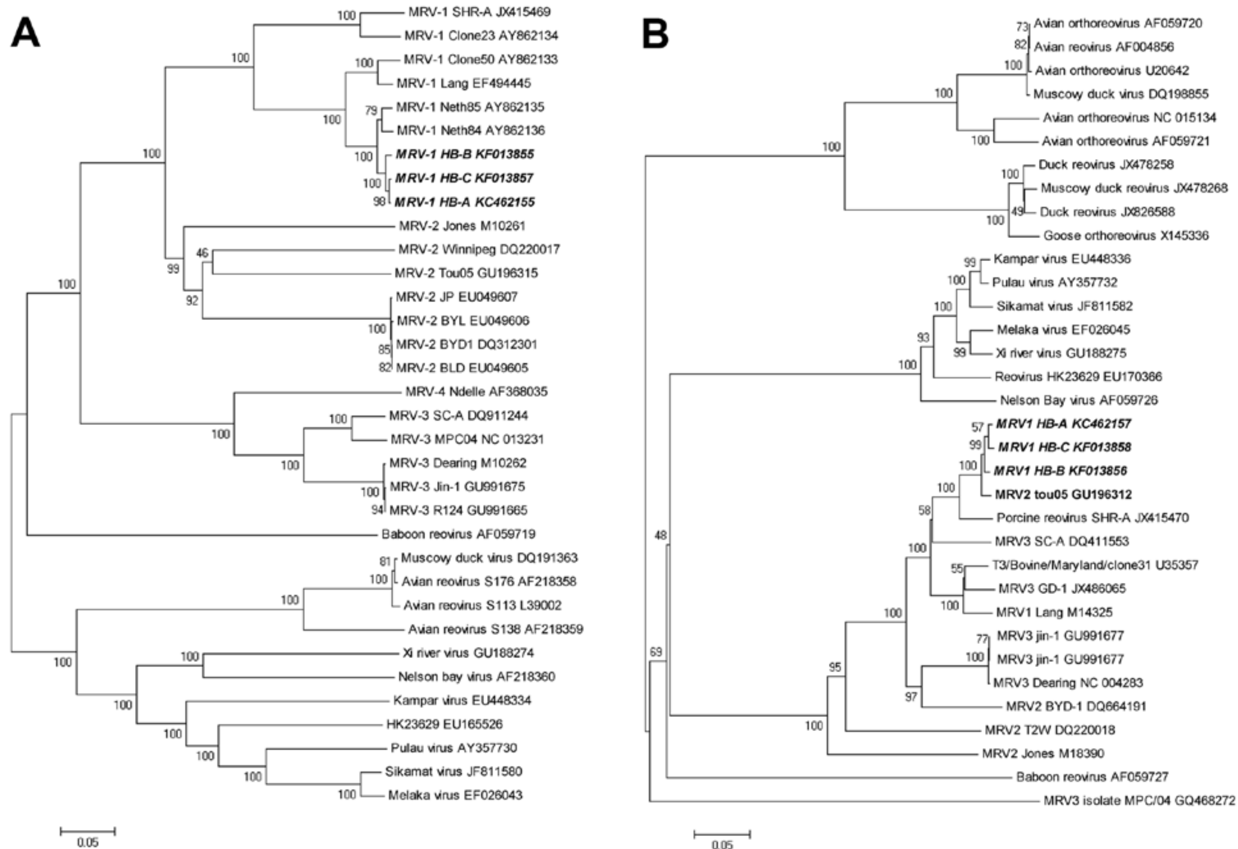

Figure 2. Phylogenetic tree and network based on the nucleotide sequences of the small (S)1 and S3 segments of orthoreoviruses. Neighbor-joining tree of multiple orthoreovirus species. Numbers at nodes indicate bootstrap values based on 1,000 replicates. Scale bar indicates nucleotide substitutions per site. A) S1 segment; B) S3 segment. MRV, mammalian orthoreovirus. Strains sequenced in this study are indicated in ***boldface italics***. An expanded version of this figure is available online (wwwnc.cdc.gov/EID/article/19/12/13-0043-F2.htm).

To further identify the virus and its phylogeny, we amplified and sequenced the MRV1-HB-A L1–L3, medium (M)1–M3, and small (S)1–S4 genes (GenBank accession nos. KC462149– KC462158). Primers used for all 10 segments are shown in the online Technical Appendix (wwwnc.cdc.gov/EID/article/19/12/13–0043-Techapp1.pdf). The nucleotide sequences obtained for each segment were compared with those of other orthoreoviruses by using the ClustalX1.83 (www.clustal.org) programs. Phylogenetic relationship was assessed by using 3 approaches: Bayesian phylogenetic trees, neighbor-joining, and split network. Topology was essentially the same for Bayesian trees, neighbor-joining, and split network (Figure 2; an expanded version of this figure is available online at wwwnc.cdc.gov/EID/

Table 1. Nucleotide identities for segments of novel mink orthoreovirus MRV1HB-A, China*

MRV1HB-A	Reovirus prototype strain, %				Human reovirus strain MRV2tou05, %	Swine reovirus strain, %	
RNA segment	T1L	T2J	T3D	T4N	Tou05	SC-A	SHR-A
L1	89.5	75.8	89.5	90.2	**97.4**	96.9	91.3
L2	86.5	73	77.2	NA	**97.7**	95.9	84.5
L3	84.3	77.5	84.5	NA	**97.2**	96	85.6
M1	94.9	70.2	94.4	NA	90	**96.2**	94.9
M2	84.7	76.6	89.7	89.1	**96.4**	95.5	90.1
M3	85.7	71.2	85.1	NA	**97.3**	96.6	85.5
S1	**91.9**	58	40.6	42	58.6	40.9	73.3
S2	**96.2**	77.2	85.4	85.6	84.7	85.9	92.7
S3	90.7	74.4	85.5	NA	**97.8**	90	93.9
S4	87.9	79.9	87.4	91.2	94.9	**98.3**	78.1

*T1L, type1 Lang; T2J, type 2 Jones; T3D, type3 Dearing; T4N, type 4 Ndelle; L, large segment; NA, not available; M, medium segment; S, small segment. **Boldface** indicates high sequence identity.

Table 2. Responses of human and animal serum samples to novel mink orthoreovirus MRV1-HB-A, China

Serum source, province	Hemagglutination inhibition, no. positive/no. tested (%)	Microneutralization, no. positive/no. tested (%)
Human		
Hebei	178/181 (98.3)	161/172 (93.6)
Jilin	116/128 (90.6)	97/117 (82.9)
Swine		
Hebei	33/66 (50)	27/59 (45.8)
Guangdong	50/56 (89.3)	44/52 (84.6)
Mink		
Hebei	48/48 (100)	46/46 (100)
Jilin	17/30 (56.7)	15/28 (53.6)

article/19/12/13-0043-F2.htm). Sequence analysis showed that the 6 segments (L1–L3, M2, M3, and S3) of MRV1-HB-A were closely related to those of the human MRV2tou05 strain and that the S1 and S2 segments were similar to those of other serotype 1 reoviruses that infect humans. The other 2 segments (M1 and S4) were closest to those of the SC-A strain, which was isolated from swine in 2006 in Sichuan, China (Table 1). The S1 segment of MRV1-HB-A is genetically similar to that of the human reovirus strains T1Neth/84 and T1Neth/85 (97% identity) isolated in the Netherlands in 1984 and 1985 (Figure 2). Nucleotide sequences of the prototype type 1 Lang and the MRV1-HB-A S1 genes shared 92% of positional identity, which provided sequence confirmation that this new isolate was a type 1 strain (Table 1). Otherwise, the S3 gene showed high identity with type 2 human reovirus Tou05 (98% identity) and porcine reovirus SHR-A (94% identity) strains (Figure 2). On the basis of these data, we conclude that the novel type 1 mink reovirus, designated MRV1-HB-A, might have originated from reassortment between human and swine strains.

Furthermore, we assessed the seroprevalence of mink reovirus antibodies in the human population and in 2 animal populations (mink and swine). Human serum samples were collected in Raoyao County People's Hospital (n = 181) of Hebei Province and Chaoyang District People's Hospital (n = 128) of Jilin Province. The human serum was donated by students, faculty members, and university workers, when they underwent annual health checkups at the 2 hospitals. From swine, 122 serum samples were collected in Hebei (n = 66) and Guangdong (n = 56) Provinces. From mink, 78 serum samples were collected in Hebei (n = 48) and Jilin (n = 30) Provinces. All samples were tested by hemagglutination inhibition and microneutralization assay as described (*5*,*6*) by using the MRV1-HB-A isolated strains as antigen. Samples with titers ≥16 were considered seropositive after 2 independent assays; lower titers were considered nonspecific reactions. For all groups, hemagglutination inhibition and microneutralization assays demonstrated similar trends, although the percentage of samples that were positive for MRV1-HB-A antibodies by microneutralization was slightly lower than by hemagglutination inhibition assay (Table 2). Because these antibodies were found in some, but not all, serum samples tested from each species and because the seropositive rate differed among regions, these antibodies probably reflect actual infection with reovirus MRV1-HB-A strain or with other strains that include a similar S1 gene.

Conclusions

Our study provides genomic evidence and molecular confirmation of a novel reovirus in mink. Although there is no direct evidence to prove the origin of MRV1-HB-A, the close genetic relationship of MRV1-HB-A with strains from humans and swine indicated a high probability that MRV1-HB-A resulted from a reassortment of human and swine strains. In view of the lack of sequence data for the reovirus from mink in public databases, addition of the complete genome sequencing information for the mink reovirus will aid in the characterization of mammalian reovirus diversity and evolution. Although MRVs were assumed to cause rather mild respiratory or gastrointestinal diseases, recent findings indicate the occurrence of higher virulent MRV strains in man and other mammals. To be prepared for the potential emergence of more virulent variants, we should carefully monitor virus evolution in real time.

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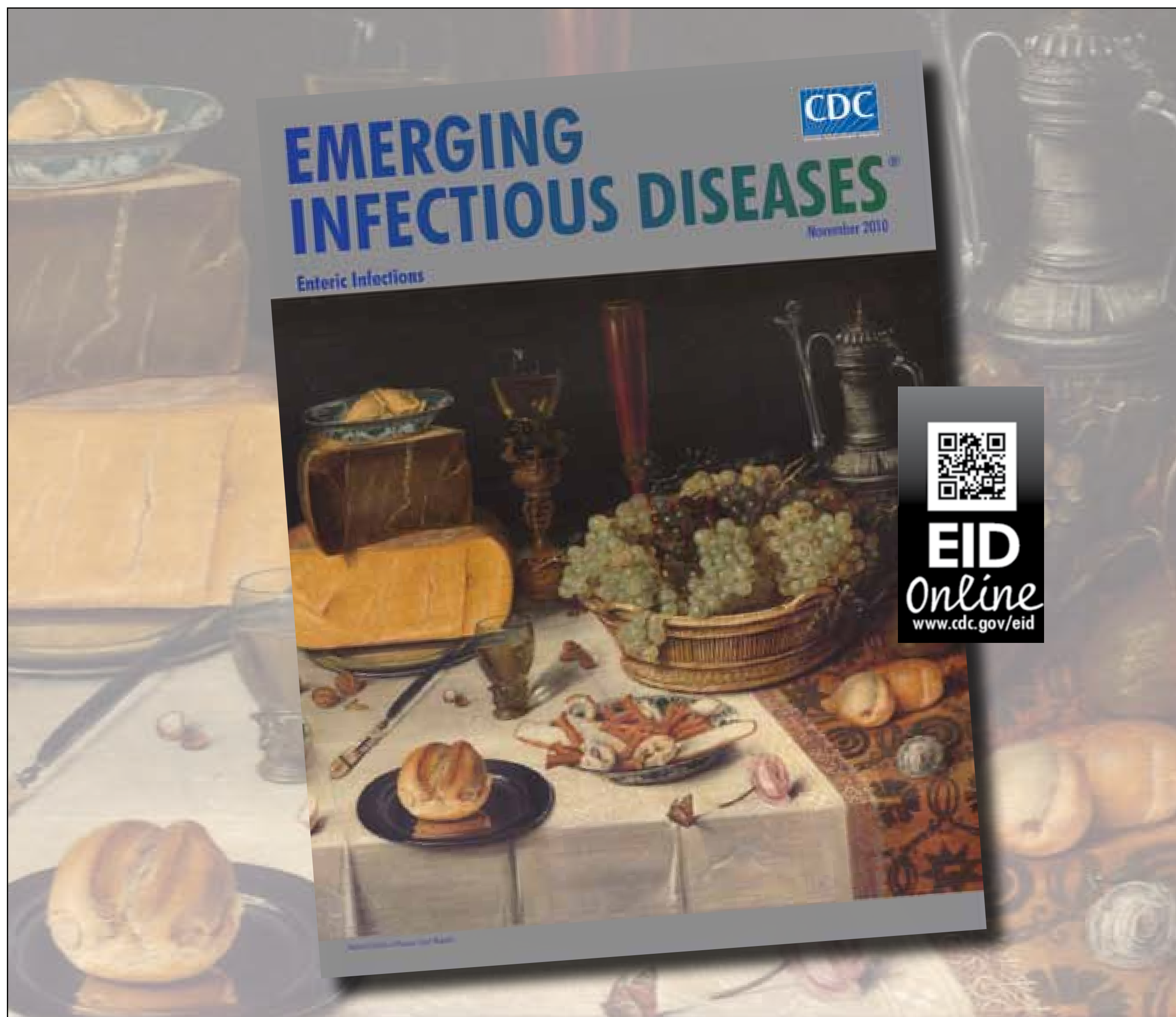

Outbreak of Human Infection with *Sarcocystis nesbitti*, Malaysia, 2012

Sazaly AbuBakar, Boon-Teong Teoh, Sing-Sin Sam, Li-Yen Chang, Jefree Johari, Poh-Sim Hooi, Harvinder-Kaur Lakhbeer-Singh, Claire M. Italiano, Sharifah F. Syed Omar, Kum-Thong Wong, Norlisah Ramli, and Chong-Tin Tan

An outbreak of fever associated with myalgia and myositis occurred in 2012 among 89 of 92 college students and teachers who visited Pangkor Island, Malaysia. The *Sarcocystis nesbitti* 18S rRNA gene and sarcocysts were obtained from muscle tissues of 2 students. Our findings indicate emergence of *S. nesbitti* infections in humans in Malaysia.

Sarcocystis spp. infections are emerging parasitic infections among travelers to potentially disease-endemic areas of Southeast Asia. More than 100 travelers acquired an acute, muscular, *Sarcocystis* spp. infection–like illness while traveling to and from Tioman Island, Malaysia, during 2011–2012 (*1*). Several cases were histologically confirmed by detection of intramuscular sarcocysts. Before these reports associated with travel to Tioman Island, <100 cases of intramuscular infection with *Sarcocystis* spp. had been reported (*2–4*) in humans. Earlier studies with tongue tissues obtained in an autopsy series suggested an infection prevalence of ≤21% among Malaysians (*5*). However, routine diagnostic examination of >1,500 limb muscle biopsy specimens in the past 20 years for various muscle diseases at the University of Malaya Medical Centre did not yield any sarcocyst-positive tissues (K.T. Wong, unpub. data). This finding suggests that human infection with *Sarcocystis* spp. is rare or that most of the infections are silent, mild and self-limited (*6*), or under-recognized.

There are >100 *Sarcocystis* spp. known and most have been isolated from muscle tissues of various intermediate hosts, including mammals, birds, and reptiles. *Sarcocystis* spp. are parasites with dual hosts to accommodate their dual life cycles. The sexual reproductive stage occurs in the definitive host, which appears to be relatively species constrained. During this stage, parasite activity is limited to the intestinal tract. In contrast, the asexual reproductive stage occurs in the intermediate host and appears to be relatively less species constrained. This stage occurs in the vascular endothelium and culminates in formation of mature muscle sarcocysts (*6*). However, *Sarcocystis* spp. infections in humans as the accidental intermediate host have been reported as intramuscular sarcocysts of unknown species (*7*).

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The Study

An outbreak investigation was undertaken after 89 symptomatic persons from Malaysia came to our institute after a college retreat during January 17–19, 2012, on Pangkor Island, Malaysia (4°13′ 52.35″N, 100°32′ 44.55″E). Ninety-two persons attended the retreat, which was held in a small hotel on the coast of the island; all outdoor activities were conducted on the beach or in the ocean. Eighty-nine symptomatic case-patients were identified with onset of fever (94%), myalgia (91%), headache (87%), and cough (40%) ≤26 days upon return. In persons who had a fever, the fever had a relapsing-remitting nature in 57% of patients.

Investigation by using magnetic resonance imaging (MRI) was prompted by development of visible swelling of the face in 9 patients and swelling of the calf muscles in 4 patients. Eight patients who had facial swelling and myalgia for 4–6 weeks underwent whole-body MRI by using the 1.5T Signa HDx MR System (GE Healthcare, Pittsburgh, PA, USA). All 8 patients showed changes in muscles of mastication, including superficial temporalis and deep temporalis, and in masseter muscles. Abnormalities were also observed in back muscles in 4 patients and in calf muscles in 2 patients. Muscle affected showed asymmetric high signal intensities on T2-weighted short T1 inversion recovery, consistent with inflammatory edema. A biopsy specimen was obtained from the temporalis muscle of 1 of these patients. Two leg muscle biopsy specimens were obtained from 2 other patients who reported specific muscle pain and had changes consistent with myositis by MRI. Mild myositis (inflammation) was observed in 3 muscle biopsy specimens examined.

Muscle tissues were ground with sterile glass beads by using a Precellys 24 homogenizer (Bertin Technologies, Montigny le Bretonneux, France) at 5,500 rpm for 30 s. Ten microliters of homogenates was inoculated into various cell cultures for virus isolation. Virus was not isolated from homogenates. RNA and DNA were also extracted from tissue homogenates by using the QIAamp Viral RNA Mini Kit and QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), respectively. PCR amplification for detection of infectious agents was performed. No specific amplification was obtained by using available primers for commonly detected viruses, including alphaviruses and other arboviruses.

However, a *Sarcocystis* sp.18S rRNA gene was detected by using 5 primer pairs described (*8*).

Amplified DNA fragments were purified and sequenced by using the BigDye Terminator v3.1 Cycle Sequencing Kit on an automated capillary DNA sequencer 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were aligned with all available *Sarcocystis* spp. 18S rRNA sequences from GenBank. A neighbor-joining phylogenetic tree was constructed by using the maximum composite likelihood method as implemented in MEGA5 (www.megasoftware.net/).

Typical sarcocysts (length ≈190 μm) were observed in cell cultures inoculated with the muscle tissue homogenates of 1 of the patients (Figure 1, panel A) and directly in the muscle tissue of another patient (Figure 1, panel B). Attempts were made to culture sarcocysts from muscle tissue homogenates in U937 and THP-1 human monocytic cell lines but no propagation of bradyzoites was obtained. Nucleic acid amplification of the 2 tissue samples consistently showed DNA fragments with expected sizes of 329–1,208 bp. One tissue sample was from the temporalis muscle of 1 patient, and the other was from the leg muscle of another patient.

Both patients reported relapsing-remitting fever (3 episodes each), myalgia, and headache. Despite the presence of myositis, neither patient had increased serum creatinine phosphokinase levels but did have increased eosinophil counts of 1.0–2.6 × 10^9 cells/L (reference range 0.02–0.50 × 10^9 cells/L).

DNA sequences obtained from 1,812-bp fragments were compared with fragments in GenBank by using BLAST (http://blast.ncbi.nlm.nih.gov/). Sequences matched 100% of those reported as *Sarcocystis nesbitti* (*9*). A phylogenetic tree constructed by using sequences *S. nesbitti* MY29365821 (GenBank accession no. HF544323) and *S. nesbitti* MY29433657 (accession no. HF544324) and those available in GenBank placed the 2 sequences in the clade with *S. nesbitti* and *S. atheridis* (*9*) (Figure 2).

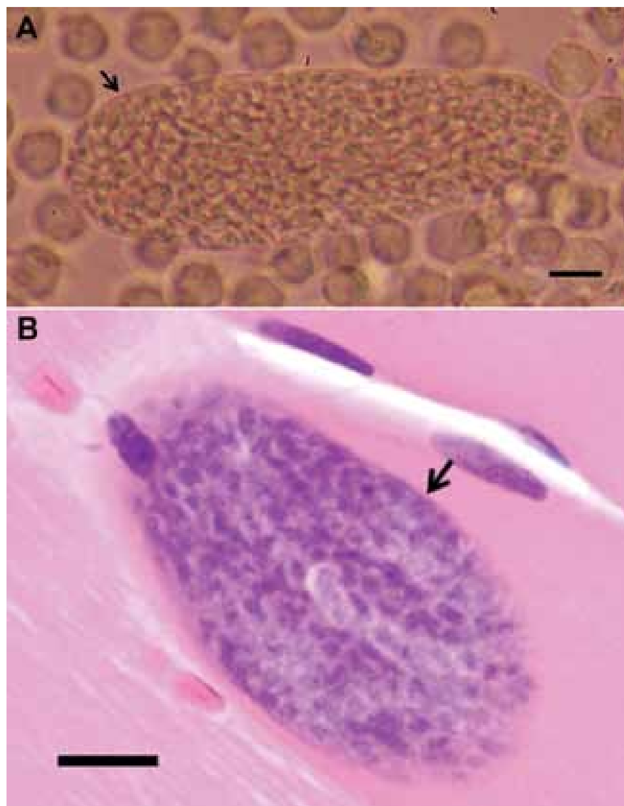

Figure 1. A) Sarcocysts isolated from persons infected with *Sarcocystis nesbetti*, Pangkor Island, Malaysia, 2012. Intact human sarcocyst (length 190 μm) with thin cyst wall (arrow) from homogenized temporalis tissue inoculated into a U937 monocytic cell culture (original magnification ×200, scale bar = 20 μm). B) Intramuscular sarcocyst enclosed by a thin smooth cyst wall (arrow) without any protrusions. Maximum cyst wall thickness is ≈0.5 μm (hematoxylin and eosin stained, original magnification ×40, scale bar = 10 μm).

Conclusions

There have been several reports of *Sarcocystis* spp. infection in Malaysia and of tourists who had traveled to Malaysia (*1,2,5,10–13*). However, the *Sarcocystis* species was not identified in any of these reports. Only *S. hominis* and *S. suihominis* have been identified as the cause of human infections. In these instances, infections were believed to be asymptomatic, although minor self-limiting gastroenteritis was possible. Humans are definitive hosts for both species but only intestinal infections (no intramuscular sarcocysts) have been observed (*6*).

We report symptomatic *S. nesbitti* infection in humans. Predominant manifestations were fever (relapsing in ≈50% of patients), myalgia, headache, and cough. Although only 2 patients were confirmed to be acutely infected with *S. nesbitti*, it was likely that the remaining students and teachers in the group had the same infection because nearly all had similar signs and symptoms with onset of illness within days of each other. In addition, 9 patients had a distinctive facial myositis, but sarcocysts could not be verified in all of them because only 3 patients agreed to provide a muscle biopsy specimen. No other microorganisms were isolated from cell cultures of blood or other body fluids of patients.

S. nesbitti was reported by Mandour in 1969 in skeletal muscles of *Macaca mulatta* monkeys (*14*). Its presence in *M. fascicularis* monkeys, but not in humans, was reported in China by Yang et al. (*15*). Similar to infection in monkeys, it is likely that humans are also accidental intermediate hosts. Its definitive host is still unknown but earlier phylogenetic analysis suggests that snakes could be a probable definitive host (*9*). Our findings highlight the emergence of *S. nesbitti* infections in humans and suggest that these infections might be endemic to Malaysia.

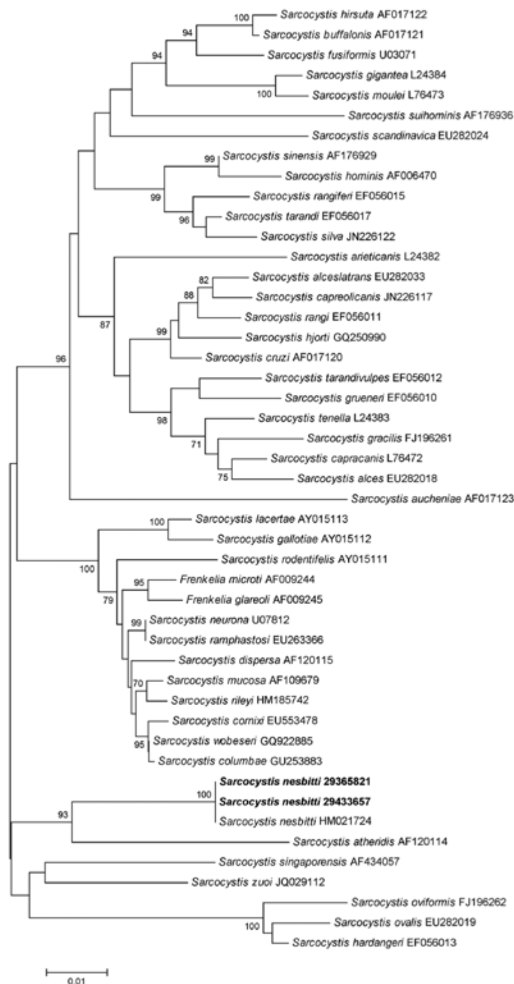

Figure 2. Neighbor-joining phylogenetic tree of *Sarcocystis* spp. 18S rRNA sequences. *Sarcocystis nesbitti* strains isolated in this study are indicated in **boldface**. Numbers at nodes indicate bootstrap values (%) for 1,000 replicates. Bootstrap values <70% are not shown. Scale bar indicates nucleotide substitutions per site.

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Transmission of Brucellosis from Elk to Cattle and Bison, Greater Yellowstone Area, USA, 2002–2012

Jack C. Rhyan, Pauline Nol, Christine Quance, Arnold Gertonson, John Belfrage, Lauren Harris, Kelly Straka, and Suelee Robbe-Austerman

Bovine brucellosis has been nearly eliminated from livestock in the United States. Bison and elk in the Greater Yellowstone Area remain reservoirs for the disease. During 1990–2002, no known cases occurred in Greater Yellowstone Area livestock. Since then, 17 transmission events from wildlife to livestock have been investigated.

Bovine brucellosis, caused by *Brucella abortus*, is a global zoonotic disease primarily infecting cattle, in which it produces abortions, retained placentas, male reproductive tract lesions, arthritis, and bursitis. In humans, brucellosis can cause recurrent fever, night sweats, joint and back pain, other influenza-like symptoms, and arthritis. In animals and humans, it can persist for long periods. During the 1930s, a state–federal cooperative effort was begun to eliminate the disease from livestock in the United States. From an initial estimated prevalence in 1934 of ≈15%, with nearly 50% of cattle herds having evidence of infection (*1*,*2*), the United States now has no known infected livestock herds outside of portions of Idaho, Wyoming, and Montana, adjacent to Grand Teton and Yellowstone National Parks. This area, referred to as the Greater Yellowstone Area (GYA), also encompasses state and federal feeding grounds in Wyoming where elk are fed during the winter. Considered a spillover disease from cattle to elk and bison, brucellosis now regularly spills back from elk to cattle. Although bison-to-cattle transmission has been demonstrated experimentally and in nature (*3*,*4*), it has not been reported in the GYA, probably because of ongoing rigorous management actions to keep cattle and bison spatially and temporally separated.

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In 1992, a court case highlighted the potential for transmission of brucellosis from free-ranging wildlife to livestock in the GYA. The litigation concerned brucellosis transmission purportedly from elk or bison to 2 cattle herds in 1988 and 1989 (*5*). Before those incidents and since ≈1961, brucellosis had been detected in 4 GYA cattle herds, and transmission was attributed to a wildlife source on the basis of epidemiologic investigations (*6*). From 1990 through 2001, no brucellosis was found in any GYA livestock despite intensive surveillance in some areas, precipitated by court action. We report a series of recent cases in which brucellosis was transmitted from free-ranging elk to domestic cattle or ranched bison as determined by epidemiologic and microbiological investigations.

The Study

During April 2002–April 2012, brucellosis was discovered in 13 beef cattle herds and 4 ranched bison herds in the GYA (Figure 1). Additionally, from comingling of cattle herds at the time of transmission and transfer of ownership of some animals between infection and detection, 3 more infected cattle herds were identified. In each of the 17 herds, infection was detected by serologic testing and confirmed by culture of tissues collected at slaughter of ≥1 animals. The source of infection of each cattle or bison herd was determined through extensive epidemiologic investigations by state and federal animal health authorities. These investigations included serologic testing of all cattle herds adjacent to or in contact with the infected herd, testing of all herds from which the infected herds had received animals in the preceding years, interviews with owners and managers to determine the history of comingling with wildlife, and comparison of DNA test results of the isolates with those from wildlife and domestic animals. The transmission event in 2002 was previously reported (*7*).

We subjected 248 *B. abortus* isolates from affected cattle and bison herds and surrounding wild elk and bison to a 10-loci variable number tandem repeat assay. We analyzed results using a minimum spanning tree model (*8*).

Cattle and bison herd sizes varied from <50 to >300 animals, and seroprevalence ranged from 0.2% to 20% (Table). For 8 herds, infection initially was detected through required testing of cattle going through markets or to slaughter. Four infected herds were detected by routine testing because of the location of herds in the brucellosis-endemic area, 1 herd was detected because of testing of area herds in proximity to a previously infected herd, and 4 herds were detected by testing required for interstate transport or change of ownership.

Conclusions

Examination of the data from these herds reveals several facts. With few exceptions, herds had low seroprevalence

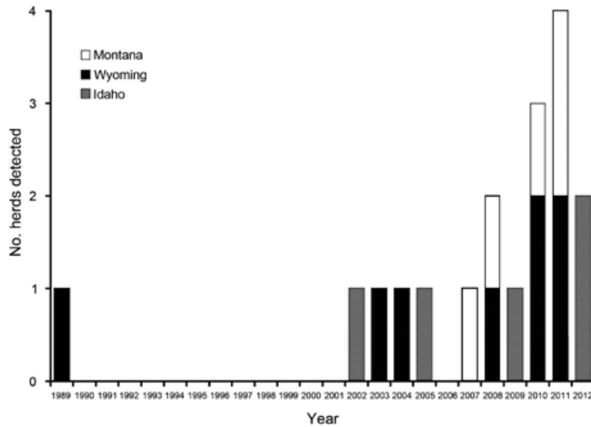

Figure 1. Number of *Brucella abortus*–positive domestic cattle and ranched bison herds (combined) detected each year, Greater Yellowstone Area, USA, 1989–2012.

at time of detection (14 of 17 herds: <10%; 11 of 17 herds: <3%). Additionally, few or no abortions were reported by herd owners or managers. These findings probably are due to rigorous surveillance and the widespread use of vaccination in GYA herds. The attenuated live vaccine, strain RB51, is efficacious in decreasing abortions but does not prevent infection (*9*). The herd with highest seroprevalence (herd no. 4) had the lowest percentage of vaccinated cattle (41%). Nearly all animals in other herds had received calfhood vaccination. In several cases, management actions may have increased risk for exposure (i.e., allowing elk to feed with cattle and placing cattle in pasture with elk during late winter or spring). In Wyoming and Idaho, proximity to elk feeding grounds varied. With 1 exception (herd no. 7), owners or investigators reported that elk were sharing the premises with cattle. Herd no. 7's owner and his son were employed at a nearby elk feeding ground, where their duties included removal of elk fetuses, which created the potential for transmission through fomites. Information obtained from state wildlife agencies indicated that in every transmission case, serologic surveillance of elk in the area showed some level of infection in that species. On the basis of the 10-loci variable number tandem repeat assay, the *B. abortus* isolates recovered from cattle and farmed bison are very closely related to—and sometimes indistinguishable from—isolates from wild elk (Figure 2).

Seventeen instances of brucellosis transmission from elk to livestock were reported during the last decade. This crescendo of interspecies transmission in all 3 GYA states and involving ranches in proximity to and remote from elk feeding grounds suggests a change or combination of changes in risk factors in the GYA ecosystem. Until the discovery of increasing prevalence in non–feeding ground elk (2006–2008) (*10*), *B. abortus* infection was not believed to have been self-sustaining in these populations (*6*). This belief was supported by high seroprevalence in populations in proximity to feeding grounds, with a marked decrease in prevalence proportional to distance from feeding grounds. In the last decade, however, seroprevalence in some non–feeding ground elk herds has increased to levels similar to those of feeding ground herds, suggesting that brucellosis is now self-sustaining in these populations (*11*).

Several factors are likely to have contributed to changes in elk distributions and the resulting increases in brucellosis in some populations and its transmission to livestock. These include population and density increases (*11*,*12*), changes in land management that created safe havens for

Table. Cattle and ranched bison herds found infected with *Brucella abortus* due to transmission from elk, Greater Yellowstone Area, USA

Herd no.	County, state	Species	Herd size	Date detected	Seropositive, %	Culture results	Distance to feeding ground, km
1	Fremont, ID	Cattle	50–100	2002 Apr	12.0	Biovar 1	50*
2	Sublette, WY	Cattle	>300	2003 Oct	9.9	Biovar 1	2.4
3†	Teton, WY	Cattle	>300	2004 Jun	1.9	Biovar 4	Adjacent
4	Bonneville, ID	Cattle	<50	2005 Aug	20.0	Biovar 1	85‡
5	Park§, MT	Cattle	>300	2007 May	0.2	Biovar 1	>100
6	Park, MT	Cattle	<50	2008 May	2.9	Biovar 1	>100
7	Sublette, WY	Cattle	>300	2008 Jun	5.5	Biovar 4	24
8	Jefferson, ID	Cattle	>300	2009 Jul	1.5	Biovar 1	85
9	Park, WY	Cattle	>300	2010 Oct	1.1	Biovar 1	>100
10	Park, WY	Bison	200–300	2010 Nov	11.5	Biovar 4	>100
12	Park, WY	Cattle	>300	2011 Feb	0.9	Biovar 1	>100
13	Park, WY	Cattle	>300	2011 Sep	1.2	Biovar 1	>100
14	Park, MT	Cattle	>300	2011 Sep	2.0	Biovar 1	>100
15	Madison, MT	Bison	>300	2011 Nov	0.2	Biovar 1	>100
16	Fremont, ID	Cattle	50–100	2012 Apr	5.8	Biovar 1	90
17	Bonneville, ID	Bison	200–300	2012 Mar	0.7	Biovar 4	40

*Elk were also intentionally fed on ranch by owner.
†Brucellosis was detected in 2 herds that were pastured together during spring of 2004. This was considered a single transmission event, and statistics are given for the combined herds.
‡Herd located 0.8 km from site where elk feeding ground had been until 2003.
§This herd was discovered infected in Carbon County, MT, in 2007, but animals had been transported from Park County in 2005. Index cow aborted in 2005 and did not calve in 2006.

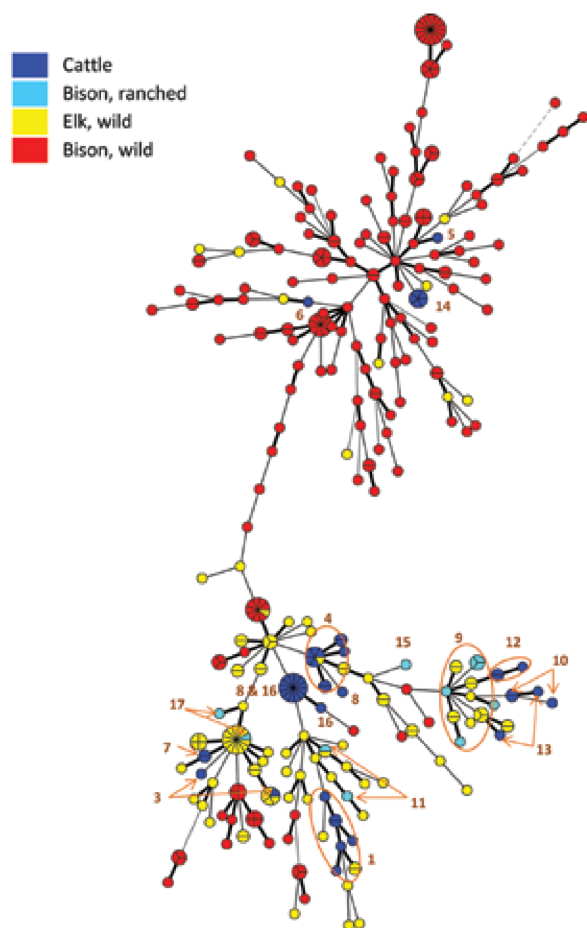

Figure 2. Minimum spanning tree generated from variable number tandem repeat (VNTR) data for 348 Brucella abortus isolates in the National Veterinary Services Laboratory database. Each sphere, or node, represents a unique VNTR type. Nodes are color coded according to the source of the isolate, and segments of nodes represent isolates from different animals with the same VNTR profile. The numbers represent the herd designations as indicated in the Table (note that herd no. 2 is not represented in this figure).

elk (*13*), and reintroduction of wolves to the GYA (*14*). Other factors that might have had local or general effects include climatic and snowfall changes (*15*), reduction of habitat by urbanization, and increased use of motorized backcountry vehicles. Changes in elk and cattle brucellosis surveillance during the last decade do not account for the disease spread in elk or increased transmission to livestock.

If brucellosis continues to increase among free-ranging elk populations remote from feeding grounds, the area to which brucellosis is endemic is likely to expand and the risk for transmission to livestock and the public will increase, in part reversing the hard-fought gains of the past 75 years in eliminating the disease in the United States. Gaining a better understanding of ecologic and sociologic changes in the GYA and their impact on the epidemiology of this wildlife–livestock–human interface disease is essential to developing effective management strategies.

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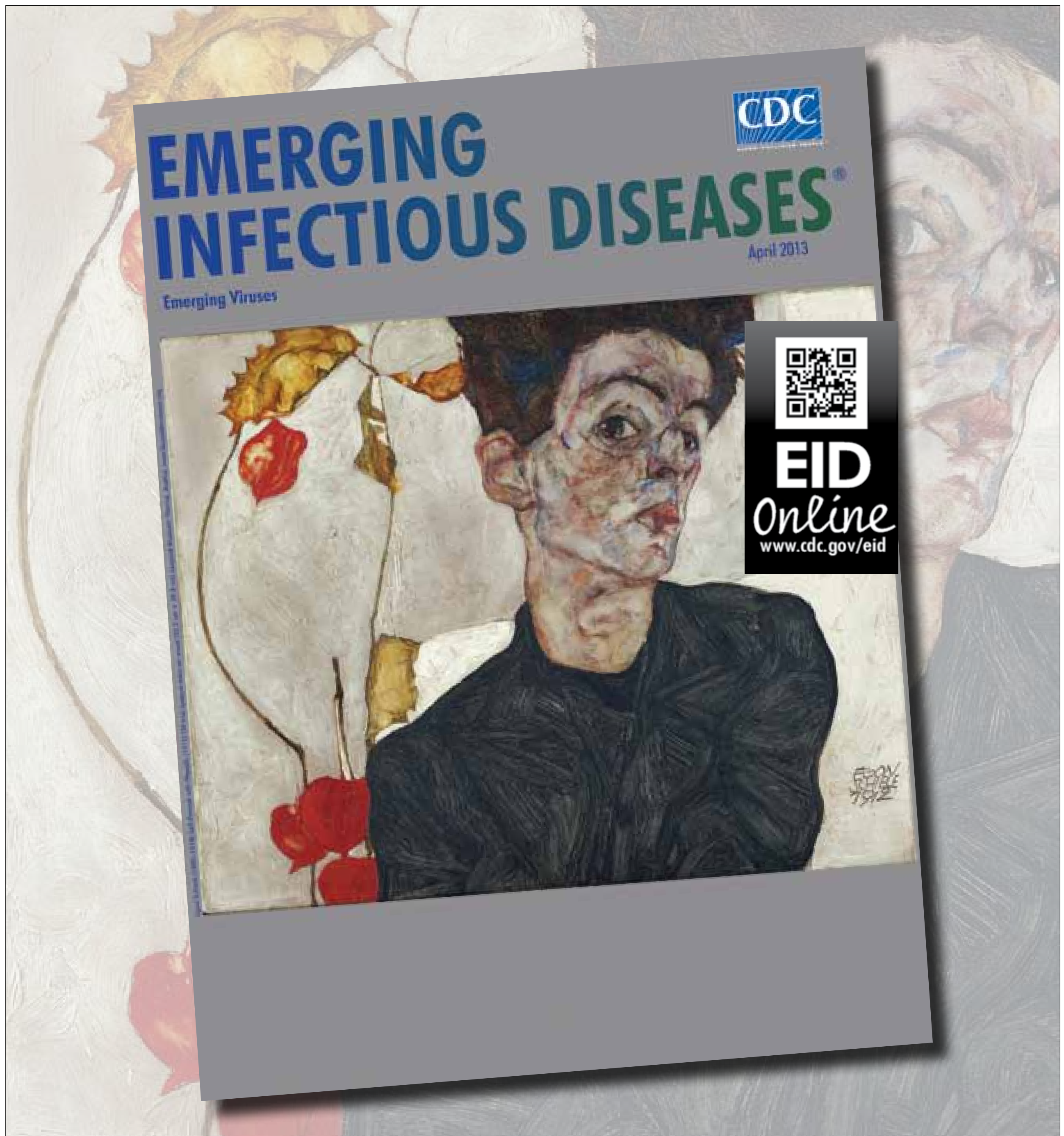

Concomitant Human Infections with 2 Cowpox Virus Strains in Related Cases, France, 2011

Corinne Ducournau, Audrey Ferrier-Rembert, Olivier Ferraris, Aurélie Joffre, Anne-Laure Favier, Olivier Flusin, Dieter Van Cauteren, Kaci Kecir, Brigitte Auburtin, Serge Védy, Maël Bessaud, and Christophe N. Peyrefitte

We investigated 4 related human cases of cowpox virus infection reported in France during 2011. Three patients were infected by the same strain, probably transmitted by imported pet rats, and the fourth patient was infected by another strain. The 2 strains were genetically related to viruses previously isolated from humans with cowpox infection in Europe.

The Study

On September 12, 2011, an 8-year-old girl (patient 1) was admitted to the regional hospital of Epinal, France, for a cutaneous lesion on the lateral part of her neck that had evolved to a necro-ulcerative rash (Figure 1, panel A). Her sister (patient 2, age unknown) had similar cutaneous lesions. The family had purchased 4 rats (*Rattus norvegicus*) from a pet shop on August 19, 2011. The rats had been imported from a breeding facility in the Czech Republic by a local pet dealer. Locomotor disorders developed in 1 rat, and it died 4 days after the purchase. Two other rats became ill during the following weeks: the first, displaying symptoms of coryza, was examined by a veterinarian on September 5; the second was examined by the same veterinarian on September 12 for a vestibular syndrome that evolved to severe respiratory failure and then death on September 15. The fourth rat died without visible signs of disease. No biologic samples were collected.

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Patient 3 was the 26-year-old female veterinarian who examined the sick rats. She kept their corpses in her office for a few days before destroying them. On September 20, she displayed cutaneous lesions similar to those of patients 1 and 2; samples of her lesions were collected 3 days later.

Patient 4 (age unknown) was the cousin of patients 1 and 2. He spent a few days in their house several weeks after the purchase of the rats but did not report any direct contact with them. On October 13, he displayed cutaneous lesions that were noticeably smaller than those of the other patients.

We conducted molecular testing of the patients' lesion samples by using 14-kDa protein gene–targeting real-time PCR for orthopoxvirus detection (*1*). All samples gave a positive result with the orthopoxvirus probe and a negative result with the variola virus-specific probe, indicating the presence of orthopoxviruses with the exclusion of variola virus. The ≈600-nt PCR products were sequenced, and a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) identified cowpox virus (CPXV; family *Poxviridae*, genus *Orthopoxvirus*) in all cases.

The clinical samples were inoculated onto Vero cells (ATCC CC-81). Samples from patients 1, 2, and 3 gave rise to massive cytopathic effect (CPE), whereas no CPE was observed in cell cultures inoculated with samples from patient 4 (Table). Therefore, the samples from patient 4 were used to inoculate 2 additional cell lines, BHK-21 (ATCC CCL-10) and MRC-5 (ATCC CCL-171). A low CPE was observed 7 days postinoculation for both cell lines. An additional passage (passage 2) was performed on Vero cells for all the isolates. At this second passage, compared with the 3 other isolates, CEPAD335 (from patient 4) produced smaller plaques on Vero cells (Figure 1, panels B, C). Negative-stain electron microscopy performed on supernatant of cell cultures infected by isolates CEPAD332 and CEPAD335 showed typical poxvirus-like particles (Figure 1, panels D, E).

After DNA extraction from cell culture supernatants, the viral genome was amplified by PCR by using primer pairs that targeted 3 additional genomic regions (*2*,*3*): hemagglutinin (HA; ≈900 nt), C18L (≈850 nt), and G1L (≈850 nt), according to CPXV strain GRI nomenclature (Figure 2) (*4*). The sequences of the corresponding amplicons were determined and were deposited into GenBank under accession nos. KC592396–KC592411. The isolates CEPAD332, 333, and 336 were identical in the C18L, G1L, and HA regions; CEPAD333 diverged slightly from CEPAD332 and CEPAD 336 in the 14-kDa region (3 substitutions out of 576 nt). The results suggested that these 3 isolates originated from a unique

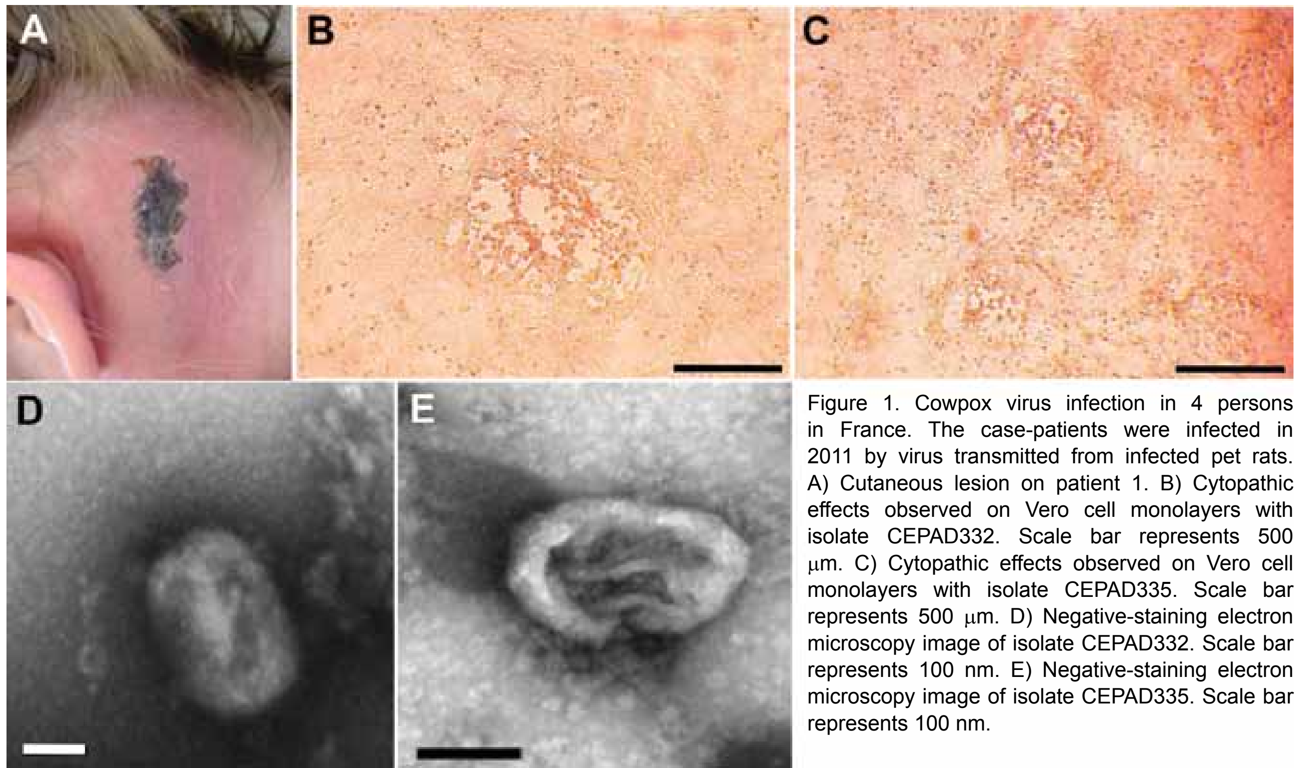

Figure 1. Cowpox virus infection in 4 persons in France. The case-patients were infected in 2011 by virus transmitted from infected pet rats. A) Cutaneous lesion on patient 1. B) Cytopathic effects observed on Vero cell monolayers with isolate CEPAD332. Scale bar represents 500 μm. C) Cytopathic effects observed on Vero cell monolayers with isolate CEPAD335. Scale bar represents 500 μm. D) Negative-staining electron microscopy image of isolate CEPAD332. Scale bar represents 100 nm. E) Negative-staining electron microscopy image of isolate CEPAD335. Scale bar represents 100 nm.

CPVX strain. By contrast, CEPAD335 clearly diverged from the 3 others in all 4 studied regions (nucleotide divergence >2.5% in each region, large nucleotide insertions in the G1L region), indicating that patient 4 was infected by a different CPXV strain.

Phylogenetic studies were conducted by comparing the sequences of the 4 isolates with CPXV reference sequences (*4*–*6*) and sequences of CPXV isolates previously isolated in Europe (*7*–*11*) (Figure 2). In the HA region, CEPAD332, 333, and 336 showed 100% identity to several strains isolated in France and Germany in 2008 and 2009 from patients also infected by imported pet rats (*7*,*9*,*11*). Because these strains have not been sequenced in other genomic regions, it was impossible to study their relationships with our isolates.

In 3 genomic regions, CEPAD335 was closely related to isolate NANCY, a CPXV isolated in France in 2001 (*4*). Only 1 nt change was observed in each C18L and HA region; in G1L, CEPAD335 sequence contained a 84 nt-long insertion compared with NANCY, but only 1 nt change was observed in their matching parts. By contrast, CEPAD335 and NANCY were found relatively distant in the 14-kDa gene phylogram (18 nt changes out of 594 nt).

Conclusions

At least 2 different CPVX strains were involved in the occurrence of 4 related human cases. Patients 1, 2, and 3 were probably infected by the same strain acquired from the pet rats, but the origin of the infection with the second strain remains unclear. We propose 2 hypotheses to explain these infections. The first hypothesis is the co-infection of the pet rat batch by the 2 CPXV strains. However, patient 4 did not report direct contact with the pet rats; furthermore, his lesions were observed at least 4 weeks after the death

Table. Phenotypic properties of cowpox virus isolates from 4 patients, France, 2011*

Patient no.	Biologic samples in different cell lines			Isolate	Morphologic characteristics of isolates after passage 2 in Vero cells
	Vero	MRC5	BHK21		
1	High CPE	ND	ND	CEPAD332	High CPE on day 3, mean plaque size 600 μm ± 100 μm
2 (sister of patient 1)	High CPE	ND	ND	CEPAD336	High CPE on day 3, mean plaque size 600 μm ± 100 μm
3 (veterinarian)	High CPE	ND	ND	CEPAD333	High CPE on day 3, mean plaque size 600 μm ± 100 μm
4 (cousin of patients 1 and 2)	No CPE	Low CPE	Low CPE	CEPAD335	Low CPE on day 7, mean plaque size 300 μm ± 50 μm

*CPE, cytopathic effect; ND, not done.

of the rats, while the CPXV incubation period is believed to be <2 weeks in humans (*12*). Alternatively, this patient might have been infected by other animals; he reported regular, close contact with horses and domestic cats that go outdoors, and horses and cats are known to transmit cowpox viruses to humans (*12*). In France and other European countries, human infection with CPXV has been known as a zoonosis transmitted mainly by feral cats and, more rarely, dairy cows. However, the outbreak of CPXV that occurred in Germany and France in 2008 in pet rat owners brought attention to this new source of infection. Several cases of pet rat–to–human CPXV transmission have been reported recently (*7*,*9*,*13*–*15*). The use of rodents as pets is likely to lead to an increase in CPXV human cases in the future, especially because persons younger than 30 years do not exhibit cross-reactive immunity conferred by smallpox vaccination, which was stopped at the end of the 1970s.

Veterinary investigations were conducted in the pet shop in France where the 4 rats in this study had been purchased. Because no animals were ill, no samples were

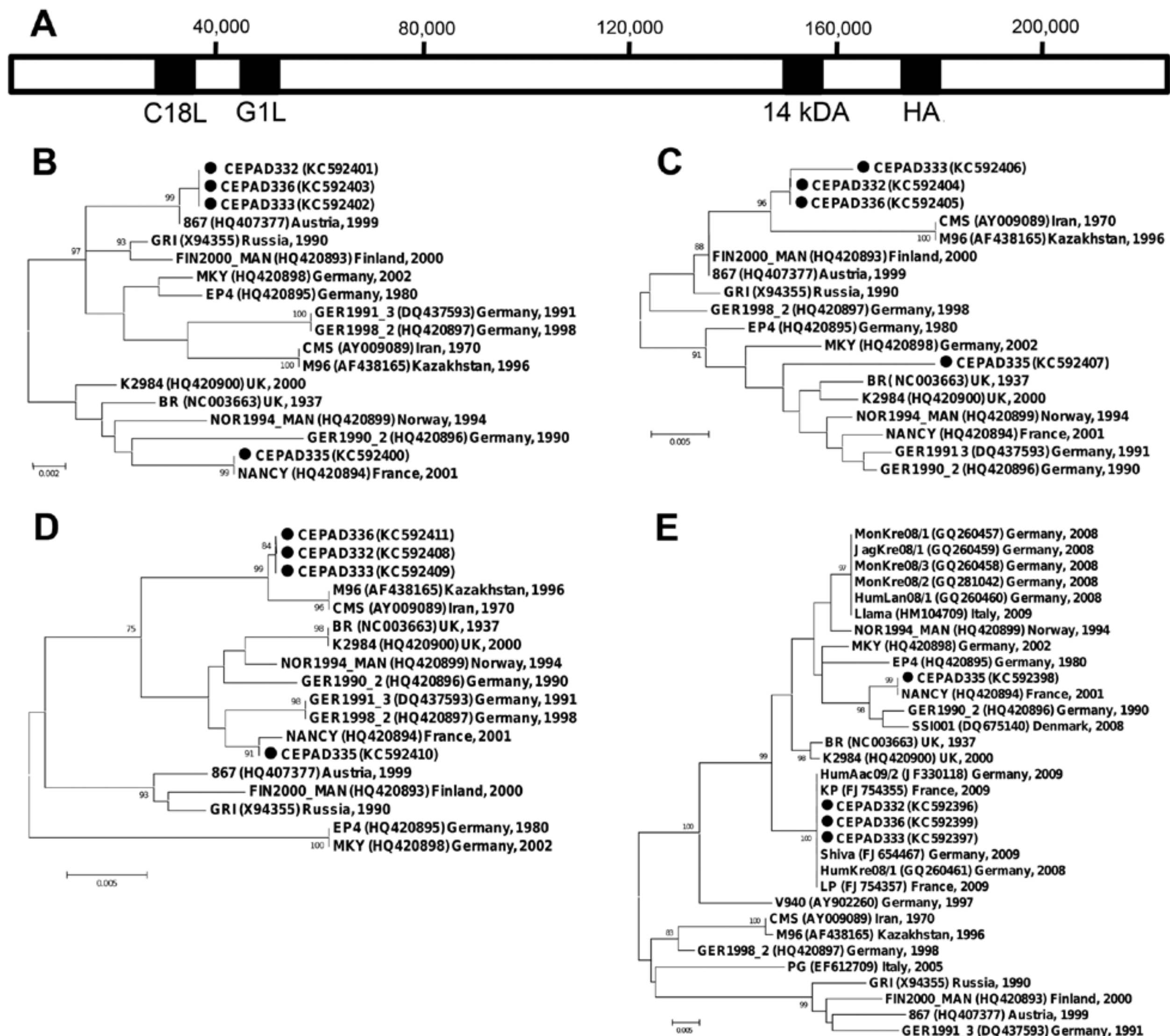

Figure 2. Schematic representation of cowpox virus (CPXV) GRI genome (A) and phylogenetic relationships between 4 genomic regions of CPXV isolates collected in France during 2011 and other CPXVs: C18L (B), 14-kDa (C), G1L (D), hemagglutinin (E). The sequenced regions are shaded in black in panel A. Nucleotide sequences were aligned by using CLC Main Workbench 6.0 software (CLC Bio,Aarhus, Denmark). Neighbor-joining phylograms were constructed in MEGA4 (www.megasoftware.net) by using the maximum composite likelihood method; the robustness of the resulting trees was assessed with 1,000 bootstrap replicates. The length of the branches is proportional to the number of base substitutions per site. The percentage bootstrap values are indicated if >70. Circles indicate the 2011 isolates from France; for other isolates, the year and country of isolation are indicated. Scale bars indicate nucleotide substitutions per site.

collected. No investigations were performed at the facility in the Czech Republic. Four previous human CPXV contaminations observed in France in 2009 were caused by contacts with infected pet rats that also originated from the Czech Republic (*9*).

Small wild rodents are believed to constitute the natural reservoir of CPXV, but this virus is able to infect a wide range of mammals, such as cats, cows, horses, elephants, and dogs (*12*). Our observation of human infections by 2 different CPXV strains genetically closely related to strains isolated years ago in France and Germany suggest the circulation of genetically stable viral strains among wild or domestic animals and their sporadic emergence among humans. Further studies regarding the molecular relationships between CPXV strains isolated from humans and from wild or domestic animals would help clarify the epidemiology of this virus.

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Ms Ducournau is a BSc research associate at French National Reference Center for Orthopoxviruses. She is interested in poxvirus diagnosis and surveillance and has developed kits for detecting poxviruses.

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Zoonotic *Onchocerca lupi* Infection in Dogs, Greece and Portugal, 2011–2012

Domenico Otranto, Filipe Dantas-Torres, Alessio Giannelli, Maria Stefania Latrofa, Elias Papadopoulos, Luís Cardoso, and Helder Cortes

Onchocerca lupi infection is reported primarily in symptomatic dogs. We aimed to determine the infection in dogs from areas of Greece and Portugal with reported cases. Of 107 dogs, 9 (8%) were skin snip–positive for the parasite. DNA sequences of parasites in specimens from distinct dog populations differed genetically from those in GenBank.

Zoonotic onchocercosis has been attributed to species that primarily infest cattle (*Onchocerca gutturosa*), horses (*O. cervicalis*), the European deer (*O. jakutensis*), and wild boars (*O. dewittei japonica*) (*1*). In their definitive hosts, all these species localize in subcutaneous tissues, muscular fasciae, or cervical ligaments, whereas in humans, *O. gutturosa* and *O. cervicalis* also have an ocular localization (reviewed in *2*).

O. lupi is a recently recognized parasite causing nodular lesions associated with ocular disease (i.e., conjunctivitis, ocular swelling, photophobia, lacrimation, discharge, exophthalmia) in dogs (*3*). The zoonotic potential of this filarioid has been suspected (*4*) but has only recently been demonstrated in a patient from Turkey (*5*). Ocular cases in humans are increasingly being reported worldwide, including in Iran (*6*), Turkey, and Tunisia (*7*). In addition, *O. lupi* infection was recently diagnosed near the spinal canal in a 22-month-old child from Arizona, USA (*2*).

Since its first description in a Caucasian wolf (*Canis lupus*) from Georgia in 1967 (*8*), *O. lupi* remained almost unknown for decades until being reported in dogs from southern Europe (Greece, Portugal) and central Europe (Germany, Hungary) (Figure 1, panel A) (reviewed in *3*). In the western United States, canine onchocercosis (*9*) has been attributed to species parasitizing other hosts (i.e., cattle, horses, or wild ungulates), but such cases were probably caused by *O. lupi*, as recently confirmed morphologically and molecularly in 2 cats (*10*) and 4 dogs (*11*).

Several aspects of the biology and ecology of *O. lupi* remain unknown and the knowledge of its actual distribution is limited to a few case reports. We conducted an epidemiologic survey to estimate the occurrence of *O. lupi* infection in dog populations from areas of Greece and Portugal where multiple (*12*) or single (*13*) cases, respectively, have been reported. The genetic make-up of the parasites in the specimens collected from both canine populations was assessed by comparing them with sequences available in GenBank.

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The Study

In June 2011 and November 2012, we sampled a total of 107 dogs of different ages, sexes, and weights from a site in Greece (site A, 23 dogs) and a site in Portugal (site B, 84 dogs). Briefly, site A was located within the boundaries of Amaxades (25°04′27″E, 41°07′12″N, altitude 56 m), a small village of ≈1,000 inhabitants between Xanthi and Komotini, on the border between Greece and Turkey. This is a traditionally agricultural and poor dry area, with tobacco and sunflower plantations among the most important cultivations. Site B was in Olhão (southern Portugal, 7°50′33″O, 37°01′42″ N, altitude 8 m), a municipality with 45,000 inhabitants representing ≈10% of the population of the Algarve, a region in which tourism is a major economic activity (Figure 1, panel A). Both sites are located along or near the seacoast, where small river streams run during the rainy season and dry out during the summer. Animals from site A were shepherd dogs living in small rural communities with sheep and goats; animals from site B were stray dogs kept according to Portuguese regulations in a shelter until they were adopted or euthanized. The shelter was surrounded by a large area of salt water in open facilities at which sea salt was collected.

Skin samples were collected by using a disposable scalpel over an area of ≈0.2 × 0.2 × 0.2 cm from inter ocular frontal area of the head and soaked at 37°C in saline solution for 1 h. Sediments (20 μL) were individually observed under light microscopy (i.e., 1 field of 18 × 18 mm coverslip). Microfilariae were counted, identified according to morphologic keys (*3,14*), and differentiated from those of filarioid species most commonly retrieved in dogs from the Mediterranean region (*15*). Briefly, microfilariae of *O. lupi* had a short flattened unsheathed body (mean length 110.1 ± 7.5 μm, width 6.8 ± 1.2 μm) with a rounded head bearing a tiny tooth on the cephalic

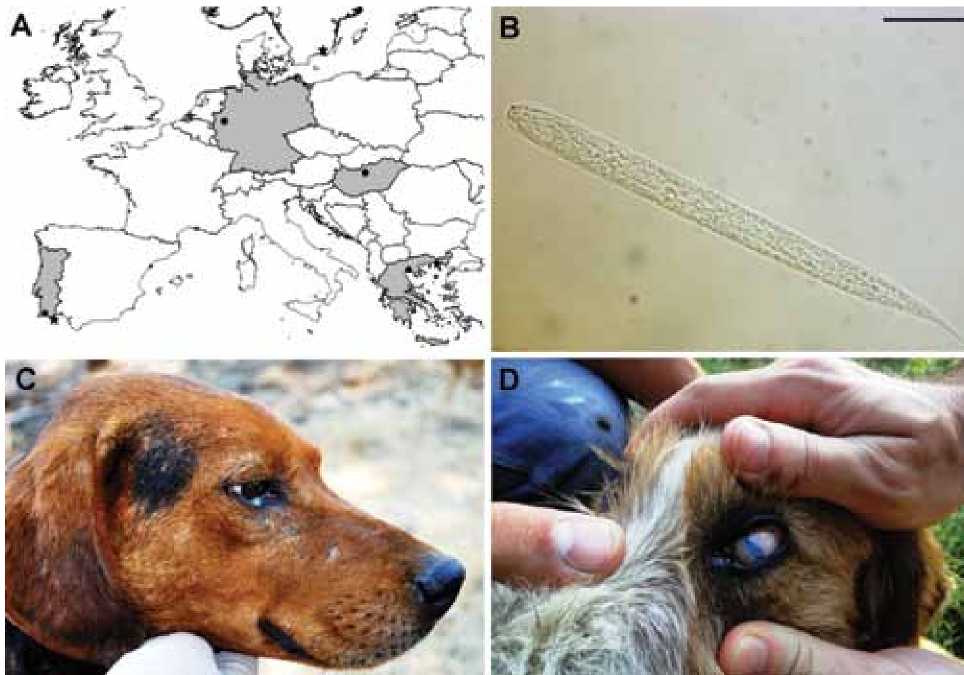

Figure 1. A) Areas (in gray) and localities (black dots) where *Onchocerca lupi* infections were reported and sampling sites (asterisk) from Greece (site A) and Portugal (site B). Scale bar = 500 km. B) Light microscopy image of microfilariae of *O. lupi* detected at the skin sediment. Scale bar = 20 μm. Original magnification ×100. Dog positive for *O. lupi* had conjunctival swelling and a purulent mucus discharge (C), or blindness with severe keratitis and uveitis (D).

edge. The body was blunt with a short bent tail of ≈11.7 μm (Figure 1, panel B).

Of 107 dogs, 9 (8%; 2 from site A and 7 from site B) were positive for *O. lupi* microfilariae, with a maximum of 480 microfilariae detected in a single sample. Animals positive for *O. lupi* at site A displayed a range of ocular alterations from conjunctival swelling and mucopurulent discharge (Figure 1, panel C) to blindness (Figure 1, panel D). Conversely, all animals from site B were asymptomatic.

After microscopic observations, microfilariae were removed with a 10-mL pipette and placed in saline solution in single tubes at –20°C, before DNA was extracted and partial (582 bp) cytochrome *c* oxidase subunit 1 (*cox*1) and 12S rDNA (304 bp) gene fragments amplified as described elsewhere (*7*). In accordance with the morphologic identification, the BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of both genes showed a high overall nucleotide homology with sequences of *O. lupi* available in GenBank (i.e., 99% for 12S rDNA: GU365879; from 98% to 100% for *cox*1 accession numbers reported in Figure 2).

All *cox*1 sequences available in GenBank for *O. lupi* were analyzed by using MEGA5 (www.megasoftware.net) and showed a low intraspecific variability (mean 0.7%, range 0%–2.1%). All *cox*1 sequences of *O. lupi* were identical according their geographic provenience (i.e., 2 in Greece, 1 in Turkey, 2 in Hungary, 9 in Portugal, and 7 in the United States) and had a high nucleotide similarity (mean 99.2%, range 99.6%–100%), except for those from Portugal, which differed considerably from the others (mean 98.2%, range 97.9%–98.2%). The phylogenetic analysis using *cox*1 sequences by MEGA5 under the neighbor-joining method confirmed that *O. lupi* clustered with those in the genus, to the exclusion of other filarioids. In particular, specimens of *O. lupi* from Portugal formed a sister clade (with a strong nodal support) with those from other origins (Figure 2). Sequences were deposited in GenBank under accession numbers KC686701–KC686702 and KC686703–KC686704 for *cox*1 and 12S rDNA, respectively).

Conclusions

Our data clearly showed that *O. lupi* infection occurred in dogs from both sampling areas (overall positivity 8%), and that dogs exhibited different clinical features that ranged from no apparent clinical sign to blindness. The lack of any ocular lesions in *O. lupi*–infected dogs from site B might be due to differences in sampling times (i.e., during summer, site A, and late autumn, site B), and thus in adult worm development and/or in the pathogenicity of the populations of parasites, which might reflect the variations documented in *cox*1 sequences of individuals from Portugal and those populations of *O. lupi* from different geographic areas. In addition, aberrant infection of adult nematodes in dogs cannot be ruled out, as recently demonstrated in a human patient from Arizona in whom *O. lupi* was found in the spinal canal (*2*). Whether asymptomatic animals can be a source of *O. lupi* infection for the vectors (which remain unknown) needs to be assessed (*16* in online Technical Appendix, wwwnc.cdc.gov/EID/articlepdfs/19/12/13-0264-Techapp1.pdf). Despite the increasing number of *O. lupi* infections reported in animals

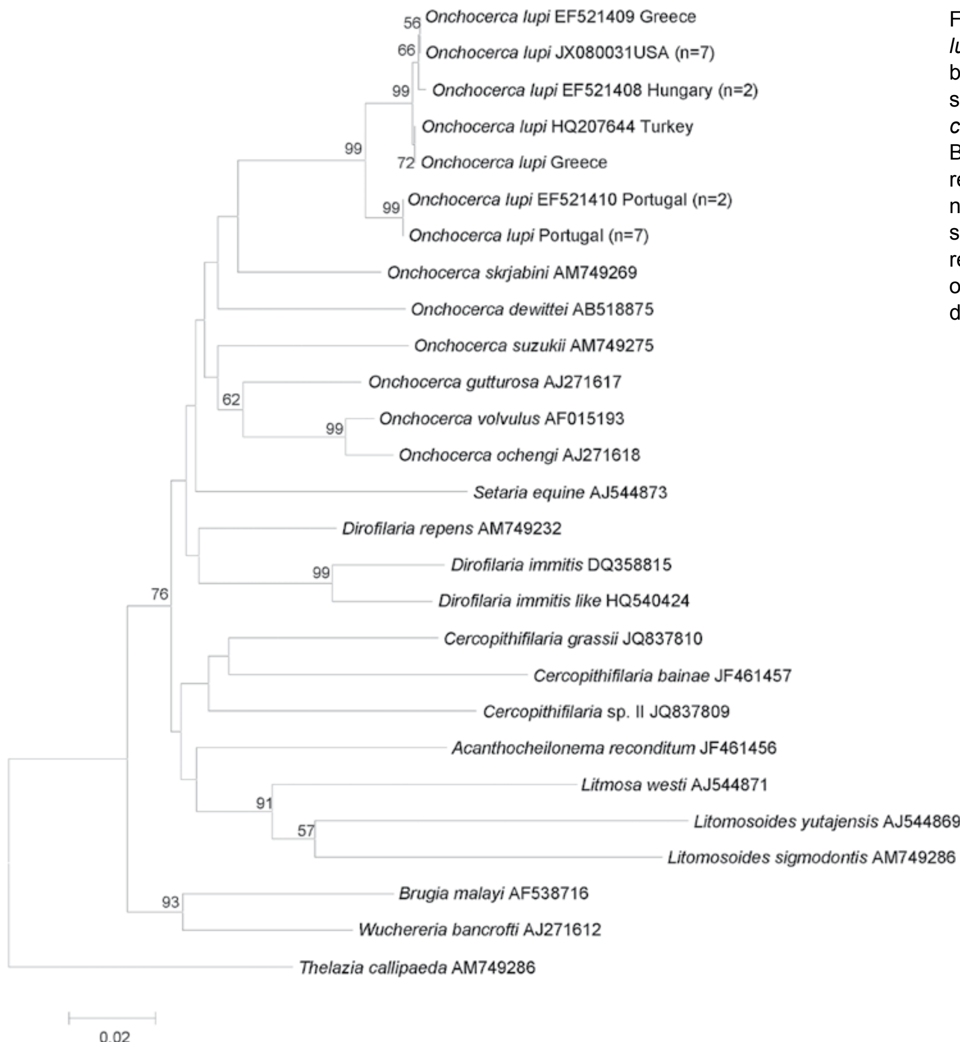

Figure 2. Phylogeny of *Onchocerca lupi* and other filarial nematodes based on cytochrome *c* oxidase subunit 1 gene sequences. *Thelazia callipaeda* was used as outgroup. Bootstrap confidence limits (8,000 replicates). GenBank accession numbers and number of haplotype sequences (in parenthesis) are reported along with their geographic origin. Scale bar indicates genetic differences.

and humans, the difficulties in achieving a reliable diagnosis through the skin-snip technique and the unwillingness of some pet owners to allow collection of a piece of skin from the animal's head, might explain the scant data on *O. lupi*. Therefore, population-based surveys should be performed to estimate the distribution of the infection in dogs and to assess the risk to humans. In addition, further studies are needed to improve understanding of the biology of this parasite, including its hosts and vectors. The reliability of the tools and procedures for diagnosing *O. lupi* infection in dogs and in humans, especially in asymptomatic individuals, also needs to be assessed. Finally, our data should alert physicians and ophthalmologists about the potential risk for *O. lupi* infection in humans and their pets (cats and dogs).

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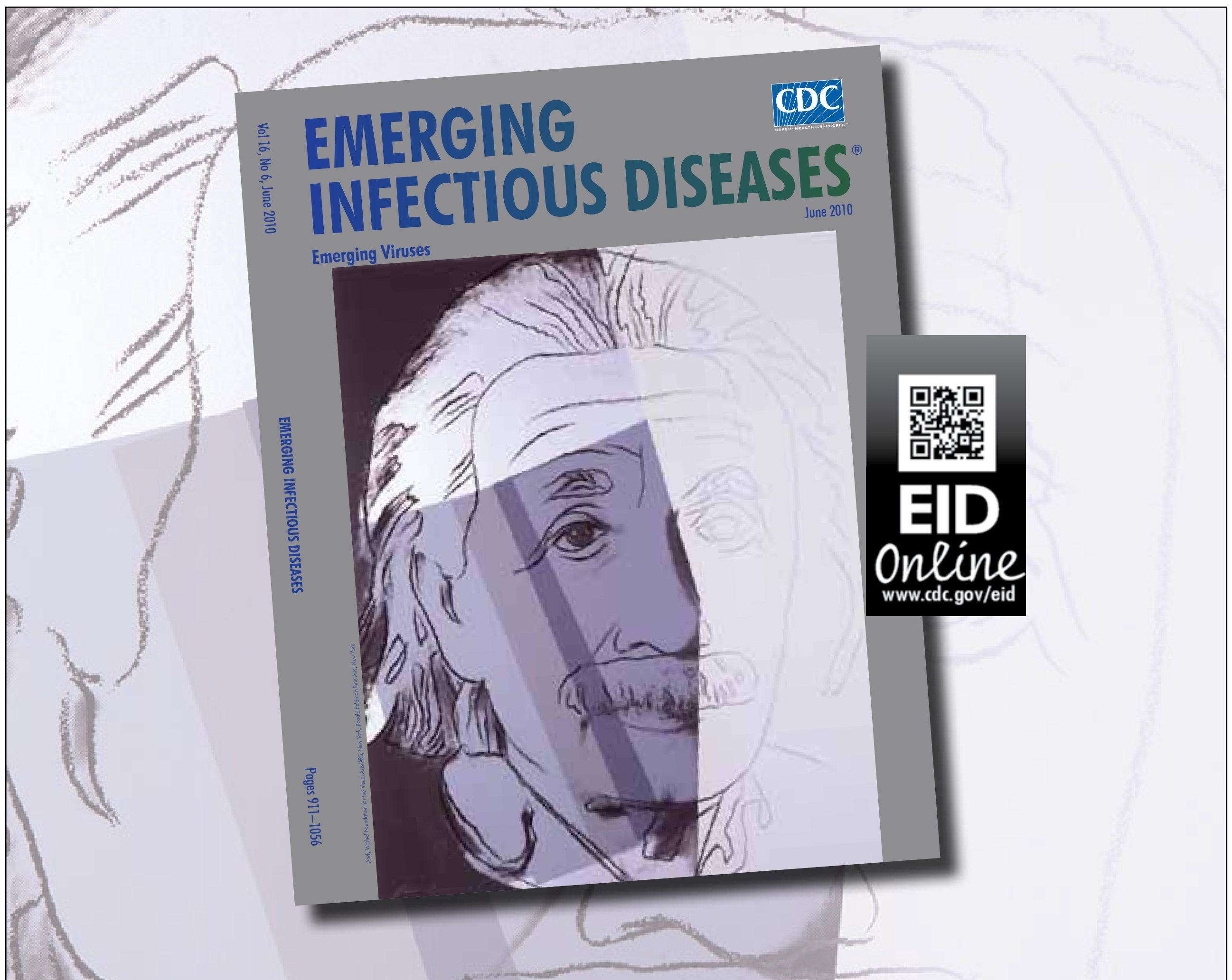

Novel Cause of Tuberculosis in Meerkats, South Africa

Sven D.C. Parsons, Julian A. Drewe, Nicolaas C. Gey van Pittius, Robin M. Warren, and Paul D. van Helden

The organism that causes tuberculosis in meerkats (*Suricata suricatta*) has been poorly characterized. Our genetic analysis showed it to be a novel member of the *Mycobacterium tuberculosis* complex and closely related to the dassie bacillus. We have named this epidemiologically and genetically unique strain *M. suricattae*.

Tuberculosis (TB) is caused by a group of distinct mycobacterial strains that might have evolved as host-adapted ecotypes (*1*) and that are collectively named the *Mycobacterium tuberculosis* complex (MTC) (*2*). In southern Africa, *M. tuberculosis* and *M. bovis* cause TB in numerous animals (*3*), the dassie bacillus infects rock hyraxes (dassies, *Procavia capensis*), and *M. mungi* infects banded mongooses (*Mungos mungo*) (*4–6*).

TB in free-living meerkats (*Suricata suricatta*) from the Kalahari Desert, South Africa, was first reported in 2002 (*7*), and its epidemiology and pathology have been comprehensively described (*8*,*9*). Mycobacterial strains isolated from these animals have been described as *M. tuberculosis* (*7*), *M. bovis* (*8*), and as a "member of the animal-adapted lineage of the MTC" (*10*), raising concerns that the occurrence of TB in these meerkats resulted from anthropogenic exposure to these pathogens and that affected meerkat populations could pose an infection risk to other wildlife, domestic animals, and humans (*8*). However, these studies used genetic analyses designed to differentiate between *M. tuberculosis* and *M. bovis* (*7*) and between these and *M. africanum*, *M. canetti*, *M. microti*, and *M. bovis* BCG (*8*) but not between these strains and the dassie bacillus or *M. mungi*. To gain greater insight into the etiology of this disease, we conducted a more comprehensive genetic analysis of mycobacterial isolates from this meerkat population.

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The Study

Permission to sample meerkats was obtained from the University of Pretoria Animal Ethics Committee. Postmortem examinations were performed on 4 meerkats from the Kalahari Meerkat Project (26°58′S, 21°49′E) that had shown visible disease. Samples from lesions typical of TB in this species (*8*) were used to establish mycobacterial cultures in the BD BACTEC MGIT 960 Mycobacterial Detection System (Becton Dickinson, Franklin Lakes, NJ, USA) (*11*). Four cultures originating from 3 animals were positive by Ziehl-Neelsen stain and were grown further on Difco Middlebrook 7H10 Agar supplemented with 10% OADC Enrichment (Becton Dickinson) for 6–8 weeks, after which DNA was extracted (*11*). However, only 1 isolate (MK172) yielded sufficient DNA for DNA fingerprinting by the IS*6110* method (*12*). PCRs were conducted by using either heat-killed liquid cultures or purified DNA as a template.

Isolates were screened for the presence or absence of 4 phylogenetically informative genomic regions of difference (RDs) (*11*), and all showed deletion of RD9 but not of RD1, RD4, and RD12. This genotype is shared by *M. africanum*, *M. orygis*, and the dassie bacillus (*2*,*11*); isolates were therefore analyzed for the presence or absence of $RD1^{das}$, a genetic marker specific for the dassie bacillus (*5*). Because this RD was deleted in all isolates, these were subsequently analyzed for the presence or absence of N-$RD25^{das}$, $RD5^{das}$, and $RDVirS^{das}$ (*5*); a G→A single-nucleotide polymorphism (SNP) in Rv1510 ($Rv1510^{1129}$); and a single-nucleotide deletion in Rv0911 ($Rv0911^{389}$) (*2*). For all isolates, N-$RD25^{das}$, $RD5^{das}$, and $RDVirS^{das}$ were deleted and $Rv1510^{1129}$ and $Rv0911^{389}$ were present, consistent with the dassie bacillus genotype (*2*,*5*). However, although the $RD5^{das}$ deletion in this bacillus has been caused by the insertion of an inverted IS*6110* sequence (*5*), for the meerkat strain, sequencing of the $RD5^{das}$ PCR product showed this region to be occupied by an IS*6110* sequence in a forward orientation, followed by a proline-proline-glutamate gene homologue.

Spoligotyping was performed according to the internationally standardized method (*13*). However, we repeatedly obtained no amplification of any spacer included in this array. We investigated the possible deletion of the direct-repeat region, the genomic region analyzed by spoligotyping, by attempting to amplify by PCR selected genetic sequences upstream and downstream thereof (Table 1). This analysis confirmed that much, if not all, of the direct-repeat region had been deleted in these isolates, together with ≈3,500 bp upstream and up to 1,700 bp downstream of this region (Table 1).

Additionally, genetic characterization was done by sequencing of fragments of the *gyrB* gene (*2*) and 16S rDNA (*14*). For all isolates, the *gyrB* sequence was consistent with

Table 1. PCR analysis of the genomic regions flanking the direct-repeat region of *Mycobacterium suricattae*

PCR target, bp	Forward primer, 5′→3′	Reverse primer, 5′ →3′	PCR result*
10–200†	TACCTACGCCACCACCTCAAG	TCAGTCTGCCGTGACTTCGG	–
966–1,518†	CCCTATGTGGATGCGTGGTTG	GGGTTTCGGGTTTGGCTTTCG	–
2,214–2,377†	GTGTCGCTGGCTGAGACC	GCTCCTTTCCATTTGCTGTC	–
3,506–3,730†	ACCGATAATCGCTTGACACC	CCCTCGTTCTCTAGCAGCAG	+
60–262‡	ACGTAACTGCCGCAACACCTC	AATATACGACATCAGCGACAA	–
335–906‡	CGGCTGCGAGTGGGCATTTAG	TCCCTGGCGGAGTTGAACGG	–
1,702–1,931‡	TATCTCCGGCTCTCTTTCCA	TCTTTAAGGACACCGCGTTC	+
2,603–2,763‡	GTTCCGATAGGCGAGAACAG	CCAGTTCGGGAAGGTAGTCA	+

*–, no product; +, successful amplification.
†Upstream of *M. tuberculosis* H37Rv direct-repeat region.
‡Downstream of *M. tuberculosis* H37Rv direct-repeat region.

that of *M. africanum*, *M. pinnipedii*, and the dassie bacillus (*2*). However, the 16S rDNA sequence differed from that of all other MTC members by having a T→G SNP at position 214 (16S $rDNA^{214}$). Analysis by mycobacterial interspersed repetitive unit–variable number tandem repeats (*15*) identified 2 strain variants in our sample set (Table 2); IS*6110* DNA fingerprint analysis (*12*) of isolate MK172 showed it to contain 21 copies of the IS*6110* insertion sequence element (Figure 1).

Conclusion

We genetically characterized the causative pathogen of meerkat TB as a novel MTC strain that has several genetic features typical of the dassie bacillus and *M. mungi* (Figure 2). However, this pathogen differs from the closely related dassie bacillus in its mycobacterial interspersed repetitive unit–variable number tandem repeats patterns (Table 2) by being a unique $RD5^{das}$ variant and by containing 21 copies of the IS*6110* insertion element (Figure 1) (compared with 10–15 copies in the dassie bacillus) (*2*). This evidence of IS*6110* copy number expansion might indicate involvement of this insertion sequence in the occurrence of other genetic deletions in this strain, including those in the direct-repeat region. Notably, in addition to the novel SNP 16S $rDNA^{214}$, the loss of the direct-repeat region spacers, which are routinely screened for by spoligotyping, distinguishes this strain from all other MTC members (*1*,*13*,*14*). As evidenced by their shared RDs and SNPs, the genetic homogeneity of multiple isolates of this distinctive strain suggests that it has undergone selective evolution, possibly through adaptation to its meerkat host (*1*). It is highly pathogenic in this species and seems to be substantially more virulent than the genetically similar dassie bacillus (*4*,*5*). As such,

Table 2. MIRU-VNTR patterns of *Mycobacterium suricattae* and representative isolates of selected members of the *M. tuberculosis* complex*

	MIRU-VNTR copy number			
Locus	*M. africanum*†	*M. mungi*†	*M. suricattae*	Dassie bacillus†
MIRU 2	2	2	2	2
VNTR 424/Mtub04	4	3	3‡, 2§	2
VNTR 577/ETR-C	5	3	5	5
MIRU 4/ETR-D	2	3	2	3
MIRU 40	2	1	2	2
MIRU 10	7	5	6	7
MIRU 16	4	3	2	3
VNTR 1955/Mtub21	4	3	3	3
MIRU 20	2	2	2	2
VNTR 2163b/QUB11b	5	–	–	7
VNTR 2165/ETR-A	6	6	–	6
VNTR2347/Mtub29	3	3	3	3
VNTR 2401/Mtub30	4	4	4‡, 5§	3
VNTR 2461/ETR-B	4	4	5	4
MIRU 23	4	4	4	4
MIRU 24	2	2	3	2
MIRU 26	4	4	4	5
MIRU 27	3	3	1	4
VNTR 3171/Mtub 34	3	3	3	3
MIRU 31/ETR-E	5	8 and 9	5	5
VNTR 3690/Mtub 39	4	–	8	5
VNTR 4052/QUB 26	6	–	3	4
VNTR 4156/QUB 4156	3	–	1	3
MIRU 39	2	2	2	2

*MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number tandem repeats; –, no amplification.
†From Alexander et al., 2010 (*4*).
‡Copy number of 3 isolates from 2 meerkats, including MK172.
§Copy number of a fourth isolate from a third meerkat.

Figure 1. IS*6110* restriction fragment length polymorphism patterns of A) a reference strain of *Mycobacterium tuberculosis* (Mt14323) with selected fragment lengths indicated in kilobases, and B) *M. suricattae* (isolate MK172).

to distinguish this epidemiologically unique strain from other MTC members, we have named it *M. suricattae* after the host species from which it has been isolated.

The identification of this bacillus in Africa is further evidence that the early evolution of the animal-adapted MTC strains occurred on this continent. Strains derived from the early diversification of the RD9-deleted lineage include *M. africanum*, which has been almost exclusively isolated in West Africa (*2*); *M. mungi*, which was isolated from African mongooses (*6*); and *M. orygis* and the dassie bacillus, which have been isolated from animals mainly originating from this continent and the Middle East (*2*,*4*,*5*).

Of these strains, *M. africanum* subtype I and the dassie bacillus share a unique common progenitor (*2*); our study confirms the shared SNP Rv1510^{1129} as a genetic marker thereof (Figure 2). Given that *M. africanum* might have an unidentified West African animal host (*1*), it might be useful to consider that other members of this lineage have become established in highly gregarious small mammal hosts, including 2 mongoose species.

This study demonstrates that the occurrence of TB in the Kalahari meerkats might not be indicative of an external infectious source of *M. tuberculosis* or *M. bovis*, as has been reported (*6*,*8*). Rather, our findings suggest that the disease is caused by an indigenous MTC member, which we have named *M. suricattae*. Our limited sample set precludes a detailed analysis of the epidemiology of this

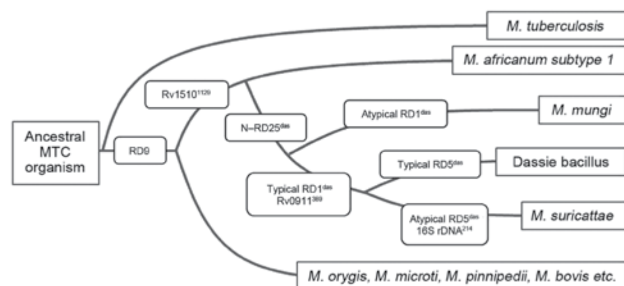

Figure 2. Phylogeny of the *Mycobacterium tuberculosis* complex (MTC) detailing relevant genetic regions of difference (RDs) and single-nucleotide differences that distinguish between *M. africanum* subtype 1 and the small African mammal–adapted members of these strains.

pathogen; however, the identification of this strain and the characterization of several of its discriminatory genetic markers will be useful for future investigations of the ecology and evolution of the African animal–adapted members of the MTC.

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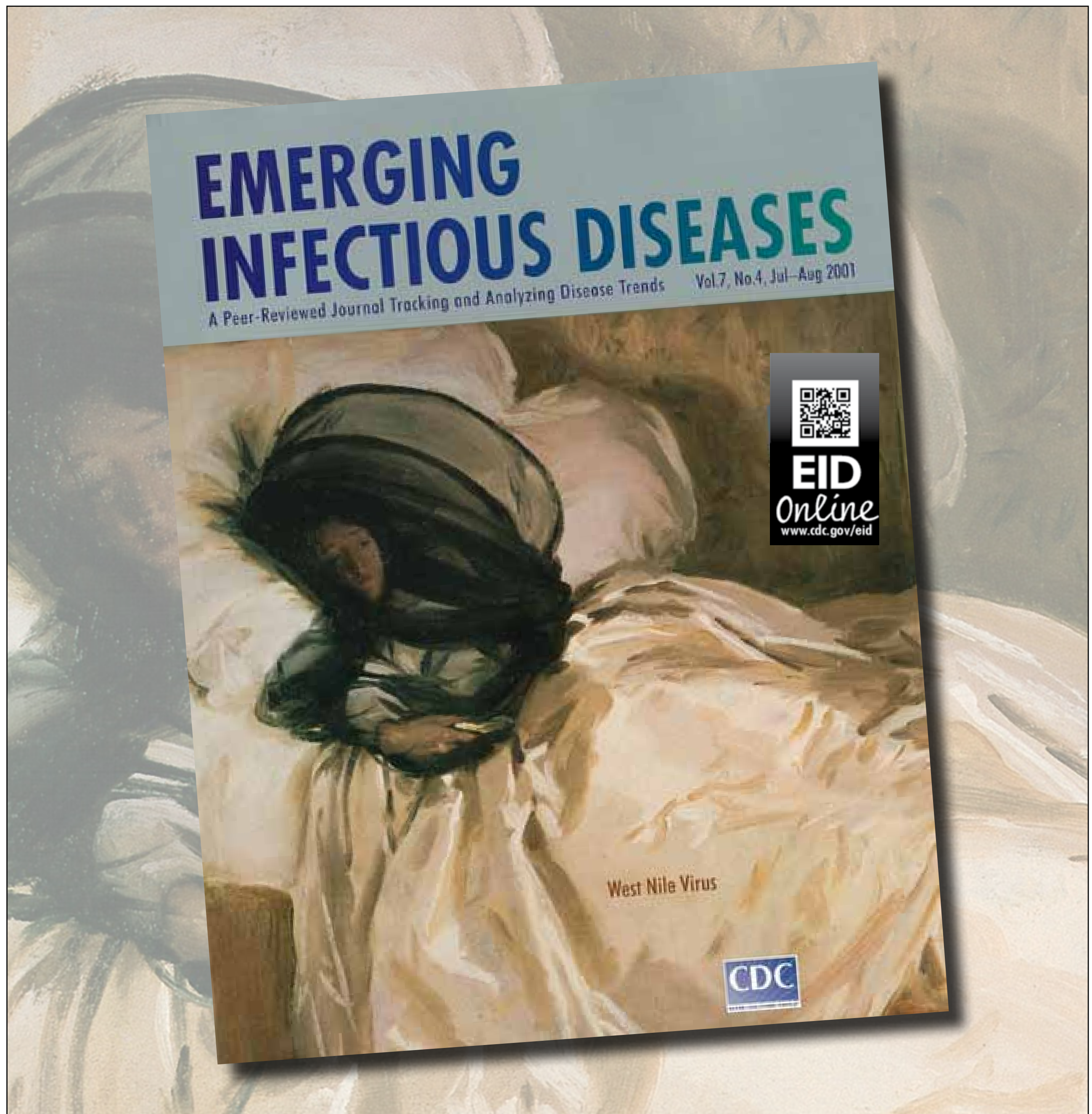

Cerebellar Cysticercosis Caused by Larval *Taenia crassiceps* Tapeworm in Immunocompetent Woman, Germany

Vasileios Ntoukas,[1] Dennis Tappe,[1] Daniel Pfütze, Michaela Simon, and Thomas Holzmann

Human cysticercosis caused by *Taenia crassiceps* tapeworm larvae involves the muscles and subcutis mostly in immunocompromised patients and the eye in immunocompetent persons. We report a successfully treated cerebellar infection in an immunocompetent woman. We developed serologic tests, and the parasite was identified by histologic examination and 12s rDNA PCR and sequencing.

Taenia crassiceps tapeworms are intestinal parasites of carnivores (final hosts), mostly foxes and dogs, in North America, Europe, and Russia. Rodents are natural intermediate hosts that harbor the cyst-like larvae (metacestodes, cysticerci) in their body cavities or subcutaneously, where the larvae proliferate by asexual budding (*1*). Prevalence among foxes in Germany and Lithuania is high (*2*), 24% and 26.4%, respectively. In contrast, prevalence in Denmark is low, only 0.2% (*3*). Although humans rarely serve as intermediate hosts, an increasing number of zoonotic infections have emerged in recent years (*1*,*4*–*12*). Infection of humans is thought to occur after consumption of food or water contaminated with infective ova shed in carnivore feces (*1*). All recognized cases involving the muscles or subcutis of humans have been associated with underlying immunosuppression (*1*,*4*–*7*,*12*), except for 1 case (Ronald Neafie, pers. comm). In contrast, intraocular infections (*8*–*10*) do not seem to require an impaired immune system (Table).

We describe a case of intracranial *T. crassiceps* tapeworm cysticercosis with severe involvement of the cerebellum. Combined surgical removal of the larvae and treatment with albendazole and praziquantel led to a complete cure in this nonimmunocompromised patient. The organism was unequivocally identified by molecular methods, thus avoiding a misdiagnosis of *Taenia solium* tapeworm cysticercosis.

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The Study

In 2011, in Regensburg, southern Germany, a 51-year-old German woman was hospitalized because of progressive headache, nausea, and vomiting. The signs and symptoms had started 2 weeks before, and intensity had been increasing ever since. At the time of admission, the patient showed cerebellar ataxia but no further neurologic deficits. She did not have fever or other symptoms. She had no known chronic preconditions or recent hospital stays and had never taken immunosuppressant drugs. She had no family history of neurologic symptoms or malignant diseases. Cranial computed tomography was performed and demonstrated a tumorous lesion (≈30 ´ 30 mm) in the right cerebellar hemisphere compressing the fourth ventricle. Magnetic resonance imaging revealed a multicystic mass with little perifocal edema (Figure 1). The patient's leukocyte count was elevated (27.4 ´ 10^9 cells), and a differential count indicated 84% neutrophils, 8% lymphocytes, and 4% eosinophils. Aspartate aminotransferase (129 U/L), alanine aminotransferase (335 U/L), and gamma glutamyl transferase (196 U/L) levels were elevated, and total plasma protein concentration was slightly lowered (4.7 g/dL). Kidney function test results, C-reactive protein levels, and gamma globulin levels were within normal limits.

Craniotomy revealed subdural and intracerebellar jelly-like tumorous tissue. The tumor, which consisted of multiple spherical masses with diameters of 2–4 mm, was resected. No infiltration of meningeal structures or the skull was evident.

Because an intracranial parasitosis or tumor was suspected, serum, tissue, and fluid from the cystic lesion were examined. Gross and histologic aspects of the excised tissue revealed typical structures for cestode larvae (Figure 2). Serum and tissue samples were sent to a reference laboratory for further examination. Serologic test results for echinococcosis, which used crude and recombinant antigen ELISAs, and indirect hemagglutination test results were negative (*11*). Commercial Western blots for cysticercosis and echinococcosis (LDBIO Diagnostics, Lyon, France) showed weak atypical bands of ≈47 kDa and 30 kDa, respectively (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/19/12/13-0284-Techapp1.pdf). For the tissue samples, cestode-specific PCRs selective for the parasite's mitochondrial 12S rRNA gene

[1]These authors contributed equally to this article.

Table. Cases of *Taenia crassiceps* tapeworm infection in humans*

Patient residence	Site of infection	Type of immunosuppression	Reference
Germany	Cerebellum	None	This article
Switzerland	Subcutis and muscle, upper limb	AIDS	(*4*)
Germany	Subcutis and muscle, forearm and hand	NHL	(*1*)
USA (Oregon)	Subcutis, shoulder	None	Ronald Neafie, pers. comm.
USA (Maine)	Eye (subretinal)	None	Ronald Neafie, pers. comm.)
France	Subcutis and muscle, arm	AIDS	(*7*)
France	Subcutis and muscle, forearm	AIDS	(*5*)
USA (Missouri)	Eye (subretinal)	None	(*9*)
France	Subcutis and muscle	AIDS	(*12*)
Austria	Eye (anterior chamber)	None	(*8*)
Germany	Subcutis and muscle, back	AIDS	(*6*)
Canada (Ontario)	Eye (anterior chamber)	None	(*10*)

* NHL, non-Hodgkin lymphoma.

(*13*) and mitochondrial cytochrome c oxidase subunit I gene (*14*) were positive. After sequencing and conducting a BLAST search (www.ncbi.nlm.nih.gov/blast/Blast.cgi) of the 380-bp and 450-bp amplicons, we found that the sequences showed 99% and 100% homology with the *T. crassiceps* tapeworm, respectively.

A crude *T. crassiceps* ELISA similar to an in-house *Echinococcus multilocularis* assay was set up (*11*) by using laboratory-kept *T. crassiceps* tapeworm larvae from another human patient (*1*). Serum samples from 10 healthy blood donors served as negative controls, and a standardized threshold index of 1.0 was calculated. Because no serum from patients with proven *T. crassiceps* tapeworm infections was available to use as a positive control, we used serum from patients with histologically confirmed cystic echinococcosis (5 patients), alveolar echinococcosis (7 patients), and peripheral cysticercosis (2 patients). All serum samples were positive, showing indices of 1.2–9.1, 1.4–6.6, and 2.2–3.3, respectively. The patient's serum, however, had an index below the threshold (0.76). When 5-µm cryosections from *T. crassiceps* tapeworm larvae were used for immunofluorescence tests, the patient's serum exhibited a weak tegumental signal (online Technical Appendix Figure 2).

After surgery, the patient was given praziquantel (600 mg twice daily) and albendazole (400 mg twice daily) as described (*1*) for 3 months. The postoperative course was uneventful, the patient recovered rapidly, and there were no clinical or radiographic signs of recurrence after a follow-up period of 18 months. Extended imaging investigations showed no further sites of infection.

When the patient was asked about potential risk factors, she indicated that she had been living with her dog near a forest in a local rural area for many years. Consumption of wild berries or mushrooms possibly contaminated by fox feces could not be excluded. The dog, which had not regularly undergone deworming, had access to the garden and the nearby forest.

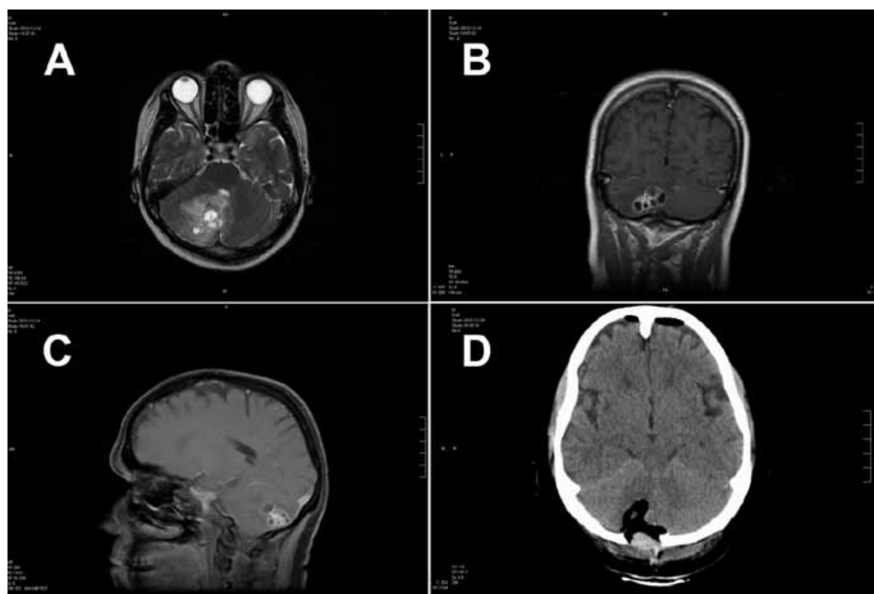

Figure 1. Magnetic resonance (MR) and computed tomographic images of the brain of a 51-year-old woman infected with *Taenia crassiceps* tapeworm larvae, Germany. A) Transverse view, T1-weighted MR image. The 30 × 30 mm parasitic lesion with perifocal edema is located in the right hemisphere of the cerebellum and caused ataxia, headache, and nausea. The fourth ventricle is compressed. B) Coronal view, T2-weighted MR image. The cyst-like appearance of the parasitic tissue is clearly visible. This lesion can be misinterpreted as cerebral echinococcosis, racemose cysticercosis caused by a *Taenia solium* tapeworm, or coenurosis. C) Sagittal view, MR image with contrast enhancing agent. D) Transverse view, computed tomographic image after surgery. No residual parasitic masses, only the parenchymal defect in the cerebellum after resection of *T. crassiceps* tapeworm larvae, are visible.

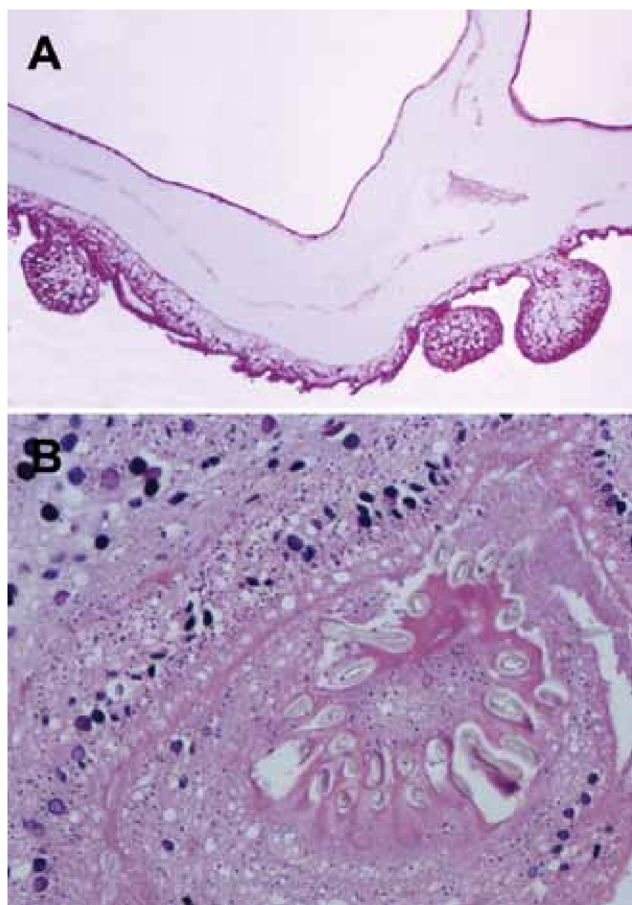

Figure 2. Histologic section through *Taenia crassiceps* tapeworm larvae removed from the cerebellum of a 51-year-old woman, Germany. A) Section through parasite body showing multiple connected bladders (asexual buddings) at the caudal end. Original magnification ×20. B) Transverse section through the parasite's protoscolex showing numerous hooklets, similar to *T. solium* tapeworm larvae. Original magnification ×40. Like the *Taenia solium* tapeworm that causes cysticercosis, and in contrast to different tapeworms that cause coenurosis (*T.* [*Multiceps*] *multiceps*, *T.* [*Multiceps*] *serialis*) or echinococcosis, the *T. crassiceps* tapeworm has only 1 invaginated protoscolex, but it is on a very long neck (*Cysticercus longicollis*). The hooklets of *T. crassiceps* tapeworms are larger than those of *T. solium* tapeworms and have a larger blade length than handle length. The small hooklets measure 123 µm; the large hooklets measure 167 µm.

Conclusions

In recent years, more reports of human infection with *T. crassiceps* tapeworms have surfaced, possibly because of increasing numbers of immunocompromised persons. The patient described here showed no evidence of an impaired immune system, and the parasite was found in an immunologically privileged site. Similarly, for patients with *T. crassiceps* larvae infection of the eye and for patients with neurocysticercosis caused by *T. solium*, immunosuppression does not seem to be a prerequisite for infection. *T. crassiceps* tapeworm larvae are apparently able to infect the same variety of human tissues as *T. solium*, but do so much more rarely. Most infections, including the case reported here, have been reported from southern Germany (*1,6*) and France (*5*,*7*,*12*). Other infections of humans have been reported from neighboring Switzerland and Austria and from North America. Similar to the distribution of alveolar echinococcosis in Europe (another larval cestode disease for which the red fox is also the final host), a contiguous area with microfoci of transmission could hypothetically be possible. Diagnosis depends on the radiographic image resembling a racemose cysticercus (because of the multicystic aspect of *T. crassiceps* tapeworm infections) and correct identification of the parasite by gross morphologic and histologic appearance by experienced pathologists or by molecular techniques. 12S rDNA PCR proved to be a useful tool that is not widely used (*13*,*14*), and its use helped avoid the misdiagnosis of *T. solium* tapeworm neurocysticercosis.

The diagnosis of *T. crassiceps* tapeworm infection is demanding for laboratories because no tests are commercially available. As described here, unusual serologic reactions displayed on tests for other larval helminthoses should raise the level of suspicion for a different causative agent. Of note, the serologic diagnosis of neurocysticercosis caused by *T. solium* can be difficult; commercial tests showed sensitivity <72% (*15*). Such a low sensitivity could hypothetically explain the negative ELISA result for the patient reported here, for whom no peripheral tissues were infected, in contrast to the control serum used. Thus, the true prevalence of human disease caused by *T. crassiceps* tapeworms could be underestimated, and future seroprevalence studies using ELISA and immunofluorescence testing can possibly shed more light on this type of infection. The source of infection for this patient remains unclear, but her dog is probably the major risk factor (*1*,*8*,*10*). As a preventive measure, carnivorous pets should undergo regular deworming.

Acknowledgments

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Dr Ntoukas is a neurosurgeon at the Krankenhaus der Barmherzigen Brüder in Regensburg, Germany, a tertiary care hospital. He specializes in brain tumor and spine surgery.

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Powassan Virus in Mammals, Alaska and New Mexico, USA, and Russia, 2004–2007

Eleanor R. Deardorff, Robert A. Nofchissey, Joseph A. Cook, Andrew G. Hope, Albina Tsvetkova, Sandra L. Talbot, and Gregory D. Ebel

Powassan virus is endemic to the United States, Canada, and the Russian Far East. We report serologic evidence of circulation of this virus in Alaska, New Mexico, and Siberia. These data support further studies of viral ecology in rapidly changing Arctic environments.

Powassan virus (POWV) is a tick-borne virus (family *Flaviviridae*, genus *Flavivirus*) with recent and increasing prevalence. The only member of the tick-borne encephalitis (TBE) serogroup of flaviviruses endemic to North America, POWV is an emerging cause of human illness and death (*1,2*). Transmitted primarily by *Ixodes* spp. ticks and maintained in enzootic cycles involving small- to medium-size mammals, POWV exists as 2 genetically divergent and spatially distinct lineages that are serologically indistinguishable: lineage I, prototype POWV and lineage II, deer tick virus (DTV) (*3*,*4*). The 2 lineages are maintained in different vector and host species.

First discovered in eastern Canada, POWV is now known to also circulate in the northeastern United States and the Russian Far East and has been documented in the western United States and Canada in wildlife and human infections (5–9). Clinical signs range from self-limiting febrile illness to severe neurologic disruption and death (2). Both lineages have been isolated from persons with fatal cases, and the incidence of human infection increased from an average of 0.7 cases/year (1958–1998) to 1.9 cases/year (1999–2007) (2,10). This apparent increase, coupled with the relatively recent discovery of lineage II and the well-documented diversity of TBE serogroup flaviviruses in the Old World, highlights the medical role of POWV and related viruses in North America.

Several TBE serogroup viruses, including POWV, also occur in the Russian Far East (*6*). Two hypotheses have emerged regarding the geographic distribution of POWV. The first hypothesis is that TBE serogroup flaviviruses in the Old and New Worlds persisted during the Pleistocene Epoch in Palearctic and Nearctic refugia (refuge areas), respectively, and then spread across continents (*11*). The second hypothesis is that POWV was introduced into Russia from North America in the 20th century (*6*,*12*). These hypotheses are not mutually exclusive, and POWV or closely related TBE serogroup viruses may be endemic to Beringia, the region surrounding the Bering Strait that connects Asia and North America. Because high latitude environments are experiencing rapid rates of change, and the distribution of POWV in North America is unclear, documenting potential sylvatic hosts of this pathogen is critical to evaluating its capacity to emerge into human populations.

The purpose of this study was to better understand the prevalence, distribution, and host specificity of POWV in western North America and Siberia. We also investigated the history and dynamics of POWV or related TBE serogroup viruses in Beringia.

The Study

Animals were collected in live traps and snap traps from sites in Siberia (2006), Alaska (2004–2005) and throughout the southwestern United States (2005–2007 (Table 1, Figure 1) under University of New Mexico Institutional Animal Care and Use Committee protocol 12–100764-MCC. Blood was collected on site during specimen processing. We screened blood samples from > 600 wild small-to-medium sized mammals representing 31 host species for POWV-specific antibodies.

Serum samples and supernatants were tested by using a strip immunoblot assay (SIA) with recombinant DTV envelope glycoprotein. Because POWV and DTV are serologically indistinguishable, this antigen binds antibodies specific for DTV, POWV, or other closely related viruses.

In brief, antigen was adhered to a nitrocellulose membrane, and strips were produced with the following antigens and markers: Coomassie blue (orientation control), specific pathogen–free mouse serum (negative control), serum from mice inoculated with DTV envelope glycoprotein (DTV-positive control), mouse IgG (IgG-positive control), and purified DTV E-glycoprotein (test). Approximately 0.25 μg of DTV envelope glycoprotein was used per 2-mm test strip. Samples were tested at a 1:200 dilution, and antibody was detected by using an alkaline phosphatase–conjugated secondary antibody (goat antimouse IgG). Colorimetric intensity was assessed and

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Table 1. Powassan virus seroprevalence in mammals captured in eastern Russia (Siberia), Alaska, and the southwestern United States*

Region	Species	Common name	No. positive/no. tested (%)	95% CI
Siberia, Russia	*Lepus timidus*	Mountain hare	0/1 (0)	0–79.35
	Microtus gregalis	Narrow-headed vole	0/2 (0)	0–65.76
	Microtus oeconomus	Tundra vole	0/12 (0)	0–24.25
	Mustela erminea	Stoat	0/1 (0)	0–79.35
	Myodes rufocanus	Gray red-backed vole	0/6 (0)	0–39.03
	Myodes rutilus	Northern red-backed vole	6/79 (7.6)	3.52–15.59
	Myopus schisticolor	Wood lemming	0/2 (0)	0–65.76
	Sciurus vulgaris	Tuft-eared squirrel	0/2 (0)	0–65.76
	Spermophilus undulatus	Long-tailed ground squirrel	0/1 (0)	0–79.35
	Tamias sibiricus	Siberian chipmunk	0/5 (0)	0–43.45
Total	NA	NA	6/111 (5.4)	NA
Central Alaska	*Microtus oeconomus*	Tundra vole	0/5 (0)	0–43.45
	Mustela vison	American mink	0/2 (0)	0–65.76
	Myodes rutilus	Northern red-backed vole	14/243 (5.8)	3.46–9.44
	Sorex cinereus	Cinereus shrew	0/8 (0)	0–32.44
	Sorex hoyi	Pygmy shrew	0/1 (0)	0–79.35
	Sorex monticolus	Montane shrew	0/6 (0)	0–39.03
	Sorex tundrensis	Tundra shrew	0/2 (0)	0–65.76
	Tamiasciurus hudsonicus	Red squirrel	0/3 (0)	0–56.15
Southern Alaska	*Myodes gapperi*	Southern red-backed vole	6/89 (6.7)	3.13–13.93
Total	NA	NA	20/359 (5.6)	NA
Southwestern USA	*Dipodomys merriami*	Merriam's kangaroo rat	0/15 (0)	0–20.39
	Dipodomys ordii	Ord's kangaroo rat	0/1 (0)	0–79.35
	Mus musculus	House mouse	0/4 (0)	0–48.99
	Neotoma albigula	White-throated woodrat	0/10 (0)	0–27.75
	Neotoma cinerea	Bushy-tailed woodrat	0/4 (0)	0–48.99
	Notiosorex crawfordi	Desert shrew	0/1 (0)	0–79.35
	Onychomys arenicola	Mearn's grasshopper mouse	0/14 (0)	0–21.53
	Perognathus flavus	Silky pocket mouse	0/3 (0)	0–56.15
	Peromyscus boylii	Brush mouse	0/6 (0)	0–39.03
	Peromyscus eremicus	Cactus mouse	0/19 (0)	0–16.82
	Peromyscus maniculatus	Deer mouse	2/33 (6.0)	1.68–19.61
	Peromyscus leucopus	White-footed mouse	0/22 (0)	0–14.87
	Peromyscus truei	Piñon mouse	2/9 (22.2)	6.32–54.74
	Sigmodon hispidus	Hispid cotton rat	0/3 (0)	0–56.15
Total	NA	NA	4/144 (2.8)	NA

*NA, not applicable.

DTV envelope glycoprotein–positive results were compared with 3+ and 1+ IgG control bands.

In Siberia and central Alaska, antibodies reacting with DTV antigen were detected exclusively in northern red-backed voles (*Myodes rutilus*) (6.2%) (Table 1). In southern Alaska, DTV-reactive antibodies were detected in the only species tested, the southern red-backed vole (*M. gapperi*) (6.7%). In the southwestern United States, DTV-reactive antibodies were found in New Mexico in 2 *Peromyscus* species mice: the piñon mouse (*P. truei*) and the deer mouse (*P. maniculatus*) (22.2% and 6.0%, respectively) that were collected sympatrically. The deer mouse is of particular interest because it is the primary host of Sin Nombre virus, the etiologic agent of hantavirus cardiopulmonary syndrome in North America (*13*).

To identify the virus responsible for serologic reactivity, we collected ticks (*Ixodes angustus)* from coastal southeastern Alaska (61.3210°N, 145.3030°W; 59.2459°N, 135.1753°W; and 55.8717°N, 132.3481°W) in 2009 from captured mammals (Table 2). Reverse transcription PCR was performed for ticks and tissues from seronegative animals collected proximally to seropositive animals and thus potentially in the acute stage of infection. No viral RNA was detected in ticks or in seronegative rodent tissue.

Conclusions

Although we used a DTV antigen because of its technical convenience, we do not believe that DTV per se is present in these rodent populations. POWV is present throughout western United States and western Canada. However, the virus responsible for the observed seropositivity in Alaska is unknown. The most likely candidate is POWV but without an isolate or sequence data, tick-borne encephalitis virus or other Eurasian flavivirus cannot be ruled out, and we cannot rule out the possibility that the virus is a flavivirus with no known vector. The utility of the SIA is partially based on known cross-reactivity of flaviviruses because it enables detection of divergent lineages. Determination of endpoint antibody titers and confirmation of POWV specificity by plaque-reduction neutralization tests were not possible because of freezer failure.

Because few wild rodent antibodies are commercially available, our methodology used anti-Mus secondary

Figure 1. Worldwide distribution of previously confirmed Powassan virus activity. Dots indicate approximate locations of known Powassan virus circulation as shown by human illness, virus isolation from animals, and Powassan virus–specific antibodies in humans or animals. Stars indicate approximate locations of antibody-positive animals, reported herein, collected during 2004–2007 and from whom samples were tested by using a strip immunoblot assay.

antibody, which may have varying sensitivity against the 31 species tested. Thus, low-level reactivity may have been missed. However, the prevalence of antibodies detected by SIA in our study is consistent with that reported from known POWV transmission foci (*14*).

These serologic results enable us to conclude that ≥1 flaviviruses antigenically similar to DTV circulate in Siberia, Alaska, and the southwestern United States (Table 1). Transmission appears to involve *Myodes* spp. voles in northern regions and *Peromyscus* mice in southern regions. Considerable overlap in the geographic ranges of these species may provide continuous populations of competent amplifying hosts from Mexico (*P. maniculatus and P. truei*) to Siberia (*M. rutilus*) (Figure 2). The seropositivity in Siberia may be from introduced POWV, native TBE virus, or other related virus. Viral RNA sequence is necessary to delineate the viral species that are circulating among *M. rutilus* in Siberia. Additional host species may be involved; considering the small sample for the current study, seropositivity rates and distributions, although consistent with expectations, may be considerably refined with increased sampling (Table 1). The incidence and host association of *Ix. angustus* ticks were similar to those of a previous report (*15*), and further vectorial studies are warranted.

Our findings augment knowledge of distribution of TBE serogroup flavivirus in the Nearctic and will guide further studies of New World TBE serogroup flavivirus ecology. Future work will focus on acquisition of viral isolates and nucleic acid sequences from *Myodes* spp. voles in Alaska and Siberia and from *Peromyscus* spp. mice in the southwestern United States.

Table 2. Ticks collected from trapped mammals in southeastern Alaska, USA, June–July 2009, and tested by reverse transcription PCR for flavivirus RNA*

Host species	No.	Adult males	Adult females	Nymphs	Lavae	Total	Average infestation
Microtus longicaudus	2	0	1	1	0	2	1.0
Microtus pennsylvanicus	1	0	0	1	0	1	1.0
Myodes gapperi	18	1	17	33	4	55	3.1
Myodes rutilus	12	0	5	9	2	16	1.3
Peromyscus keeni	21	2	16	33	26	77	3.7
Peromyscus maniculatus	5	0	2	3	0	5	1.0
Sorex cinereus	3	0	3	12	0	15	5.0
Sorex monticolus	10	0	0	18	22	40	4.0
Synaptomys borealis	1	0	0	10	0	10	10.0
Tamiasciurus hudsonicus	6	0	8	2	2	12	2.0
Total	79	3	52	122	56	233	2.9

*Several individual ticks (1 adult male, 3 adult females, and 12 nymphs) were not tested by reverse transcription PCR because of desiccation during storage. No larvae were tested. Infestation rate was calculated by dividing the total number of ticks by the total number of individuals for each mammalian species.

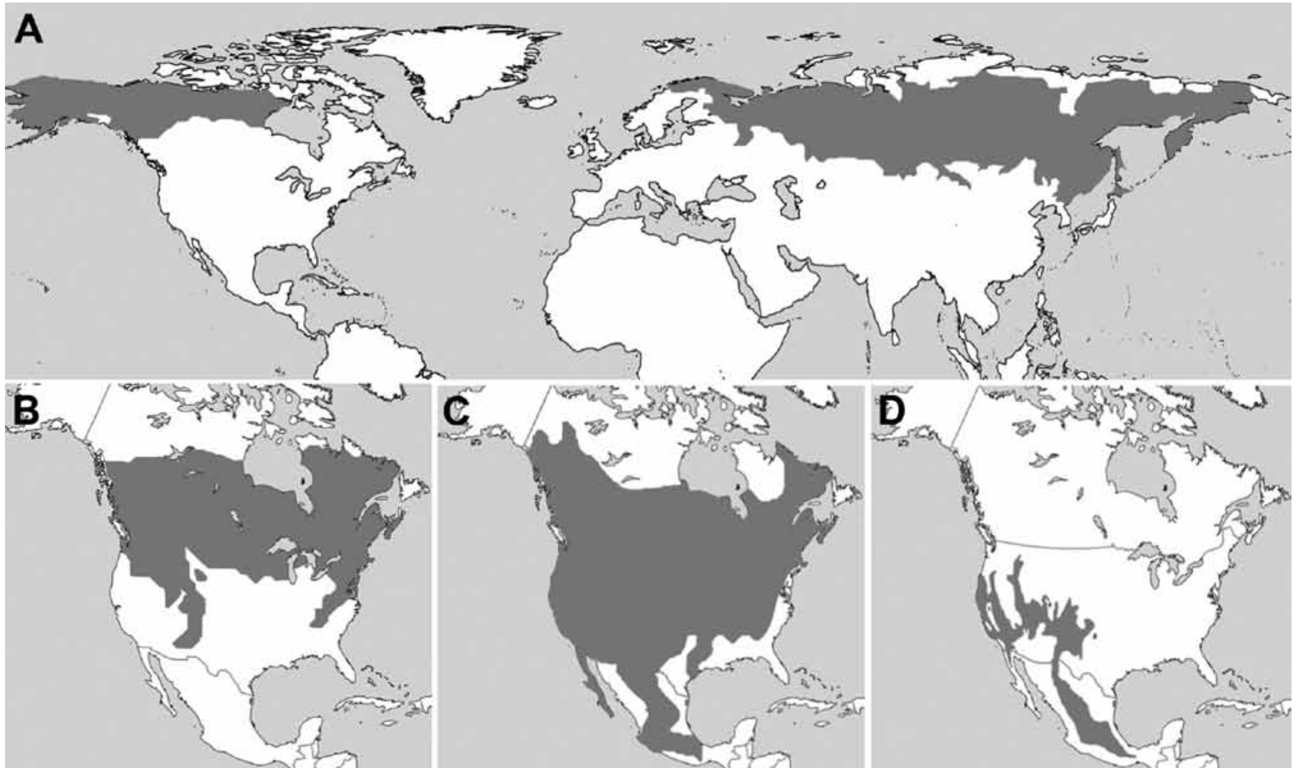

Figure 2. Ranges (gray) of A) northern red-backed vole (*Myodes rutilus*), B) southern red-backed vole (*M. gapperi*), C) deer mouse (*Peromyscus maniculatus*), and D) piñon mouse (*P. truei*), United States, Russia, and Canada. Major range overlap between the 4 species found with deer-tick virus–reactive antibodies suggests that the responsible virus may have access to competent amplifying hosts throughout North America. Panel A was based on the International Union for Conservation of Nature and Natural Resources Red List (www.iucnredlist.org/) and panels B–D were based on the Smithsonian National Museum of Natural History, North American Mammals (www.mnh.si.edu/mna/main.cfm).

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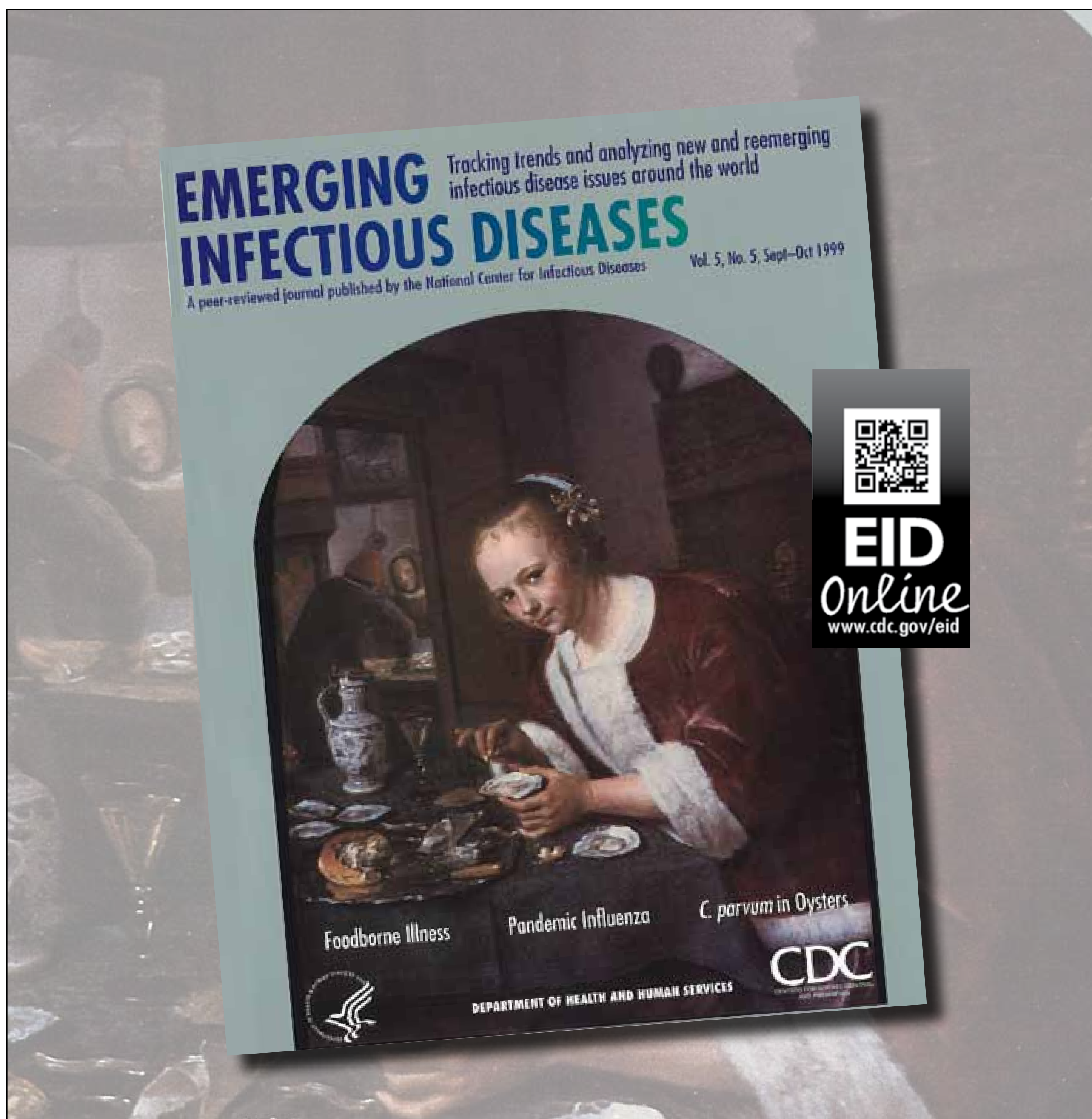

Reemergence of Vaccinia Virus during Zoonotic Outbreak, Pará State, Brazil

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In 2010, vaccinia virus caused an outbreak of bovine vaccinia that affected dairy cattle and rural workers in Pará State, Brazil. Genetic analyses identified the virus as distinct from BeAn58058 vaccinia virus (identified in 1960s) and from smallpox vaccine virus strains. These findings suggest spread of autochthonous group 1 vaccinia virus in this region.

Over the past decade, several exanthematous vaccinia virus (VACV) outbreaks that affected dairy cattle and rural workers have been reported in Brazil. During outbreaks, lesions developed on teats and udders of dairy cattle and caused a decrease in milk production (*1,2*). Infected milkers usually had lesions on their hands; the infection was apparently transmitted by unprotected contact with infected cattle (*1,2*).

Molecular studies have shown that autochthonous VACVs from Brazil (VACV-BR) can be divided into 2 groups: group 1 and group 2 (*3,4*). Group 1 includes isolates Cantagalo, Araçatuba, Passatempo, Guarani P2, Mariana, and Pelotas 2; group 2 includes isolates Guarani P1, Pelotas1, and BeAN58058 (BAV). This molecular dichotomy is also reflected in certain biologic properties of the isolates, including virulence in the BALB/c mouse model and plaque phenotype in BSC-40 cells (*1*). Although each VACV strain has unique genetic characteristics, most of them are similar to each other within the same group, especially those belonging to group 1; they most likely share a common ancestor. Although some researchers believed that VACV vaccine strains could have spread from humans to domestic animals and adapted to the rural environment (*2*), recent studies have suggested an independent origin for VACV isolates from South America, which is distinct from vaccine strains used in South America during the World Health Organization vaccination campaign. (*3,4*).

Despite emergence of VACV in the past decade, VACV was also isolated during the 1960s and 1970s during government efforts to investigate emerging viruses in forests in Brazil (*5–7*). One of those isolates, BAV, was obtained in 1963 from the blood of a rodent in Pará State in the Amazon region of Brazil that belonged to the genus *Oryzomys* (*6,7*). BAV was characterized during the 1990s, and restriction pattern and nucleotide sequence data supported its classification as a VACV (*6*). However, since its isolation, VACV circulation has not been reported in Pará State, even after VACV outbreaks in southeastern Brazil (*1*).

In this report, we describe reemergence VACV during a severe exanthematous outbreak in Pará State, 47 years after isolation of BAV. Our molecular data showed that this new VACV isolate clusters with group 1 VACV-BR isolates, which is the same VACV clade related to most viruses that caused zoonotic outbreaks in rural areas of Brazil in the past decade.

The Study

The outbreak was reported in July 2010 in Bom Jesus do Tocantins County (5°2′ 60²S, 48°36′36²W), Pará State, in the Amazon region of Brazil (Figure 1, Appendix, wwwnc.cdc.gov/EID/article/19/12/13-0589-F1.htm).

Dairy cattle and workers were affected. However, the source (index case) of this outbreak was not identified. At the study site, 44 lactating dairy cows became sick and had painful vesicular lesions on teats, udders, and inner thighs that rapidly progressed to ulcerative lesions and scabs (Figure 2, panels B and D). Two animals had extensive necrosis because of secondary infections, which led to loss of teats. Lesions were also observed on lips, muzzles, oral cavities, and tongues of calves (Figure 2, panel D). Three dairy workers became sick during the outbreak after direct contact with sick animals and had typical orthopoxvirus (OPV) lesions on their hands, forearms, and abdomen (Figure 2, panel A). Pain in the lesion region, fever, and fatigue were also reported by sick patients.

We collected 4 scabs and 44 serum samples from the 44 sick animals and 3 serum samples from the 3 dairy workers. Serum samples were tested by using 50% plaque reduction neutralization tests as described (*8*). Neutralizing antibodies were detected in 40 (90.0%) bovine and in 3 (100%) human samples, and titers ranged from 20 to 640 neutralizing units/mL. Scabs were macerated in buffer and centrifuged. Supernatants were diluted 1:100 in phosphate-buffered saline and used in a nested PCR specific for the C11R viral

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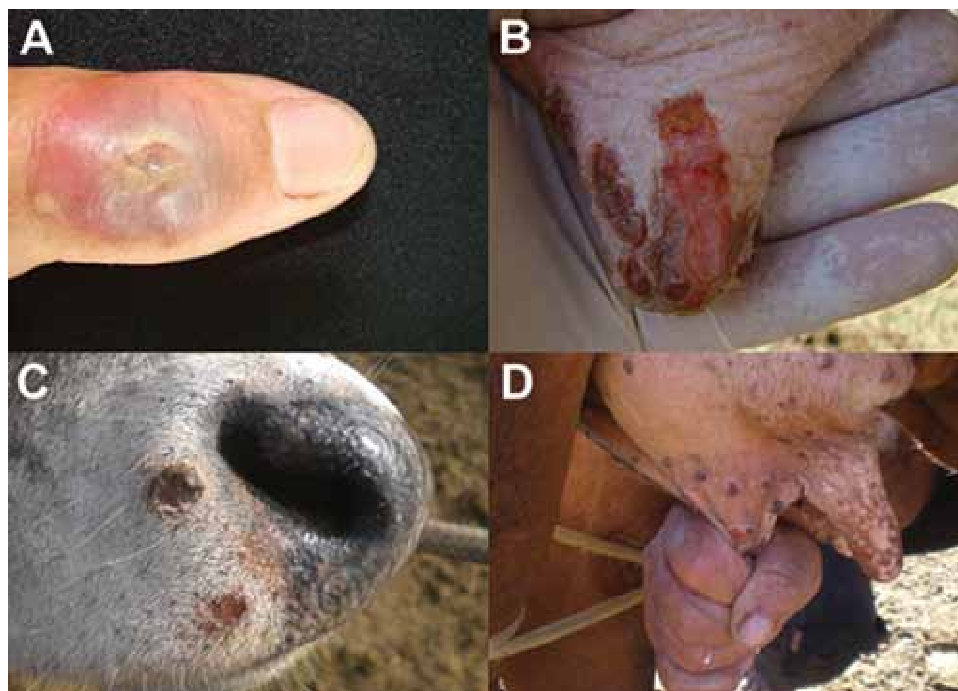

Figure 2. Exanthematic lesions caused by vaccinia virus (VACV) infection during this outbreak. A) Vesicular lesion on milker's finger that advanced to an ulcerative stage. B and D) Typical lesions on teats and udder of a dairy cow infected by VACV at different stages, ranging from ulceration to scabs. C) Lesions on a calf's muzzle probably caused by VACV infection during suckling.

growth factor gene as described (*9,10*). OPV-specific fragments from 2 scab samples were amplified. Samples were also subjected to virus isolation in Vero cells.

We isolated virus from 1 of the samples that was positive for viral growth factor by nested PCR. Negative results for VACV by PCR and virus isolation might have been caused by loss of virus titers and DNA degradation during sample transportation. After a typical poxvirus cytopathic effect was observed, virus was plaque purified and placed on Vero cell monolayers for viral amplification. This new VACV isolate was named Pará virus (PARV).

To investigate the relationship between PARV and BAV, virus gene A56R (hemagglutinin) was amplified and sequenced (*11*). The A56R gene is traditionally used for phylogenetic analysis. In addition, PARV A26L (A-type inclusion body) was also sequenced (*12*). The PCR fragments obtained were directly sequenced in both orientations and in triplicate by using a Mega-BACE 1000 Sequencer) (GE Healthcare, Little Chalfont, UK). Sequences were aligned with OPV sequences from GenBank by using ClustalW (www.ncbi.nlm.nih.gov/pmc/articles/PMC308517), and alignments were manually checked with MEGA version 4.0 software (Arizona State University, Phoenix, AZ, USA).

Optimal alignment of the A56R gene showed high identity among PARV and several group 1 VACV-BR isolates (average identity 99.8%), including VACV-TO CA (GU322359) (identity 99.9%), an amplicon obtained from blood of an Amazon monkey in Tocantins State, Brazil, in 2002 (Figure 3). PARV and BAV showed 98.3% identity with each other. PARV also showed a signature deletion of 18 nt that was also present in A56R sequences of other group 1 VACV-BR isolates

Phylogenetic trees of the A56R (Figure 3, panel A) or A26L (Figure 3, panel B) genes were constructed by using the neighbor-joining method, 1,000 bootstrap replicates, and the Tamura 3-parameter model (MEGA version 4). PARV sequences clustered with several group 1 VACV-BRs isolated during several bovine vaccinia outbreaks in Brazil. In both trees, PARV clustered in group 1 VACV-BR, whereas BAV clustered in group 2.

Conclusions

Our results confirm circulation of a new group 1 VACV-BR isolate in Pará State in the Amazon region of Brazil in the same location where BAV (group 2 VACV-BR) was isolated (*6,7*). In recent years, Bovine vaccinia outbreaks in southeastern Brazil rapidly spread to neighboring states (*1*). Epidemiologic studies suggest that movement of sick humans and the animal trade are the main causes of this circulation (*1*). The relevance of VACV circulation in the context of bovine vaccinia outbreaks has been discussed (*13*).

Several isolates belong to group 1, which is most commonly isolated from sick cattle or cow milkers; some isolates were detected in peridomestic rodents and wild monkeys (*8,13*). Therefore, although our data demonstrated that PARV does not cluster with BAV, it is not possible to phylogenetically define which group 1 isolate specifically generated PARV or caused the outbreak because of limited number of available gene sequences from VACV-BR

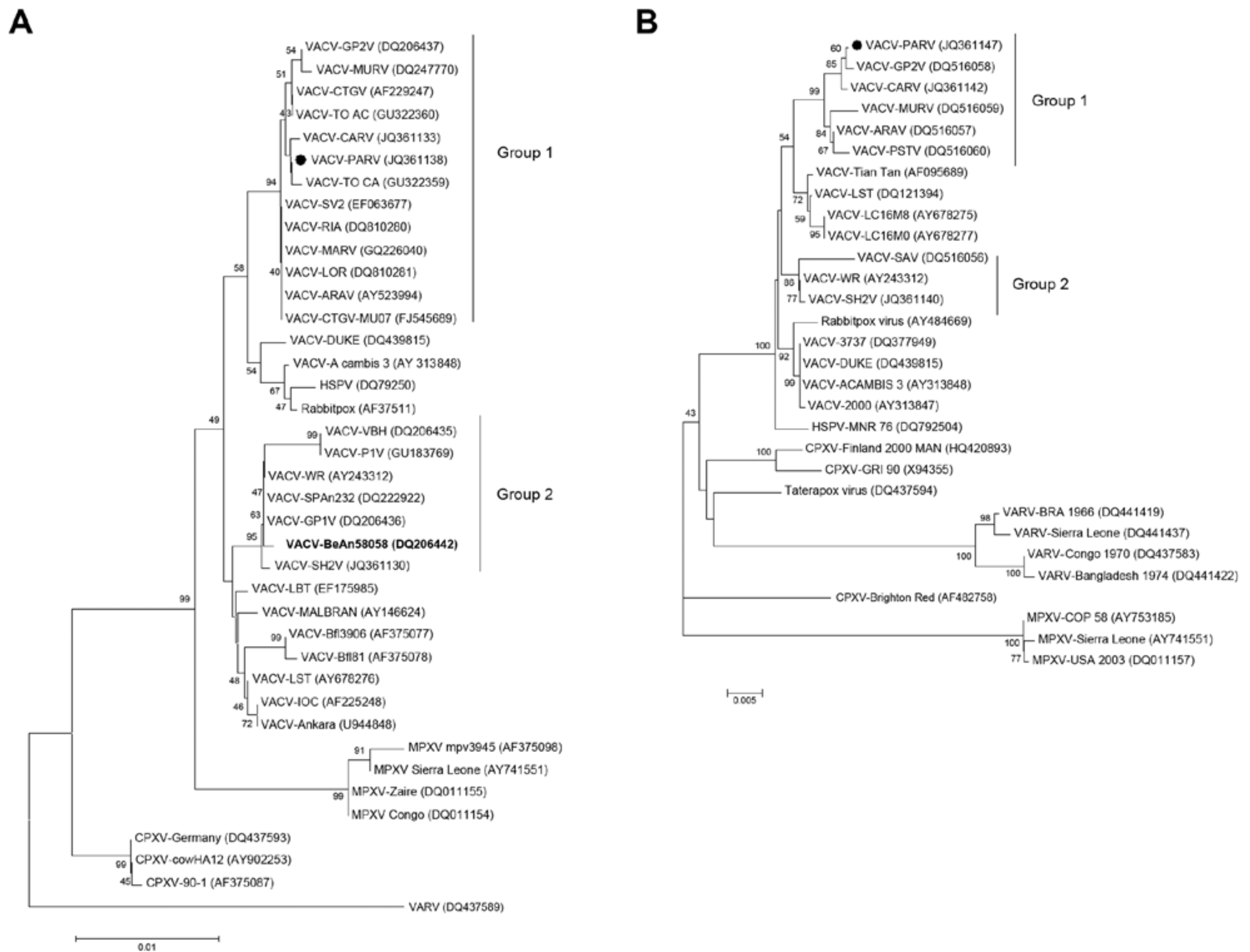

Figure 3. Phylogenetic trees based on orthopoxvirus nucleotide sequences of A56R (A) and A26L (B) genes of vaccinia virus (VACV), Pará State, Brazil. Pará virus (PARV) clusters with VACV group 1 from Brazil. Phylogenetic analysis showed that PARV (black dots) clustered in the VACV-BR-G1 clade and that BeAN58058 virus (BAV) clustered in the VACV-BR-G2 clade. A26L sequence was obtained only from PARV. Trees were constructed by using the neighbor-joining method, the Tamura-Nei model of nucleotide substitutions, and bootstrap of 1,000 replicates in MEGA version 4.0 software (Arizona State University, Phoenix, AZ, USA). In panel A, BAV is shown in **boldface**. GenBank accession numbers are indicated in parentheses. Values along the branches indicate bootstrap values. Scale bars indicate nucleotide substitutions per site. GP2V, Guarani P2 virus; MURV, Muriae virus; CTGV, Cantagalo virus; CARV, Carangola virus; MARV, Mariana virus; ARAV, Araçatuba virus; HSPV, horsepox virus; GP1V, Guarani P1 virus; MPXV, monkeypox virus; PSTV, Passatempo; CPXV, cowpox virus; VARV, variola virus.

isolates. Nevertheless, we believe the presence of this new isolate in Pará State likely resulted from virus spread from Tocantins, Maranhão, or Mato Grosso, 3 neighboring states of Pará State, which had Bovine vaccinia outbreaks in recent years, and not from reemergence of BAV (*1*).

Although group 2 VACV-BR isolates, including BAV, have not been detected in the Amazon region of Brazil in recent years, we believe that these viruses may be silently circulating or associated with bovine vaccinia outbreaks. As in other regions in Brazil, VACV outbreaks are underreported in the Amazon region because of its large size and the natural complexity involved in surveillance of zoonotic diseases. Our results reinforce the need for studies on VACV diversity and its transmission chain, which would be useful for the Amazon region in Brazil.

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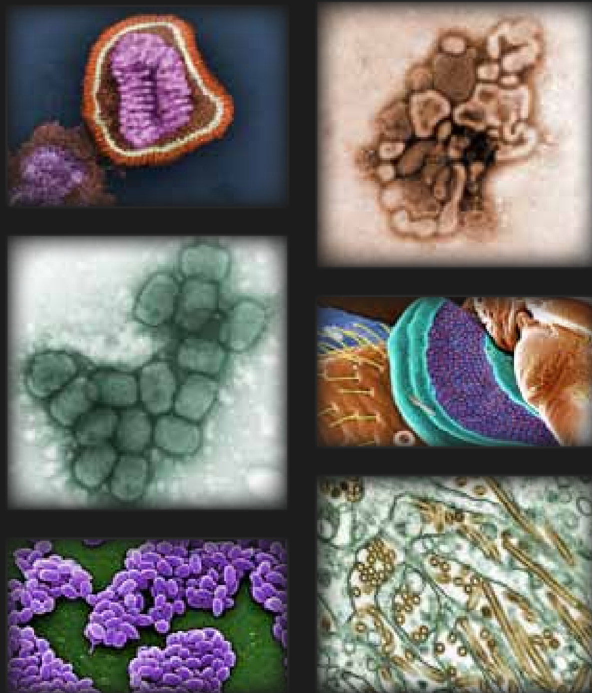

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Novel Variants of Clade 2.3.4 Highly Pathogenic Avian Influenza A(H5N1) Viruses, China

Min Gu, Guo Zhao, Kunkun Zhao, Lei Zhong, Junqing Huang, Hongquan Wan, Xiaoquan Wang, Wenbo Liu, Huimou Liu, Daxin Peng, and Xiufan Liu

We characterized 7 highly pathogenic avian influenza A(H5N1) viruses isolated from poultry in China during 2009–2012 and found that they belong to clade 2.3.4 but do not fit within the 3 defined subclades. Antigenic drift in subtype H5N1 variants may reduce the efficacy of vaccines designed to control these viruses in poultry.

Infection with the Asian lineage of highly pathogenic avian influenza (HPAI) A(H5N1) virus (prototype strain A/goose/Guangdong/1/96 [Gs/GD]) has resulted in substantial losses in the poultry industry and poses a threat to public health worldwide. According to the World Health Organization (WHO)/World Organisation for Animal Health (OIE)/Food and Agriculture Organization of the United Nations (FAO) H5N1 Evolution Working Group, 10 distinct clades of these viruses (0–9) were initially designated in 2008 to characterize the phylodynamics of the hemagglutinin (HA) gene of the Gs/GD-like viruses circulating during 1996–2007 (*1*). On the basis of these nomenclature criteria, new second-, third-, and fourth-order clades have been identified within the previously defined clades in the phylogenetic analyses that were updated in 2009 and 2011 (*2*,*3*). Therefore, as HPAI A(H5N1) virus continues to undergo substantial evolution, extensive genetic divergence is expected to periodically accumulate to form novel monophyletic groups. To identify continued divergence of clade 2.3.4 other than the recognized subclades 2.3.4.1, 2.3.4.2, and 2.3.4.3, we characterized 7 HPAI A(H5N1) viruses isolated from poultry during 2009–2012 in China (*2*).

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The Study

As part of continuous national avian influenza virus surveillance, we performed a monthly collection of cloacal swabs from various poultry species (chicken, duck, goose, quail, and pigeon) at a wholesale live-bird market (LBM) in Yangzhou, Jiangsu Province, in eastern China. Birds offered for retail sale in the LBM were mainly from local farms in Jiangsu and the neighboring provinces in eastern China; some were transported from regions in southern or northern China. Virus isolation and identification were conducted as described (*4*). During December 2009–September 2012, avian influenza virus isolates belonging to 8 HA subtypes (H1, H3–H6, H9–H11) were identified; 7 of the isolates were subtype H5N1 (Table 1).

To characterize these 7 isolates, we sequenced the HA genes to determine clade distribution. In the reconstructed phylogenetic tree (Figure) using reference sequences retrieved from the GenBank database and partially recommended by WHO/OIE/FAO (*2*), the 7 isolates belonged to clade 2.3.4 (the "Fujian-like" sublineage), which has been prevalent in China since 2005 (*5*). However, none of the isolates could be further classified into previously identified subclades 2.3.4.1, 2.3.4.2, or 2.3.4.3. Six of the viruses closely resembled A/peregrine falcon/Hong Kong/810/2009, and the remaining virus was highly homologous with recent H5 viruses bearing various neuraminidase (NA) subtypes (N1, N2, N5, and N8).

According to WHO/OIE/FAO guidelines (*1*–*3*), new clades (including subclades) were specified not only with a bootstrap value of ≥60 at the clade-defining node in which sequences monophyletically arose from a common ancestor but also with average between-clade and within-clade nucleotide divergences of >1.5% and <1.5%, respectively. Apart from subclades 2.3.4.1, 2.3.4.2, and 2.3.4.3, we found 3 additional monophyletic categories—the A/peregrine falcon/Hong Kong/810/2009-like viruses, the HPAI subtype H5N5–like reassortants, and the HPAI subtype H5N2/H5N8–like reassortants—that grouped clearly within the tree (Figure). The bootstrap values and average within-clade and between-clade distances for these 3 groups were 81, 1.0%, 4.2%; 100, 1.0%, 5.2%; and 100, 1.3%, 5.3%, respectively.

Because of the compulsory vaccination practice against HPAI in China (*6*), we examined serologic cross-reactivity between the 7 subtype H5N1 isolates and the diagnostic antigen of the widely used inactivated reassortant H5N1/PR8 vaccine Re-5 (Table 1). Although Re-5 derived its HA and NA genes from a clade 2.3.4 representative virus A/duck/Anhui/1/2006, the hemagglutination inhibition (HI) titers of Re-5 antiserum against the 7 subtype H5N1 viruses were as much as 6–7 $\log_2$ lower than that against the homologous antigen. In contrast, the antiserum of A/chicken/northern China/k0602/2010 (k0602) showed limited reaction to Re-5 and A/chicken/eastern China/AH/2012. Moreover,

Table 1. Results of HI assays using Re-5 and k0602 antiserum for 7 avian influenza A(H5N1) viruses isolated in China, 2009–2012*

Isolate	Isolation date	Antibody titer, $\log_2$	
		Re-5	k0602
A/chicken/Jiangsu/WJ/2009(H5N1)	2009 Dec	4	8
A/chicken/Jiangsu/XZ/2010(H5N1)	2010 Mar	4	8
A/chicken/northern China/k0602/2010(H5N1)	2010 May	4	**10**
A/chicken/Shandong/k0603/2010(H5N1)	2010 Jun	4	10
A/chicken/eastern China/ZG56/2011(H5N1)	2011 Dec	4	5
A/chicken/eastern China/JX/2011(H5N1)	2011 Dec	3	7
A/chicken/eastern China/AH/2012(H5N1)	2012 Sep	4	1
Re-5 diagnostic antigen†	NA	**10**	4

*Re-5 and k0602 antiserum were generated by vaccinating specific-pathogen free chickens with the commercial Re-5 vaccine (Qingdao Yebio Bioengineering Co., Ltd, Qingdao, China) and the oil-emulsified inactivated A/chicken/northern China/k0602/2010(H5N1) vaccine, respectively. HI titers against the homologous antigen/virus are shown in **boldface**. HI, hemagglutination inhibition; NA, not applicable.
†Qingdao Yebio Bioengineering Co., Ltd, China.

antigenic variation also existed among the 6 A/peregrine falcon/Hong Kong/810/2009-like viruses, as highlighted by the HI assay using k0602 antiserum (Table 1).

To explore whether these antigenic variations can be translated into protection efficacy difference in vivo, we selected A/chicken/northern China/k0602/2010 (k0602) and A/chicken/Shandong/k0603/2010 (k0603) viruses to evaluate the bivalent inactivated reassortant H5N1/PR8 vaccine Re-4/Re-5 (the HA and NA genes of Re-4 are from a clade 7 virus A/chicken/Shanxi/2/2006). This vaccine has been extensively used to control the prevalence of clade 2.3.4 and clade 7 viruses in China since 2008 (*6*). In addition, a reassortant rk0602 virus, which carries the HA and NA genes of k0602 virus and the internal genes of PR8, was recovered by using reverse genetics and the inactivated rk0602 vaccine was applied to evaluate the homologous protection. Four-week-old specific-pathogen free chickens were vaccinated with Re-4/Re-5 or the rk0602 vaccine and readily developed specific antibodies against the component viruses by day 28 after vaccination (Table 2). The birds were then intranasally challenged with $10^{6.0}$ 50% egg infectious dose of k0602 or k0603 virus. During the 10-day observation period, the Re-4/Re-5 vaccinated birds displayed clinical signs including severe depression, ruffled feathers, huddling, decreased feed and water consumption, and diarrhea; moreover, only 14.3% (1/7 birds in the k0602 group) and 10% (1/10 birds in the k0603 group) of the challenged chickens survived,

Figure. Phylogenetic tree of the hemagglutinin (HA) genes of the diverged avian influenza H5 subtype clade 2.3.4 variants from China and reference sequences retrieved from the GenBank database and partially recommended by the World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization of the United Nations H5N1 Evolution Working Group The neighbor-joining tree was generated by using MEGA 5.1 software (www.megasoftware.net). Numbers above or below the branch nodes denote bootstrap values ≥60% with 1,000 replicates. Numbers on the right are existing (2.3.3, 2.3.4.1, 2.3.4.2, 2.3.4.3, 2.5) and proposed (2.3.4.4, 2.3.4.5, 2.3.4.6) virus subclades. Black triangles indicate the 7 variants identified in this study; GenBank accession numbers for their HA genes are KC631941–KC631946 and KC261450. Scale bar indicates nucleotide substitutions per site.

Table 2. Efficacy of vaccines against highly pathogenic avian influenza virus A(H5N1) clade 2.3.4 variants in chickens, China*

Virus and vaccine type	HI titer ±SD, log_2			Challenge test results, by swab type, no. positive birds/no. tested (mean titer ±SD)†				No. surviving birds/total no.
				3 dpc		5 dpc		
	Re-4	Re-5	k0602	Tracheal	Cloacal	Tracheal	Cloacal	
k0602								
Re-4/Re-5	8.10 ±0.97	6.20 ±0.31	2.25 ±0.45	6/6 (3.79 ±1.46)	4/6 (2.25 ±2.39)	2/3 (2.17 ±2.40)	2/3 (2.92 ±2.55)	1/7
rk0602	2.10 ±0.37	3.27 ±0.33	9.35 ±0.75	0/10	0/10	0/10	0/10	10/10
Control‡	ND	ND	ND	ND	ND	ND	ND	0/5
k0603								
Re-4/Re-5	7.70 ±0.26	5.70 ±0.29	1.93 ±1.21	8/8 (3.15 ±1.30)	8/8 (3.50 ±0.25)	3/3 (3.50 ±0.43)	3/3 (2.33 ±0.58)	1/10
rk0602	2.05 ±0.85	2.75 ±0.57	9.25 ±0.71	0/10	0/10	0/10	0/10	10/10
Control‡	ND	ND	ND	ND	ND	ND	ND	0/5

*Chickens were immunized with the Re4/Re5 or the inactivated rk0602 vaccine (the HA and NA genes of rk0602 were derived from subtype H5N1 k0602 virus; the internal genes were from PR8), and HI antibody titers were determined on day 28 postvaccination. HI, hemagglutination inhibition assay; dpc, days postchallenge; ND, not done.
†Birds were challenged with $10^{6.0}$ 50% egg infectious dose (EID_{50}) of k0602 or k0603 virus; virus titers are expressed as log_{10} EID_{50}/0.1 mL.
‡Two groups of 5 mock-vaccinated chickens served as controls; all died within 3 dpc.

reflecting poor protection by the Re-4/Re-5 vaccine. In addition, shed virus was detected in tracheal and cloacal swabs from most of the tested chickens on 3 and 5 days postchallenge. By contrast, the rk0602-vaccinated chickens all survived the challenge, and no virus was recovered from tracheal and cloacal samples (Table 2).

Conclusions

The location of the 7 HPAI A(H5N1) virus variants in the HA gene tree (Figure) suggests that novel monophyletic subclades other than the previously identified 2.3.4.1, 2.3.4.2, and 2.3.4.3 subclades continue to emerge within clade 2.3.4. As a result of our findings, we suggest that these groups should be assigned new fourth-order clades of 2.3.4.4, 2.3.4.5, and 2.3.4.6 to reflect the wide divergence of clade 2.3.4 viruses.

In China, 1 of the 6 countries to which subtype H5N1 virus is endemic (*7*), multiple distinct clades (2.2, 2.5, 2.3.1, 2.3.2, 2.3.3, 2.3.4, 7, 8, and 9) were identified by surveillance during 2004–2009 (*5*). In particular, clades 2.3.2, 2.3.4, and 7 viruses have gained ecologic niches and have continued circulating by further evolving into new subclades (*2*). In addition, various NA subtypes of H5 viruses (H5N5, H5N8, and H5N2) bearing the genetic backbone of clade 2.3.4 A(H5N1) viruses have been detected in ducks, geese, quail, and chickens (*8–12*). These findings highlight the importance of periodic updates of the WHO/OIE/FAO classification of Asian A(H5N1) viruses by continuous surveillance to better understand the dynamic nature of the viral evolution.

Our findings have implications for the effectiveness of vaccination of chickens against HPAI A(H5N1) viruses. The results of cross-HI assays (Table 1) and vaccine efficacy experiments (Table 2) indicate antigenic drift in subtype H5N1 variants, as compared with the vaccine strain specifically designed to control the prevalent clade 2.3.4 virus infection in poultry. Although previous studies by Tian et al. (*13*) and Kumar et al. (*14*) proposed that vaccinated chickens with HI antibody titers of >4 log_2 could be protected from virus challenge, our data demonstrate that vaccine efficacy is substantially influenced by antigenic matching between the vaccine strain and circulating viruses in preventing the replication and transmission of influenza virus, especially when the induced antibodies are of moderate titers.

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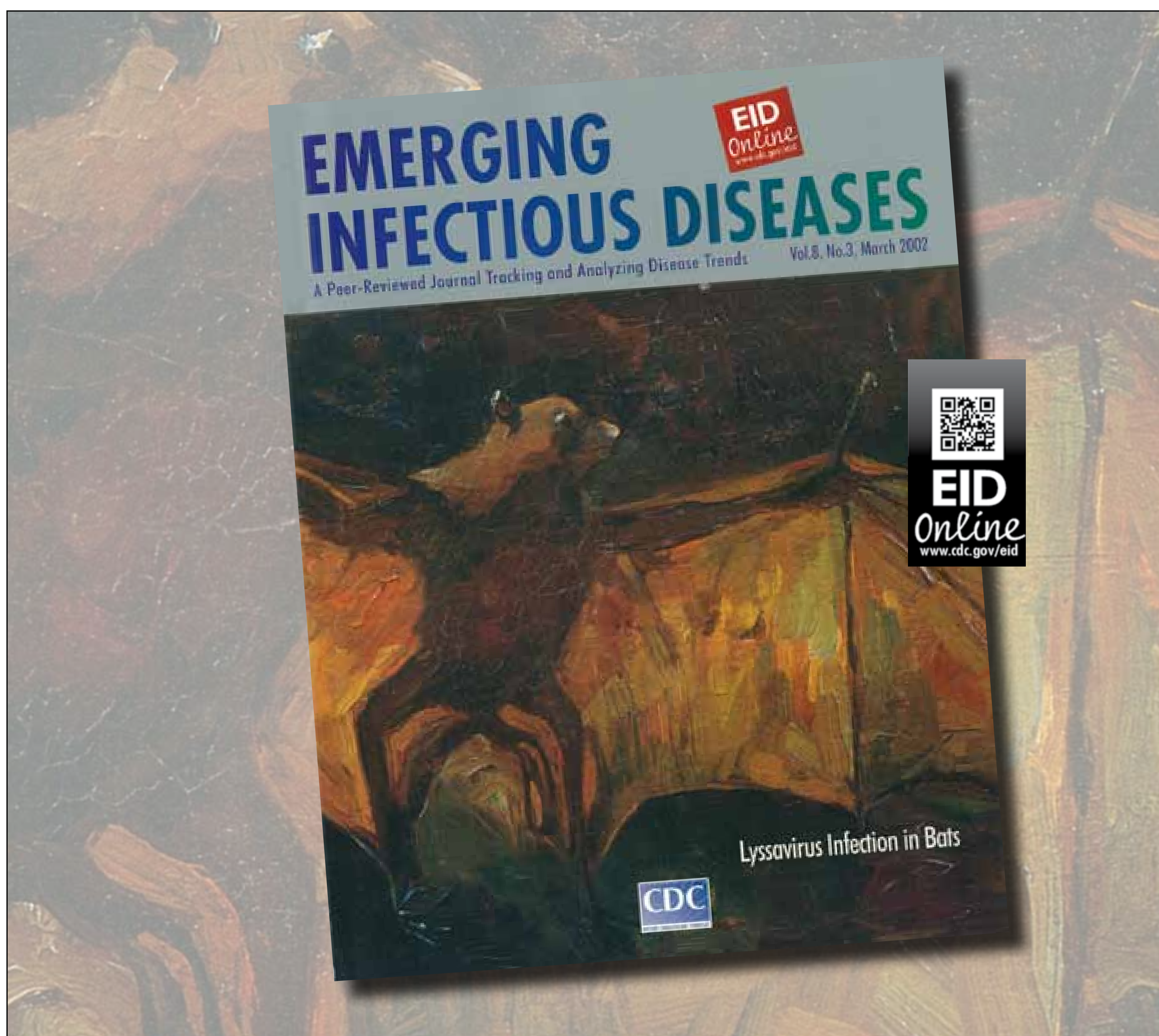

Rift Valley Fever in Namibia, 2010

Federica Monaco, Chiara Pinoni, Gian Mario Cosseddu, Siegfried Khaiseb, Paolo Calistri, Umberto Molini, Alec Bishi, Annamaria Conte, Massimo Scacchia, and Rossella Lelli

During May–July 2010 in Namibia, outbreaks of Rift Valley fever were reported to the National Veterinary Service. Analysis of animal specimens confirmed virus circulation on 7 farms. Molecular characterization showed that all outbreaks were caused by a strain of Rift Valley fever virus closely related to virus strains responsible for outbreaks in South Africa during 2009–2010.

Rift Valley fever virus (RVFV; family *Bunyaviridae*, genus *Phlebovirus*) is an enveloped RNA virus transmitted mainly by mosquitoes. This virus causes severe disease in humans and animals. The virus was identified in 1930 along the shores of Lake Naivasha in the Great Rift Valley in Kenya (*1,2*). Although direct transmission through contact with infected tissue might occur and could play a major role in human infection (*3*), mosquitoes still represent the most common way the virus is spread. Mosquito of several species (mainly *Culex* and *Aedes* spp.) have been considered vectors and reservoirs of the virus (*4–6*).

In 2010, South African veterinary authorities reported to the World Organisation for Animal Health 489 Rift Valley fever (RVF) outbreaks during the epidemic season; >14,000 cases and 8,000 deaths of animals occurred (*7,8*). The epidemic started on January 2010 in the eastern Free State Province and progressively spread west to Western Cape and Northern Cape Provinces and reached the border with Namibia. In Namibia, although virus circulation has been demonstrated in humans (*9–11*), little information is available on the distribution and the molecular characterization of RVFV circulating there. We conducted a study to identify and characterize RVFV strains that caused disease outbreaks in Namibia in 2010.

The Study

During May 9–July 30, 2010, ovine and caprine flocks showing clinical signs compatible with RVFV infection were reported to Namibian Veterinary Service. Blood samples were collected from live animals, and liver, spleen, heart, uterus, kidney, and brain samples were obtained from dead animals. Samples were sent to the Central Veterinary Laboratory in Windhoek, Namibia, for laboratory analysis. Tissue samples (100 mg) were homogenized by using a mortar and sterile quartz pestle and diluted 1:10 in phosphate-buffered saline containing antimicrobial drugs (100 U/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL gentamicin, 50 U/mL nystatin). Tissue debris was removed by low-speed centrifugation.

RNA was purified from blood samples and supernatants of homogenized tissues by using the High Pure Viral Nucleic Acid Extraction Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. RVFV RNA was identified in samples by using the specific one-step reverse transcription PCR (RT-PCR) described by Battles and Dalrymple (*12*), which is specific for 369-nt region of the medium (M) segment of RVFV RNA. Laboratory tests confirmed circulation of RVFV on 7 farms in the Hardap and Karas regions (Figure).

Aliquots of samples were shipped to the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise in Teramo, Italy, where virus isolation was conducted on samples positive for virus by RT-PCR by infecting Vero E6 cell (ATCC CRL-1586 VERO C1008) monolayers (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/19/12/13-0593-Techapp1.pdf). RT-PCR amplicons from virus-positive samples were purified by using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) and used for direct sequencing. Sequencing was performed by using the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA). Excess dye was removed by using Cleanseq (Beckman Coulter, Inc., Brea, CA, USA). Nucleotide sequences were determined by using DNA sequencer ABI PRISM 3100 (Applied Biosystems). Amplification and sequencing were repeated twice to avoid introduction of artificial substitutions. Raw sequence data were assembled by using Contig Express (Vector NTI suite 9.1; Invitrogen, Carlsbad, CA, USA), and a 328-nt fragment of the Gn glycoprotein coding sequence were obtained after deletion of primer sequences.

Seven sequences were obtained, 1 from each of the 7 outbreaks. Sequences showed 100% similarity at nucleotide and amino acid levels. The entire sequence of the M segment of 2 isolates collected (1 in Hardap and 1 in Karas) (online Technical Appendix Table 1) was generated after amplification of 9 overlapping sections. RT-PCR primers used are shown in online Technical Appendix Table 2. Because sequences were 100% identical, the RVFV isolate (Namibia 2010), was considered representative of all isolates. The Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov) was used to identify

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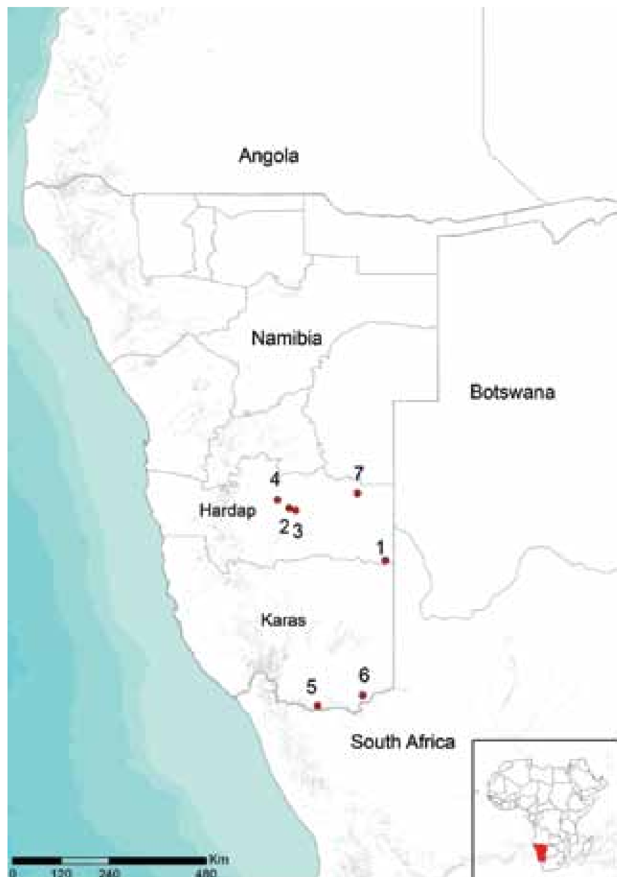

Figure. Location of farms in Namibia with Rift Valley fever virus infection, 2010. Circles and numbers indicate outbreaks from which virus circulation was determined.

homologous regions in sequence databases. Sequences were aligned by using ClustalW (www.clustal.org/) and BioEdit Sequence Analysis Editor version 7.0.5.3 (*13*). Phylogenetic analysis was conducted by using the entire sequence of the M segment from Namibia 2010 and all homologous sequences available in GenBank (online Technical Appendix, Figure 1). Because of absence of entire sequences from strains that co-circulated in South Africa and Namibia in 2009–2010, we performed phylogenetic analysis of a 490-nt fragment by using a selection of reference strains that had been isolated in different years or countries (online Technical Appendix Table 3) using the maximum-likelihood method in MEGA version 5 (*14*) with bootstrap support (1,000 replicates) (online Technical Appendix Figure 2). The unique sequence generated was submitted to GenBank under accession no. KC935380.

Overall diversity of partial M segment sequences was low, and bootstrap values for tree nodes were weak in some instances. Phylogenetic analysis showed that isolate Namibia 2010 belongs to the same group of RVFV strains isolated in South Africa in 2009 (SA404/09) and 2010 (SA85/10, SA1224/10, SA373/10, SA1221/10, SA276/10, SA276/10, SA106/10, SA404/09, SA423/10, SA482/10, SA71/10, and SA54/10). The cluster corresponds to lineage H of RVFV identified by Grobbelaar et al. (*11*). SPU77/04, which was isolated from a human in Namibia in 2004, is closely related. The number of nucleotide differences between sequences of this group was low (0–3 nt), Isolate Namibia 2010 showed 100% nt identity with SA54/10 and a 1-nt difference with SA85/10, SA482/10, SA71/10, SA106/10, SA404/09, and SA423/10.

Conclusions

The high degree of sequence identity of related RVFV strains that co-circulated in South Africa and Namibia in 2004–2010 suggests that these strains probably originated from a virus population that circulated between these 2 countries. Molecular data suggest that RFV outbreaks in Namibia in 2010 were caused by possible disseminated infections from South Africa. This hypothesis is further supported by the temporal and geographic location of the outbreaks. Clinical signs were first observed at the beginning of May in southeastern Hardap near the border with South Africa (Figure). The Auob River runs through this area, crosses the border with South Africa, and enters Kalahari National Park. Four outbreaks occurred in central Hardap (Figure) during the second half of May and the beginning of June in an area near the Auros River and an artificial lake in Hardap that supplies a broad system of water (irrigation) channels. During June 3–14, additional spread of virus was observed in the southern part of Karas near the border with South Africa where 2 outbreaks were confirmed (Figure), again near a water source, the Oranje River, which is the border between Namibia and South Africa.

The large RVF epidemic in South Africa in 2010 was attributed to heavy rainfall during January–February 2010 (*15*). In Namibia, evidence of intense rainfall was not recorded in the regions where disease outbreaks occurred in 2010 (online Technical Appendix Figure 3). This finding indicates that reactivation of local virus circulation is unlikely. Our findings suggest that control measures along borders of Namibia and other countries should be reinforced and that collaborations between veterinary and public health authorities should be strengthened to reduce the effects of future outbreaks.

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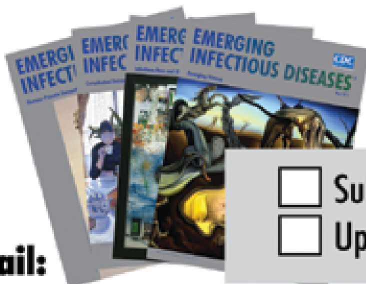

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Hepatitis E Virus Variant in Farmed Mink, Denmark

Jesper S. Krog, Solvej Ø. Breum, Trine H. Jensen, and Lars E. Larsen

Hepatitis E virus (HEV) is a zoonotic virus for which pigs are the primary animal reservoir. To investigate whether HEV occurs in mink in Denmark, we screened feces and tissues from domestic and wild mink. Our finding of a novel HEV variant supports previous findings of HEV variants in a variety of species.

Hepatitis E virus (HEV, family *Hepeviridae*) is a main cause of acute liver inflammation in humans. It is a nonenveloped RNA virus with a positive-sense genome of ≈7.2 kb. In 1997, HEV was discovered in pigs (*1*), and several studies have since shown that HEV is endemic in pigs and that pigs probably are a major animal reservoir. HEV traditionally had been divided into 4 primary genotypes (G1–G4). G1 and G2 have been found only in humans. G3 has been found globally in a wide range of mammals, including humans, pigs, deer, rabbits, and mongooses. G4, like G3, has an animal reservoir and has been found in humans, pigs, and wild boars (*2*).

Along with the human and porcine variants, avian HEV (aHEV) has been characterized. It is widespread globally and has been proposed to comprise 3 genotypes (*3*). Since 2010, several novel HEV variants have been described in red foxes, cutthroat trout, rats, bats, and ferrets (*4–8*). All new variants clearly differed from HEV G1–G4, aHEV, and each other. HEV is highly prevalent among pigs in Denmark; 92% of herds are seropositive, and ≈50% of investigated herds had pigs positive for HEV RNA (*9*). Because HEV is highly prevalent in swine in Denmark, animals fed offal from Danish slaughterhouses will be exposed to HEV. Production of mink fur is a major industry in Denmark, and mink are routinely fed a mixed diet, which often includes swine offal. Inappropriate heat-treated swine offal has previously been shown to be the source of swine-related influenza A virus infection in mink (*10*,*11*). Thus, we aimed to investigate whether mink in Denmark are infected with HEV G1–G4 or other HEV variants by screening fecal and tissue samples from domestic and wild mink.

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The Study

Initially, we screened 85 fecal samples collected during 2006 through mid-2012 from farmed mink by nested PCR; a broad panel of HEV variants was detected (*6*). One sample was positive, and subsequent sequencing and phylogenetic analysis showed that this virus represented a new HEV variant. To screen more samples for this new virus, we developed a specific real-time reverse transcription PCR (RT-PCR) (online Technical Appendix, wwwnc.cdc.gov/EID/articlepdfs/19/12/13-0614-Techapp1.pdf). The initially tested 85 fecal samples and an additional 233 fecal samples from farmed mink, together with liver and fecal samples from 89 wild mink, were tested with this new and more sensitive assay. We identified 4 positive samples, all from farmed mink. In addition, screening with an HEV real-time RT-PCR (*9*) specific for G1–G4 found none positive. The HEV-infected mink were all submitted for diagnostic examination; all had histories of diarrhea in the herd. Three of the positive samples were from herds having mink enteritis virus. Lipidosis, Aleutian mink disease virus, and catarrhal enteritis also were diagnosed in the mink (online Technical Appendix Table).

The 4 samples positive for the novel HEV variant were collected during 2008–2011 from herds across Jutland, Denmark, with a minimum distance of 80 km between the herds. The 4 PCR products obtained by the nested PCR, covering a region of 261 bp of the *RdRp* gene, were cloned and sequenced (GenBank accession nos. KC802090, KC802091, KC802092, and KC802093). The sequences were 98%–100% identical, with only 1 nonsynonymous mutation, resulting in a neutral amino acid change from isoleucine to valine (online Technical Appendix Figure). The high homology in this region is not surprisingbecause the gene encodes the RNA polymerase. We initially tried to uncover a larger fragment by primer walking, but the limited amount of material prohibited this.

On the basis of the 261-bp fragment, we analyzed the phylogenetic relationship of this novel mink HEV variant to variants found in other animals (Figure). The mink HEV variant clustered with HEV variants found in ferrets and rats, which grouped in a separate branch that was clearly distinct from other previously described HEV variants. At nucleotide level, the mink HEV variant was ≈65% identical to the closest classical HEV genotype (G3 and G4) and 76% and 69% identical with ferret and rat HEVs, respectively. At the amino acid level, the homologies were more pronounced, showing ≈87% and ≈78% identity with ferret and rat HEV variants, respectively. The grouping of the HEV reference sequences in the analysis was identical with results of previously performed phylogenetic analysis on full-length sequences (*12*).

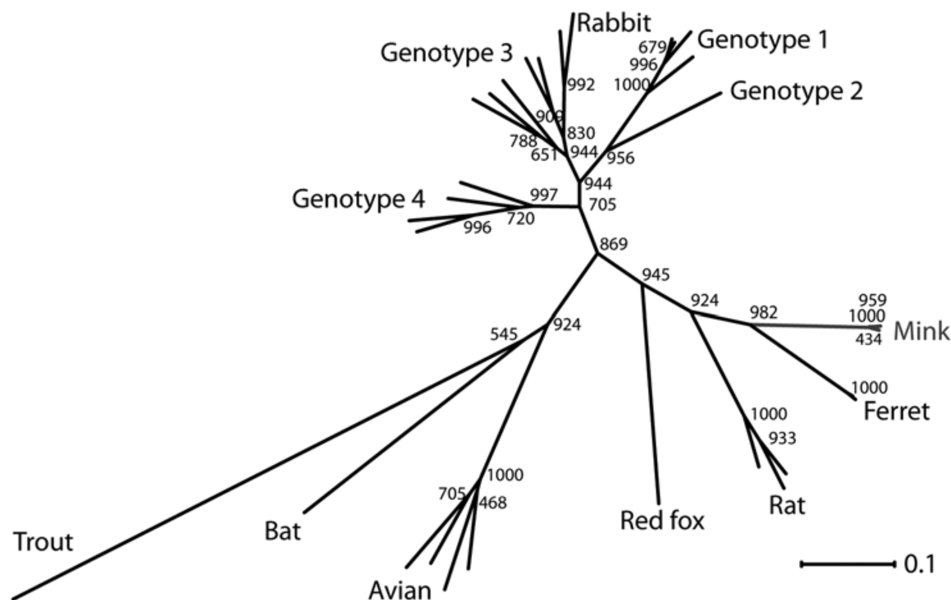

Figure. Phylogenetic tree showing the relationship between the novel mink hepatitis E virus (HEV), other HEV variants, and the 4 known HEV genotypes based on 261 bp of the *RdRp* gene. The CLC Main Workbench software (CLC bio, Aarhus, Denmark) was used for the phylogenetic analysis. Alignments were made by using MUSCLE algorithm (www.drive5.com/muscle/) and phylogenetic tree was made by using distance-based method with the neighbor-joining algorithm and bootstrap value of 1,000. Phylogenetic analysis with other methods showed similar results. Scale bar indicates nucleotide substitutions per site.

Conclusions

We detected a variant of HEV in 4 farmed mink from 4 geographically distinct locations in Denmark during a 3-year period, which indicates that the virus has been circulating among mink. Phylogenetic analysis showed that the virus was clearly distinct from, but closely related to, ferret and rat HEV variants recently reported from Germany and the United States (*6*,*7*,*13*).

It has not been possible to infect primates with rat or avian HEV variants (*13*,*14*). Thus, because of the phylogenetic resemblance of mink HEV with these nonzoonotic HEV variants, there are no indications that mink HEV can infect humans, although no human samples have been tested specifically for this virus. The zoonotic potential of HEV has been documented only in the case of G3 and G4, which were not found in mink. However, considering the relatively high HEV seroprevalence in humans, the possibility of other variants being zoonotic and cross-reacting with HEV G1–G4 in serologic assays cannot be ruled out.

Mink that tested positive for this new variant were from herds that had mink enteritis virus, hepatic lipidosis, Aleutian mink disease virus, and catarrhal enteritis, all factors that could explain the clinical and pathologic findings of the mink infected with HEV (*15*). However, it cannot be ruled out that the mink HEV variant contributed to the clinical signs of the mink HEV-positive animals. To determine whether the virus is indeed capable of inducing clinical signs in mink, the animals need to be experimentally infectedd. However, the rat and ferret HEV variants induced almost no histologic signs in rats after experimental infection, and the ferrets were described as not showing overt clinical signs (*7*,*13*). So far, only chickens infected with aHEV and humans infected with HEV G1–G4 have been described as being clinically affected by HEV infections. The possibility exists that the HEV variants recently reported in a variety of different species, including the 1 reported here, could evolve into disease-causing pathogens in animals and possibly also humans.

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Dr Krog was a research assistant at the National Veterinary Institute of Denmark, and the work presented here is part of a thesis clarifying the zoonotic aspects of hepatitis E virus in Denmark. His primary research interests include HEV infection dynamics in pigs, food safety, and viral contamination of the environment.

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Wild Birds and the Urban Ecology of Ticks

Dr. Sarah Hamer, Assistant Professor and Veterinary Ecologist with the College of Veterinary Medicine at Texas A&M University, discusses her investigation of ticks on wild birds in urban Chicago.

http://www2c.cdc.gov/podcasts/player.asp?f=8626456

Historical Prevalence and Distribution of Avian Influenza Virus A(H7N9) among Wild Birds

Sarah H. Olson, Martin Gilbert, Ming Chu Cheng, Jonna A.K. Mazet, and Damien O. Joly

We examined 48 published studies for which sample sizes could be ascertained to determine the historic prevalence of influenza A(H7N9) virus in wild bird populations and reviewed GenBank data to further establish its distribution. Low prevalence (0.0093%) in Asia suggests > 30,000 samples would be required to detect the H7N9 subtype in wild birds.

Beginning in February 2013, and ongoing at publication of this article, infections with the zoonotic virus, influenza A(H7N9), have caused serious illness in humans in provinces of southeastern China. On April 4, the China Animal Disease Control Centre announced that the virus had been detected in samples collected from a pigeon and chickens at a market in Shanghai (*1*,*2*). On April 17, the virus was detected in a sample from a wild pigeon in Nanjing, Jiangsu Province (*3*). Chen et al. concluded that humans were infected by domestic birds (*1*); no human-to-human transmission was detected or suspected (*4*). The structure of the hemagglutinin (HA) protein in the virus and the lack of reports of severe disease in poultry indicate that the virus exhibits characteristics of low pathogenicity in birds (*5*,*6*). Recent phylogenetic analysis indicates that the HA segment of the H7N9 subtype is closely related to a strain that was isolated from domestic ducks in Zhejiang, China, in 2011. The neuraminidase (NA) gene of the H7N9 subtype is closely related to that of a strain that was isolated from wild bird samples in South Korea in a location adjacent to a domestic bird production facility; additionally, 6 internal genes are closely related to those of an A(H9N2) virus isolated from a brambling (*Fringilla montifringilla*) sample during 2012 in Beijing, China (*7*,*8*).

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Little information exists on the status of A(H7N9) virus in wild birds to assess their potential as sources of human infection and disseminators of the virus to new areas. Here we report the historic distribution and prevalence of H7N9 subtypes among wild birds preceding this outbreak. This subtype was not known to cause disease in humans until the outbreak during February in China. We also examine the prevalence of individual H7, N9, and H9N2 subtypes in Asia. Finally, we estimate the sample size necessary to detect this low pathogenicity strain of avian influenza virus in wild birds.

The Study

To determine prevalence of H7, N9, H7N9, and H9N2 subtypes, we reviewed 48 peer-reviewed avian influenza surveillance studies in which sample sizes were stated and subtypes were nonselectively detected by using sequence analysis, reverse transcription PCR, or hemagglutination inhibition and neuraminidase inhibition assays. Data from these studies are summarized in the online Technical Appendix (wwwnc.cdc.gov/EID/article/19/12/13-0649-Techapp1.pdf). These included 9 studies conducted in Asia, 12 in Europe, 4 in Africa, 3 in Australia, 17 in North America, and 3 in Latin America. Extended datasets from peer-reviewed studies in Mongolia and Taiwan were provided by M. Gilbert and M.C. Cheng, respectively. The studies sampled birds during 1976–2012.

To further establish the geographic distribution of known H7N9 subtypes, we reviewed GenBank records downloaded on April 26, 2013, for HA or NA segments isolated from birds (*9*). We included a partially sequenced HA gene (1,676 bp [GenBank accession no. JN244232]) from A/wild bird/Korea/A3/2011 in our comparison (Table) after evaluating the published phylogenetic trees (*8*).

Apparent prevalence was calculated as the (no. positive samples)/(no. tested) × 100%. The regional estimate for Asia was an unweighted calculation based on the sum of all positive samples and all tested birds, irrespective of detection biases that may have arisen from different wild bird surveillance systems. We determined the minimum sample size to detect at least 1 positive sample based on a 0.05 level of significance (*10*)

Influenza H7N9 subtypes have been identified among wild birds globally (but not necessarily sequenced or submitted to GenBank) by isolation and by using reverse transcription PCR. The H7N9 subtype has been reported among wild birds from Delaware (USA)/Alberta (Canada), Guatemala, Spain, Sweden, Egypt, Mongolia, and Taiwan (online Technical Appendix Table 1). In these 48 studies, subtype H7N9 has not been detected in wild birds in these locations in Asia: Russia (combined sample size 7,353),

Table. GenBank nucleotide sequences of H7N9 samples, country of origin, hosts, and wild or domesticated status*

HA GenBank accession no.	NA GenBank accession no.	Year	Host (family/genus/species)	Location	Status
KC899669	KC899671	2013	Chicken (*Gallus gallus*)	China	Domestic
GU060482	GU060484	2009	Goose (Anatidae)	Czech Republic	Domestic
HQ244415	HQ244417	2009	Goose (Anatidae)	Czech Republic	Domestic
CY067670	CY067672	2008	Blue-winged teal (*Anas discors*)	Guatemala	Wild
CY067678	CY067680	2008	Blue-winged teal (*Anas discors*)	Guatemala	Wild
AB813056	ND	2011	Mallard (*Anas platyrhynchos*)	Japan	Unknown
AB481212	AB481213	2008	Wild duck (Anatidae)	Mongolia	Wild
JN244232†	JN244223	2011	Wild bird	South Korea	Wild
ND	JX679164	2008	Wild duck (Anatidae)	South Korea	Wild
HQ244409	HQ244407	2008	Common teal (*Anas creccca*)	Spain	Wild
AY999981	ND	2002	Mallard (*Anas platyrhynchos*)	Sweden	Wild
CY024818	CY024820	2006	Blue-winged teal (*Anas discors*)	USA, Ohio	Wild
JX899805	ND	2011	Goose (Anatidae)	USA, Nebraska	Unknown
JX899803	ND	2011	Guinea fowl (Galliformes)	USA, Nebraska	Domestic
CY133649	CY133651	2011	Northern shoveler (*Anas clypeata*)	USA, Mississippi	Wild
EU684261	ND	2000	Ruddy turnstone (*Arenaria interpres*)	USA, Delaware	Wild
CY127253	CY127255	1995	Ruddy turnstone (*Arenaria interpres*)	USA, Delaware	Wild
CY014786	CY014788	1988	Turkey (*Meleagris* spp.)	USA, Minnesota	Wild/domestic‡

*HA, hemagglutinin sequence; NA, neuraminidase sequence; ND, no data were available for this variable.
†Partial sequence.
‡Insufficient information was provided to determine status.

Japan (4,335), South Korea (28,214), or China (158) (online Technical Appendix Table 2); furthermore, when subtype H7N9 was detected in Asia, its prevalence was low (online Technical Appendix Table 2).

In countries within Asia, <0.1% of samples from wild birds tested positive for any H7 subtype; <0.05% tested positive for any N9 subtype; <0.01% tested positive for an H7N9 strain, and <0.02% tested positive for an H9N2 strain (online Technical Appendix Table 2). Assuming an apparent prevalence of 0.01%, we estimate that ≥30,000 birds would have to be sampled to detect 1 bird that was H7N9-positive with a 95% probability. To similarly detect 1 bird that was positive for H7, N9, or the H9N2 subtype in Asia, >4,000, 7,000, or 19,000 samples from birds, respectively, would be required.

Since 1988, the HA- and NA-producing genes of avian influenza subtype H7N9 have been deposited in GenBank 12 times, mainly representing isolates collected from wild bird hosts (Table). In Asia, before this outbreak, an H7N9 strain was sequenced from a wild bird in South Korea that was sampled during 2011 in a migratory bird habitat adjacent to duck farms (*7*) and also during 2011 in a sample from a mallard duck of unknown status from Japan. In 2008, the other H7N9 strain sequences collected in Asia were from a wild duck that was sampled in South Korea and from a wild bird sampled in Mongolia. All virus sequences were obtained from ducks and domestic geese, with the exception of a chicken in China and the following from birds in the United States: a turkey in Minnesota, a guinea fowl in Nebraska, and ruddy turnstones (*Arenaria interpres*) sampled in Delaware during 1995 and 2000. Eight of the complete HA and NA genetic sequences are attributed to wild birds, 3 are attributed to domestic birds, and 1 is attributed to a bird that could not be identified as wild or domestic because insufficient information was available.

Conclusions

Variation in the methods used in each study makes a precise calculation of H7N9 subtype prevalence in all wild birds impossible to determine, but given the available data, we conclude that the occurrence of the H7N9 subtype in wild bird populations is rare. We also conclude that sample sizes adequate to detect the virus among wild birds will be in the tens of thousands. Publishing the sample size and genus and species of wild birds tested in China will provide a better estimate of the prevalence among these birds related to this outbreak, especially because wild song birds have been hypothesized to be a possible reservoir (*11*). Wild birds are recorded as the predominant source of H7N9 sequences, but this may be an outcome of sampling bias. Because virologists typically focus on highly pathogenic strains in humans and domestic birds, and an H7N9 subtype was not recognized as highly pathogenic, the H7N9 strains were not was not tested for as frequently in wild birds. The HA/NA subtype concept we used for this analysis is archaic, omitting the contributions of internal protein genes to the biology of a virus; unfortunately, it is the only widespread typing system available for influenza viruses. Subsequently, the best historic prevalence estimate of the circulating internal genes is based on the H9N2 subtype.

Infection with the H7N9 subtype may prove challenging to control by culling birds, because infected domestic flocks may be asymptomatic. In wild bird populations, low pathogenicity strains are likely to be sustained longer than highly pathogenic strains, which have been unable to persist in wild populations in the absence of introductions

from a domestic reservoir (*12*). Further research should focus on identifying sequences within the new H7N9 genome that are linked to increased human pathogenicity and transmissibility and on conducting surveillance to detect these markers in viruses carried by both domestic and wild birds (*13*).

In summary, we present evidence that wild bird surveillance for the novel influenza A(H7N9) virus will require large sample sizes. Given the low likelihood of detection, risk-based surveillance is recommended. Ruling out wild birds as a continuing source of infection for domestic birds or humans will be critical to informing strategies to control the spread of this emerging zoonotic disease.

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Lack of MERS Coronavirus Neutralizing Antibodies in Humans, Eastern Province, Saudi Arabia

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We used a lentiviral vector bearing the viral spike protein to detect neutralizing antibodies against Middle East respiratory syndrome coronavirus (MERS-CoV) in persons from the Eastern Province of Saudi Arabia. None of the 268 samples tested displayed neutralizing activity, which suggests that MERS-CoV infections in humans are infrequent in this province.

The emergence of the Middle East respiratory syndrome coronavirus (MERS-CoV, formerly termed EMC coronavirus [*1*]) could pose a serious threat to public health (*2*). As of September 2013, a total of 108 laboratory-confirmed infections (with 50 deaths) caused by MERS-CoV have been reported to the World Health Organization (WHO), most from Saudi Arabia (*3*), but data are limited on MERS-CoV seroprevalence in humans (*4*).

We recently developed a lentiviral vector system to study host cell entry mediated by the spike protein of MERS-CoV (MERS-S) (*5*). This system mimics key aspects of MERS-CoV cellular entry and enables sensitive and quantitative detection of neutralizing antibodies, which are known to be generated in infected patients (*5*). We used this system to determine the presence of MERS-CoV neutralizing antibodies in serum and plasma samples obtained from patients at King Fahd Hospital of the University in Alkhobar, Saudi Arabia. The hospital is a referral hospital that serves the Eastern Province of Saudi Arabia, including the Dammam and Alhasa governorates, from which several MERS cases were reported, according to the Ministry of Health of Saudi Arabia and a recent study (*6*); no MERS patients were seen at King Fahd Hospital. Blood collection for this study was approved by the University of Dammam ethics committee, and informed, written consent for participation was received for all study participants.

The Study

Two collections of patient samples were analyzed. The first collection consisted of 158 serum samples taken from children hospitalized for lower respiratory tract infections during May 2010–May 2011. The samples came from 77 female and 81 male patients with a median age of 11.6 months (range 7.3 months to 9 years). The second sample collection consisted of 110 plasma samples from men with a median age of 28 years (range 19–52 years) who donated blood at the hospital during December 2012.

Analysis of MERS-S–driven transduction of target cells revealed that none of the samples investigated contained neutralizing antibodies against MERS-S (Figure 1). As a control, a subset of the samples was analyzed for inhibition of cellular entry mediated by the G protein of vesicular stomatitis virus (VSV-G), an animal virus that does not circulate in Saudi Arabia, and the spike protein of the human coronavirus NL63 (NL63-S), a globally circulating coronavirus. None of the samples robustly inhibited VSV-G–dependent entry, whereas most samples markedly reduced entry-driven by NL63-S (Figure 1), as expected (*7*). Experiments using serum samples of known neutralizing capacity confirmed that our neutralization experiments were sensitive and specific (Figure 2). Thus, serum samples obtained from a patient infected with MERS-CoV potently inhibited MERS-S but not VSV-G– or NL63-S–driven entry, whereas the reverse observation was made with serum samples reactive against NL63-S (Figure 2). In sum, none of the samples from children with respiratory infections and none of the samples from healthy adult men showed detectable amounts of MERS-S–neutralizing antibodies, but most neutralized NL63-S–driven host cell entry.

Conclusions

Our results suggest that the estimated MERS-CoV seroprevalence in the area served by King Fahd Hospital was $<2.3\%$ in children during 2010–2011 and $<3.3\%$ in male adults in 2012 (upper limits of the 95% CIs for 0/158 and 0/110, respectively, by Fisher exact test). Our analysis of samples from children might have underestimated seroprevalence

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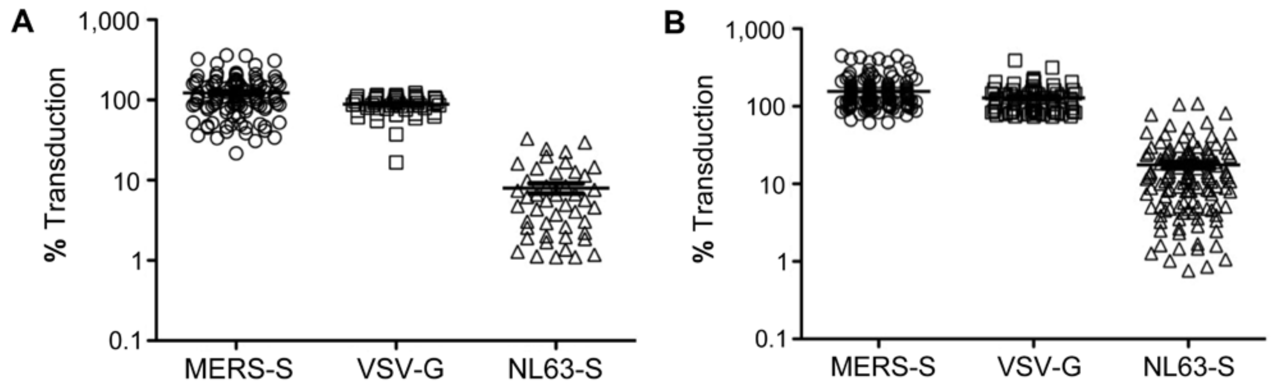

Figure 1. Neutralizing activity of serum and plasma samples obtained from patients at King Fahd Hospital of the University in Alkhobar, Saudi Arabia. A) Lentiviral vectors encoding luciferase and bearing the indicated viral glycoproteins were incubated with 1:20 dilutions of plasma from healthy adults, obtained during December 2012, and then added to target cells. Transduction efficiency was measured by quantification of luciferase activities in cell lysates and is shown relative to transduction of cells in the absence of serum, which was set at 100%. All 110 plasma samples available were tested for neutralization of Middle East respiratory syndrome coronavirus spike protein (MERS-S)–dependent transduction; subsets were also tested for neutralization of transduction driven by the G protein of vesicular stomatitis virus (VSV-G) (46/110) and the S protein of human coronavirus NL63 (NL63-S) (46/110). B) Analysis conducted as described for panel A using 158 serum samples from children with lower respiratory tract infections, obtained during May 2010–May 2011. All samples were analyzed for neutralization of MERS-S–mediated transduction; subsets were also tested for neutralization of transduction driven by VSV-G (76/158) and NL63-S (123/158). Horizontal lines indicate mean ±SEM.

because if they were hospitalized for MERS-CoV infection, a virus-specific antibody response might have developed after sample collection. Moreover, although infection of young children has been reported (*8*), the average age of MERS patients is 50 years. Our findings using samples from adult men argue against the extensive spread of MERS-CoV within this group in the Eastern Province of Saudi Arabia during 2012, which is noteworthy given recent reports of asymptomatic MERS-CoV infections (*9,10*).

We cannot rule out that other diagnostic methods that are not limited to detection of neutralizing antibodies might have identified positive samples in our collection. Future analyses are required to determine MERS-CoV seroprevalence in larger patient collectives and in animal species, such as dromedary camels, that could transmit the virus to humans (*4,11*).

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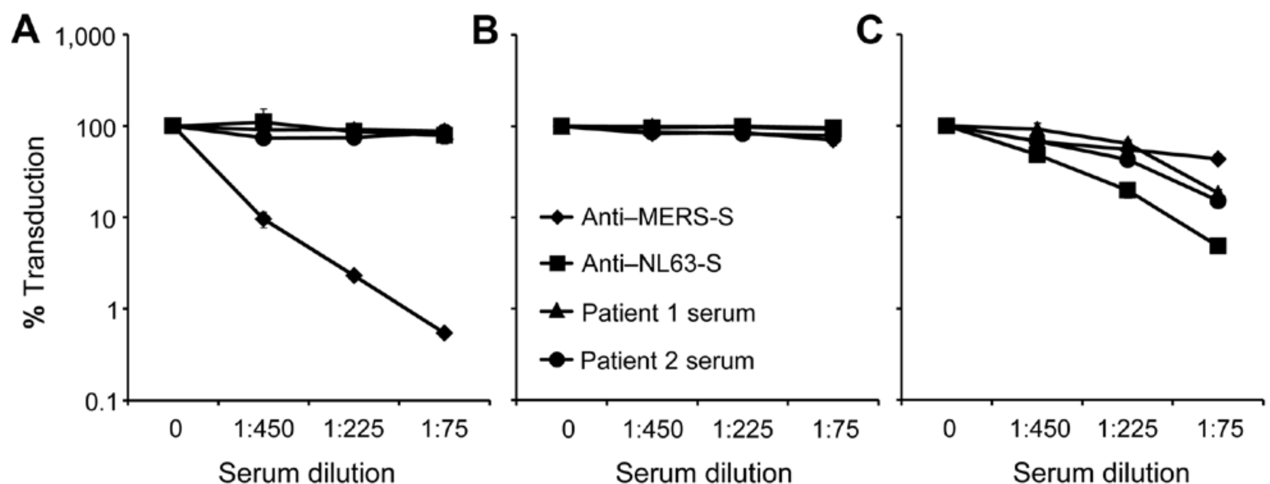

Figure 2. Analysis of serum samples with known neutralizing activity. Neutralization of transduction driven by the Middle East respiratory syndrome coronavirus spike protein (MERS-S) (A), G protein of vesicular stomatitis virus (B), and S protein of human coronavirus NL63 (NL63-S) (C) were determined as described for Figure 1, except that serum with known reactivity to MERS-S and NL63-S and serum from 2 patients at King Fahd Hospital of the University in Alkhobar, Saudi Arabia, that neutralized NL63-S–mediated transduction (Figure 1, panel A) were analyzed. Transduction of target cells in the absence of serum was set at 100%.

Ms Gierer is a third-year PhD candidate in the Georg-August University School of Science of the University of Göttingen, Göttingen, Germany. Her research is focused on coronavirus interactions with host cells, particularly at the stage of viral entry.

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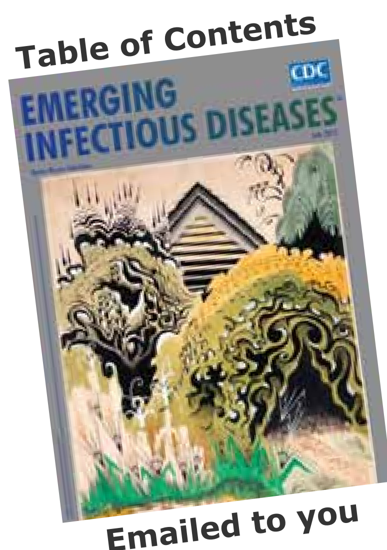

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Peste des Petits Ruminants Infection among Cattle and Wildlife in Northern Tanzania

Tiziana Lembo, Christopher Oura, Satya Parida, Richard Hoare, Lorraine Frost, Robert Fyumagwa, Fredrick Kivaria, Chobi Chubwa, Richard Kock, Sarah Cleaveland,[1] and Carrie Batten[1]

We investigated peste des petits ruminants (PPR) infection in cattle and wildlife in northern Tanzania. No wildlife from protected ecosystems were seropositive. However, cattle from villages where an outbreak had occurred among small ruminants showed high PPR seropositivity, indicating that spillover infection affects cattle. Thus, cattle could be of value for PPR serosurveillance.

Peste des petits ruminants virus (PPRV) is a highly contagious morbillivirus (genus *Morbillivirus*, family *Paramyxoviridae*) that is closely related to rinderpest virus. PPRV primarily affects sheep and goats in Africa, Middle East, and Asia but can infect a wide range of other domestic and nondomestic species (*1–4*). For example, cattle have been found to be seropositive for the virus (*1*,*2*), and PPRV was isolated from subclinically infected cattle 3 weeks after virus transmission from experimentally infected goats (R.K. Singh, pers. comm.). This finding points to the need for further investigation of the role of cattle in peste des petits ruminants (PPR) disease outbreaks. In addition, little is known about natural patterns of PPR in free-ranging African wildlife. Morbilliviruses can switch hosts, and new ecologic niches created by the eradication of rinderpest may provide opportunities for PPR emergence in new hosts (*4*,*5*).

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First identified in West Africa in the 1940s, PPR is now widespread across much of sub-Saharan Africa. High rates of death from PPR can have dramatic economic consequences, especially in rural African communities whose livelihoods rely on small ruminant livestock production (*6*). The potentially devastating effect of new introductions has raised considerable concerns for local and regional economies (*6*). PPR was first confirmed in Tanzania in 2008 in the country's northern regions. However, the virus, which was introduced into Tanzania by southward spread from neighboring countries, was probably in Tanzania long before official confirmation of the disease (*7,8*).

Capitalizing on the momentum resulting from the eradication of rinderpest, international agencies, including the Food and Agriculture Organization and the World Animal Health Organization, have focused attention on PPR; the disease has been identified as the next livestock disease candidate for eradication (*9*), and large-scale PPR interventions are being implemented in much of Africa. Epidemiologic surveillance is critical in global disease elimination and was considered key to the eradication of rinderpest (*10*). Although participatory surveillance was essential in assessing the levels and effect of cattle vaccination (*10*), wildlife serosurveillance was the primary tool for detecting the presence or absence of circulating rinderpest virus in the final stages of the eradication process (*11*). Grassroots-level surveillance is also likely to play a critical role in PPR control (*7*). However, critical questions remain about the role that species other than sheep and goats may play in PPRV persistence (*2*,*3*,*12*); the value of serosurveillance in control efforts; and, more specifically, whether cattle and wildlife species are useful indicators of virus circulation. To address these questions, we carried out serologic investigations for evidence of PPR infection in cattle and wildlife in northern Tanzania. The cattle lived in mixed cattle–small ruminant livestock systems in an area where a PPR outbreak had occurred in 2008, and the wildlife populations lived in protected-area ecosystems across a broader geographic area.

The Study

Serum samples from cattle living in close proximity to sheep and goats were available as part of epidemiologic studies involving randomly selected pastoralist households in Ngorongoro District, an area of the Serengeti ecosystem in northern Tanzania where the 2008 PPR outbreak had been confirmed (Figure) (*7*). The sampling was conducted in early to mid-2011 and included serum samples from cattle ≥3 years of age (as determined on the basis of incisor tooth eruption) and from cattle ≤2 years of age; the older cattle were alive during the 2008 outbreak, and the

[1]These authors contributed equally to this article.

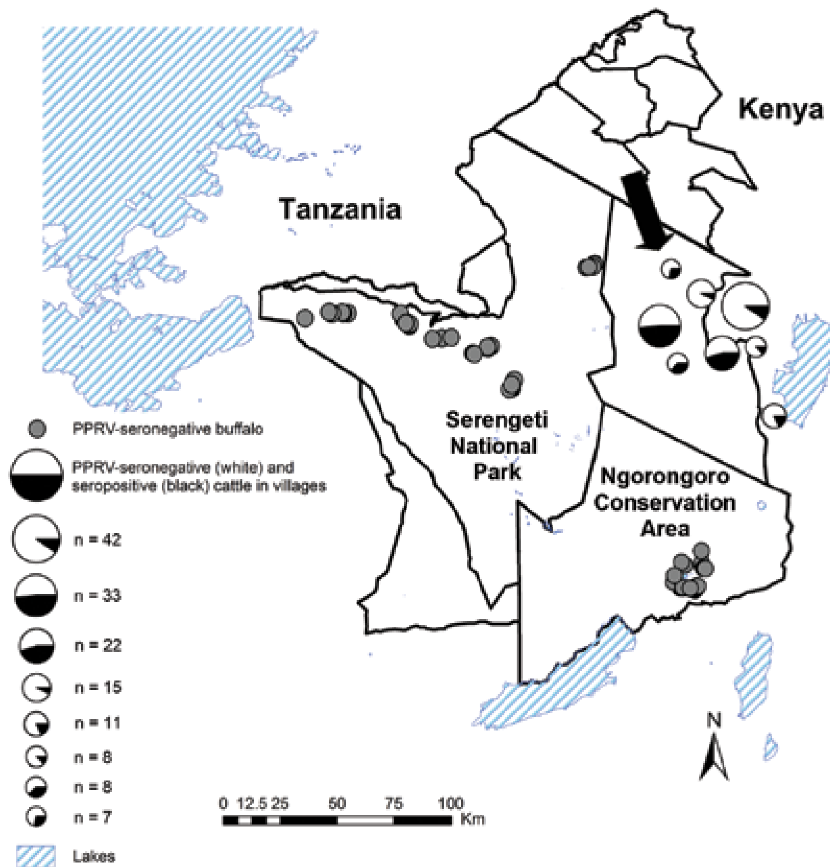

Figure. Areas in northwestern Tanzania where seroprevalence of antibodies to peste des petits ruminants virus (PPRV) was studied in cattle and buffalo. Cattle were sampled in 2011; all had been alive during a 2008 PPRV outbreak among small ruminants. Arrow shows location of 1 village affected during the 2008 outbreak (*7*). Buffalo were sampled during 2010–2012 in Serengeti National Park and Ngorongoro Conservation Area; the locations of PPRV-seronegative buffalo are shown.

younger cattle were born after the outbreak (Table 1). In the area, large-scale PPR vaccination of sheep and goats was initiated in early 2011 but did not include cattle. The last rinderpest vaccination campaign in Tanzania was carried out in 1997 (*13*).

Serum samples from buffalo (*Syncerus caffer*) and gazelle (*Eudorcas thomsonii* and *Nanger granti*) came from an archived serum bank and were made available through the Tanzania Wildlife Research Institute–Messerli Foundation Wildlife Veterinary Programme in Seronera, Tanzania. The samples had been collected during wildlife immobilization operations conducted for rinderpest surveillance, research activities, and conservation management (Table 2). Wildlife samples originated from several ecosystems, including the Serengeti ecosystem (Figure). Age information based on incisor tooth eruption was available for a subset of buffalo sampled in the Serengeti National Park and Ngorongoro Conservation Area during 2011–2012 (Table 1).

PPRV antibodies were detected by using the anti-hemagglutinin PPRV C-ELISA (Biological Diagnostic Supplies Limited [BDSL], Dreghorn, UK; www.bdsl2000.com/diagnostic-kits/ppr.aspx). Samples with positive results (i.e., inhibition value >50%) were confirmed as positive by using the anti-nucleoprotein PPRV C-ELISA (IDvet, Grabels, France; www.id-vet.com/produit/id-screen-ppr-competition/). The assays were performed and analyzed according to the manufacturers' instructions.

The screening for PPRV antibodies in cattle showed that 26.7% of the samples from cattle that were alive during the 2008 PPR outbreak were seropositive, and 5.9% of those from cattle born after the outbreak were seropositive. Seroprevalence in village cattle ranged from 7% to 48% (Figure). No detailed clinical information was available for the period of the outbreak.

Except for 1 borderline positive buffalo sample (inhibition value 56.6%), no seropositive samples were detected among samples from 266 buffalo, 59 Thomson's gazelles, and 6 Grant's gazelles. The borderline seropositive buffalo was from the Arusha ecosystem and would have been alive during the 2008 PPR outbreak. PPR-seronegative buffalo included older animals (i.e., ≥4 years) from Serengeti National Park (n = 20) and Ngorongoro Conservation Area (n = 85) that were alive at the time of the 2008 outbreak and younger animals from Serengeti National Park (n = 10) and Ngorongoro Conservation Area (n = 35).

Conclusions

Our findings show higher rates of PPR seropositivity than found in previous studies and confirm that cattle

Table 1. Seroprevalence of peste des petits ruminants virus antibodies in cattle and buffalo sampled in northern Tanzania

Animal, age, y	No. positive (%)	No. sampled
Cattle*		
<1	0	41
1–2	7 (9.0)	78
3	8 (17.8	45
4	12 (35.3)	34
5	6 (17.6)	34
6	9 (42.9)	21
≥7	4 (33.3)	12
Unknown	0	1
Total	46 (17.3)	266
Buffalo†		
1	0	5
2	0	17
3	0	23
4	0	10
5	0	26
6	0	11
≥7	0	58
Total	0	150

*Cattle in villages were sampled during 2011.
†*Syncerus caffer.* Buffalo in Serengeti National Park and Ngorongoro Conservation Area were sampled during 2011–2012. Ages were available only for a subset of buffalo.

are susceptible to PPR (*1*,*2*). These data support the view that in pastoral communities of northern Tanzania, where small ruminants and cattle co-exist, cross-species transmission of PPRV from small ruminants to cattle is likely to occur frequently.

Two broad conclusions can be drawn from these results. First, cattle are likely to be helpful indicators of PPRV circulation in mixed livestock communities and are therefore a useful population for surveillance. The study indicates that surveillance in cattle may also prove helpful in areas where PPR mass vaccination campaigns in sheep and goats have been implemented and would add value to existing syndromic surveillance networks. This conclusion is supported by the detection of seropositive young cattle (1–2 years of age) in more recent years at a time when no clinical cases were reported in small ruminants in the area. Although transmission of the live attenuated PPR vaccine strain in the field cannot be completely ruled out, there is currently no evidence for vaccine strain transmission either in the field or through experimental infection studies (*14*). Second, the high potential for cross-species transmission of PPRV from small ruminants to cattle in areas where these species live in close proximity suggests that monitoring such livestock communities would be useful for detecting any changes in the apparent pathogenicity of PPRV, including the possible emergence of PPR as a disease in cattle populations.

This preliminary study provided no evidence for PPR infection of wild ruminants within northern Tanzanian ecosystems. However, wildlife are known to be susceptible to PPR (*3*,*12*), and seropositive African buffalo and antelopes have been found in other locations (*15*). This study had limitations that prevent us from drawing definitive conclusions about infection patterns in African wildlife: sampling was largely carried out within wildlife-protected areas where there is limited opportunity for contact with sheep and goats, and only a small number of potential wildlife hosts were sampled. Seroprevalence data from other African buffalo populations (R. Kock, pers. comm.) suggest that larger sample sizes may be required from each host population to detect seropositivity and for our conclusions to be more representative. Thus, we recommend further studies to monitor wildlife infection in populations living in closer proximity to livestock.

Acknowledgments

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Table 2. Number of wildlife samples from northern Tanzania tested for peste des petits ruminants virus antibodies in northern Tanzania, 2008–2012

Ecosystem, species	No. sampled per year: Before 2008	2008	2010	2011	2012	Total no. sampled
Arusha, buffalo*	0	0	0	0	24	24
Katavi, buffalo*	0	0	23	0	0	23
Ngorongoro Conservation Area						
Buffalo*	0	0	0	48	95	143
Thomson's gazelle†	8	0	0	0	19	27
Grant's gazelle‡	6	0	0	0	0	6
Serengeti						
Buffalo*	2	3	14	22	10	51
Thomson's gazelle†	7	0	2	0	23	32
Tarangire, buffalo*	0	0	0	25	0	25
Total	23	3	39	95	171	331

**Syncerus caffer.*
†*Eudorcas thomsonii.*
‡*Nanger granti.*

for International Development, and the Scottish Government through the Combating Infectious Diseases of Livestock for International Development initiative, which supported the collection of cattle and buffalo samples in Tanzania (project BB/H009302/1) and PPR serologic analyses at The Pirbright Institute (project BB/HH009485/1). The Messerli Foundation provided support for collection of additional buffalo and gazelle samples in Tanzania.

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Surveillance for Avian Influenza A(H7N9), Beijing, China, 2013

Peng Yang, Xinghuo Pang, Ying Deng, Chunna Ma, Daitao Zhang, Ying Sun, Weixian Shi, Guilan Lu, Jiachen Zhao, Yimeng Liu, Xiaomin Peng, Yi Tian, Haikun Qian, Lijuan Chen, and Quanyi Wang

During surveillance for pneumonia of unknown etiology and sentinel hospital–based surveillance in Beijing, China, we detected avian influenza A(H7N9) virus infection in 4 persons who had pneumonia, influenza-like illness, or asymptomatic infections. Samples from poultry workers, associated poultry environments, and wild birds suggest that this virus might not be present in Beijing.

On March 31, 2013, three human infections with a novel avian influenza A(H7N9) virus were identified in Shanghai and Anhui Provinces in southeastern China (*1,2*). As of June 30, 2013, a total of 132 patients infected with this virus were reported in mainland China; 43 of these patients died (*3*). After detection of this novel virus, 6 targeted surveillance and sampling programs were implemented in Beijing, China, to identify possible cases.

The Study

The study was approved by the Institutional Review Board of the Beijing Center for Disease Control and Prevention (CDC). During April 1–June 30, samples were collected during surveillance and sampling programs in Beijing. Patients with pneumonia of unknown etiology in all hospitals were reported to the correspondent district CDC. Respiratory specimens were collected and sent to the district CDC for testing for avian influenza A(H7N9) virus by real-time PCR. Suspected positive specimens were verified by the Beijing CDC.

Surveillance was conducted in 23 sentinel hospitals and 17 collaborating laboratories during April 22–June 30. Surveillance participants were defined as patients with influenza-like illness (ILI) ≤3 days after illness onset (*4,5*). Pharyngeal swab specimens were collected from surveillance participants and sent to collaborating laboratories. Specimens were first screened by real-time PCR to identify influenza A and B viruses. Samples positive for influenza A virus were tested for influenza A(H3N2) virus, influenza A(H1N1)pdm09 virus, and influenza A(H7N9) virus by real-time PCR. Specimens positive for seasonal influenza viruses were subjected to virus isolation.

Persons in close contact with patients infected with influenza A(H7N9) virus or infected animals were medically observed for 7 days after the most recent exposure during April 1–June 30. Pharyngeal swab specimens were collected from close contacts on each day during medical observation and tested for influenza A(H7N9) virus by real-time PCR.

Poultry workers from 5 districts in Beijing were sampled during April 19–28. Pharyngeal swab specimens were collected from poultry workers and sent to the correspondent district CDC for detection of influenza A(H7N9) virus by real-time PCR.

At the same time that poultry workers were sampled, environmental samples were collected from associated poultry environments. These environmental samples were sent to district CDC laboratories for detection of influenza A(H7N9) virus.

Samples were collected from wild birds in 317 parks and 10 wetland natural reserve regions in Beijing (Figure) during May 3–10. Ten fecal samples were collected from each park, and 20 fecal samples were collected from each region. Samples were sent to the correspondent district CDC laboratories for detection of influenza A(H7N9) virus.

Thirty-nine patients with pneumonia of unknown etiology were reported, and 1 of these patients was infected with influenza A(H7N9) virus. This case was in a 7-year-old girl who had pneumonia, which was confirmed on April 12. The girl's mother and a 4-year-old acquaintance (boy) were positive for influenza A(H7N9) virus but were asymptomatic. This cluster involved 2 families of chicken dealers who lived in Beijing. On April 4, both families had purchased live chickens from the same batch, which had been transported from Tianjin, China. Samples from environments of places of residence of both families were positive for influenza A(H7N9) virus.

In sentinel hospital and laboratory-based surveillance, 3,526 pharyngeal swab specimens were subjected to PCR. One specimen was positive for influenza A(H7N9) virus and 73 specimens were positive for seasonal influenza viruses. The patient infected with influenza A(H7N9) virus was a 6-year-old boy who resided in Beijing. On May 21, fever, sore throat, and headache developed in this patient. He visited a sentinel hospital during May 21–24 and recovered on May 23. He did not have pneumonia or any history of exposure to birds or other animals. He had visited the hometown of his family in Shandong Province during May 14–15.

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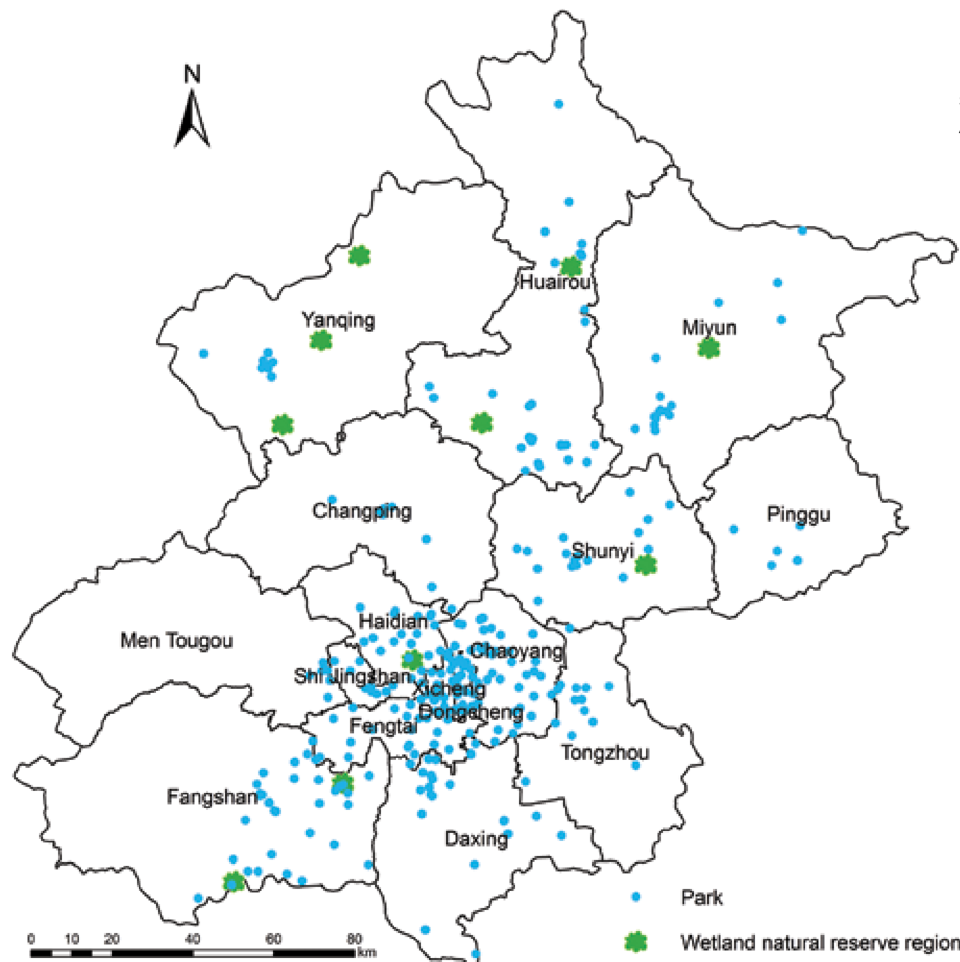

Figure. Distribution of 317 parks (dots) and 10 wetland natural reserve regions (leaves) in which surveillance for avian influenza A(H7N9) virus was conducted, Beijing, China, 2013.

A total of 1,422 poultry workers were recruited into this surveillance study, and 14 (0.98%) had ILI symptoms ≤2 weeks before recruitment. All workers were sampled but none were positive for influenza A(H7N9) virus. From the environments of poultry workers, 679 samples were collected; all were negative for influenza A(H7N9) virus. However, 3 samples from 2 other locations were positive for influenza A(H9N2) virus. In addition, 3,401 fecal samples of wild birds were collected; all were negative for influenza A virus.

Conclusions

Clinical manifestations of human infections with influenza A(H7N9) virus found in Beijing included pneumonia, ILI, and asymptomatic infection. Although patients infected with this virus in China have so far had lower respiratory tract infections (*6,7*), our findings suggest that infection with influenza A(H7N9) virus could cause a wide spectrum of clinical illness.

The first patient infected with influenza A(H7N9) virus in Beijing was found by surveillance for pneumonia of unknown etiology, which was initially designed for finding patients with severe acute respiratory syndrome or influenza A(H5N1) virus infection. Although hospital-based surveillance is less efficient in finding mild and asymptomatic infections, it may be the most feasible approach for identifying severe cases of infection with influenza A(H7N9) virus.

The second patient infected with influenza A(H7N9) virus, the 6-year-old boy, was found through sentinel hospital and laboratory-based surveillance. Before emergence of influenza A(H7N9) virus, surveillance was conducted by using cell culture–based virus isolation techniques. To increase assay sensitivity and rapidity, real-time PCR preceding virus isolation was adopted to replace the strategy of only using virus isolation. In addition, because only specimens positive for seasonal influenza viruses were subjected to virus isolation, this procedure could help avoid the risk for propagating influenza A(H7N9) virus from unknown specimens, as is caused by conducting virus isolation directly in biosafety level 2 laboratories. Our findings and those of another report (*8*) demonstrated that patients infected with influenza A(H7N9) virus only had ILI. Therefore, the strategy of PCR preceding virus isolation should

be the preferred option during sentinel hospital and laboratory-based surveillance.

Live poultry is the major source of avian influenza A(H7N9) (*6, 9–11*). In addition, migratory birds may participate in multiple reassortment events for emergence of H7N9 subtype virus (*1,12*). Therefore, the role of wild birds in transmission of avian influenza A(H7N9) virus to poultry or humans should not be ignored. In our samples from poultry workers, associated poultry environments, and wild birds, influenza A(H7N9) virus was not found, which suggests that this virus might not be present in Beijing.

In conclusion, human infections with H7N9 virus can cause a wide spectrum of clinical illnesses. Surveillance of patients with pneumonia of unknown etiology is preferred for early detection of severe cases. PCR is recommended for screening in sentinel hospital and laboratory-based surveillance of influenza A(H7N9).

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Investigating *Listeria* Outbreaks

Dr. Emily Cartwright, Infectious Disease fellow at Emory University and former EIS Officer with CDC's Division of Foodborne, Waterborne, and Environmental Diseases discusses foodborne *Listeria* outbreaks.

http://www2c.cdc.gov/podcasts/player.asp?f=8626870

Concurrent Parasitic Infections in a Renal Transplant Patient

To the Editor: Protozoan pathogens, including *Entamoeba histolytica*, *Giardia*, *Cryptosporidium*, *Cyclospora*, *Cystoisospora*, and microsporidia such as *Enterocytozoon bieneusi*, are well-known agents of diarrhea and a major public health problem in developing countries. Infection with *Cyclospora cayetanensis* and *E. bieneusi* can occur in immunocompromised and immunocompetent persons. Severe diarrhea and weight loss along with anorexia, nausea, and low-grade fever occur in immunocompromised persons, particularly those with HIV/AIDS and transplant recipients who are taking immunosuppressive drugs (*1*,*2*). However, transient diarrhea occurs in immunocompetent persons, notably in travelers returning from countries with poor hygienic standards (*1*–*3*).

We report on a kidney transplant recipient who had uncontrollable diarrhea and weight loss in whom *C. cayetanensis* and *E. bieneusi* were detected in biopsy specimens; the diarrhea resolved after treatment with drugs that act specifically on these 2 parasites. The patient was a 55-year-old man from the Dominican Republic living in New York, NY, USA; he had a history of long-term diabetes, coronary disease, and alcoholism. He had undergone a cadaveric renal transplant 14 months earlier and had an uneventful posttransplant course. After returning from visiting family in the Dominican Republic, he sought treatment for acute, profuse watery diarrhea in early November, 2009. He had >10 watery bowel movements daily that were associated with a 20-lb weight loss. His symptoms persisted for 2 months, and he required 2 hospitalizations for the diarrhea.

Results of 4 repeat fecal specimen tests (routine diagnostic microscopy and culture) were negative for parasites. Colonoscopy findings were normal; because of evidence of leukocytes in the feces and elevated fecal fat level, however, he received empirically prescribed metronidazole. Because his diarrhea and weight loss persisted, an upper endoscopy was performed, which revealed the presence of microsporidia. He then received albendazole for 3 weeks without substantial benefit.

The biopsy specimens were sent to the Centers for Disease Control and Prevention (Atlanta, GA, USA) for further analysis. Biopsy slides were stained with hematoxylin and eosin and with Gram chromotrope (*4*) and examined by microscopy. The Gram chromotrope–stained slide revealed oval spores, pinkish-red in color, measuring ≈1 μm (*5*). These spores were supra nuclear in position and were consistent with *E. bieneusi* (Figure, panel A). The tissue sections were scraped from the slides, DNA was extracted, and conventional PCR was performed by using *E. bieneusi*–specific primers as described (*5*); the sizes of the amplified product in the tissue DNA specimen and in the *E. bieneusi* control specimen were identical (Figure, panel B), confirming the presence of *E. bieneusi*. On further microscopic examination of the Gram chromotrope and the hematoxylin and eosin–stained slides, oval bodies (8–10 μm) were seen. A few of these oval bodies exhibited 4 spindle-shaped structures which were identified provisionally as merozoites of a coccidian parasite (Figure, panel C). Others had morula-like internal structure (Figure, panel D). We hypothesized that the coccidian parasite could either be *C. cayetanensis* or *Cystoisospora hominis*. Because the parasites, in various stages, were just beneath the surface of the epithelium, rather than deep within the

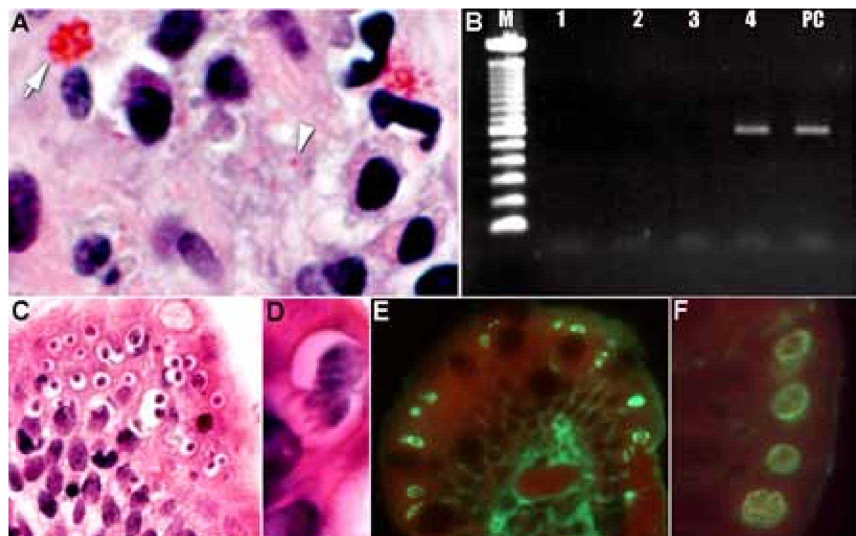

Figure. Tissue specimens from a kidney transplant recipient with concurrent parasitic infections after traveling to the Dominican Republic. A) Tissue section stained with Gram chromotrope. Note the apical location of a cluster of *Enterocytozoon bieneusi* spores at arrow (original magnification ×1,000) and single spore at arrowhead. B) Agarose gel showing PCR amplification of *E. bieneusi* 18S rDNA in the scraped section, as in panel A (M, 100-bp ladder; lane 1, DNA lysate diluted 1:5; lane 2, 1:10, lane 3, 1: 50 and lane 4, 1:100 of DNA lysate; lane 5 PC, positive control specimen). C) Tissue section stained with hematoxylin and eosin, demonstrating numerous sites in which *Cyclospora* spores are in developing stages (original magnification ×100). D) Higher power image of *Cyclospora* spores, showing the developing meronts (original magnification ×1,200). E) Immunofluorescent reactivity (dots in periphery) of the various life cycle stages of *Cyclospora* with a positive anti-*Cyclospora* serum sample (original magnification ×200). F) Note the bright fluorescence of the various parasite stages just below the apical (luminal) surface of the epithelial cells (original magnification ×1,000). A color version of this figure is available online (wwwnc.cdc.gov/EID/article/19/7/12-0926-F1.htm).

epithelium, we believed this organism to be a *Cyclospora* sp. rather than a *Cystoisospora* sp. We searched the serum bank of the Division of Parasitic Diseases, Centers for Disease Control and Prevention, and identified a serum sample from a person with a case of *C. cayetanensis* cyclosporiasis. An indirect immunofluorescence test was performed by using this serum on a deparaffinized section of the tissue biopsy specimen. Different stages of the coccidian organism were labeled brightly and produced apple-green fluorescence against a red counterstain (Eriochrome Black T), indicating that the parasite could possibly be a *Cyclospora* sp. (Figure, panels E, F). We considered that the *Cyclospora*-positive serum sample obtained from this particular patient may not be species-specific, since he might have also been infected with *Cystoisospora*. Therefore, we performed a real-time PCR assay that can distinguish *C. cayetanensis* from other coccidian parasites to identify the parasite definitively (*3*). DNA recovered from tissue in paraffin sections was successfully amplified and detected with this assay (data not shown), confirming the presence of *C. cayetanensis*.

The patient's illness was treated with albendazole for *E. bieneusi* infection and with trimethoprim and sulfamethoxazole for *C. cayetanensis* infection. The patient's diarrhea subsided after 1 week, and several subsequent fecal samples were negative for microsporidia spores and *Cyclospora* oocysts. His immunosuppressive medications were reduced, and he remained diarrhea-free for the following 3-year period of April 2010 to April 2013.

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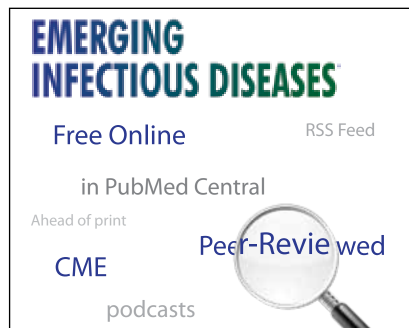

Vaccinia Virus in Household Environment during Bovine Vaccinia Outbreak, Brazil

To the Editor: Several exanthematic vaccinia virus (VACV) outbreaks have affected dairy cattle and rural workers in Brazil and Asia, and have caused economic losses and affected health services (*1–3*). VACV, the prototype of the genus *Orthopoxvirus* (OPV), exhibits serologic cross-reactivity with other OPV species and was used during the smallpox eradication campaign (*1*). Several VACV strains have been isolated during bovine vaccinia outbreaks in Brazil and have been characterized by molecular and biologic methods (*3,4*). Bovine vaccinia infections in humans are frequently related to occupational contact with sick animals during milking but have never been shown to be associated with fomites or indoor environments (*1,3*).

In August 2011, a bovine vaccinia outbreak was reported in Carangola County, Minas Gerais State, Brazil. During this outbreak, several farms were affected, and the outbreak involved humans and dairy cattle. A 41-year-old man (patient 1) who worked on a farm (20°36′30.7″S, 42°17′53.9″W) was hospitalized. He had painful lesions on the hands, high fever, lymphadenopathy, malaise, and fainting episodes. This patient reported recent contact with sick animals on the farm during milking.

At the same time, a 57-year-old man (patient 2), the owner of the farm, had a lesion on the right hand. This infection was also related to occupational exposure. Some days after the appearance of the hand lesion, this patient presumably inoculated himself at the site of an abrasion he had recently received on his nose. This resulted in

development of a large and painful lesion. This patient reported milking cows daily. He had been vaccinated against smallpox before 1977.

A total of 5 humans and 15 cows were involved in this outbreak on 5 farms. Clinical samples were obtained from the 2 patients and from 3 sick cows. Dried swab specimens from lesions were soaked in 200 mL of phosphate-buffered saline containing penicillin (200 U/mL), amphotericin B (4 μg/mL), and gentamicin (100 μg/mL); homogenized, and centrifuged at 2,000 × *g* for 3 min. The supernatants were used for molecular diagnosis and virus isolation (*3,5,6*). Supernatants were tested by using OPV-specific PCRs that targeted the C11R gene, which encodes viral growth factor (*vgf*), and the A26L gene, which encodes A-type inclusion (ATI) protein. Samples from the 2 patients were positive for *vgf* and ATI (*7*). At least 1 sample (blood or scabs) from each sick animal was also positive by PCR.

To assess the risk for virus spread in indoor environments, we collected swab specimens from several objects, including doorknobs, bathroom surfaces, and the pillow of patient 2. The pillow was positive for *vgf* and ATI by PCR.

To isolate the virus, we infected monolayers of BSC-40 cells cultured in a 6-well plate with sample supernatants and incubated the cells at 37°C for 72 h or until a cytopathic effect was detected (*3,5,6*). We isolated virus from a sample from patient 1 and from an environmental sample (the pillow of patient 2), which showed positive results in the molecular diagnostic assays.

To confirm that the isolated VACV was the OPV involved in this outbreak, we sequenced partial fragments of the A56R and A26L genes from the isolated virus. Fragments obtained were directly sequenced in both orientations in triplicate (MegaBACE

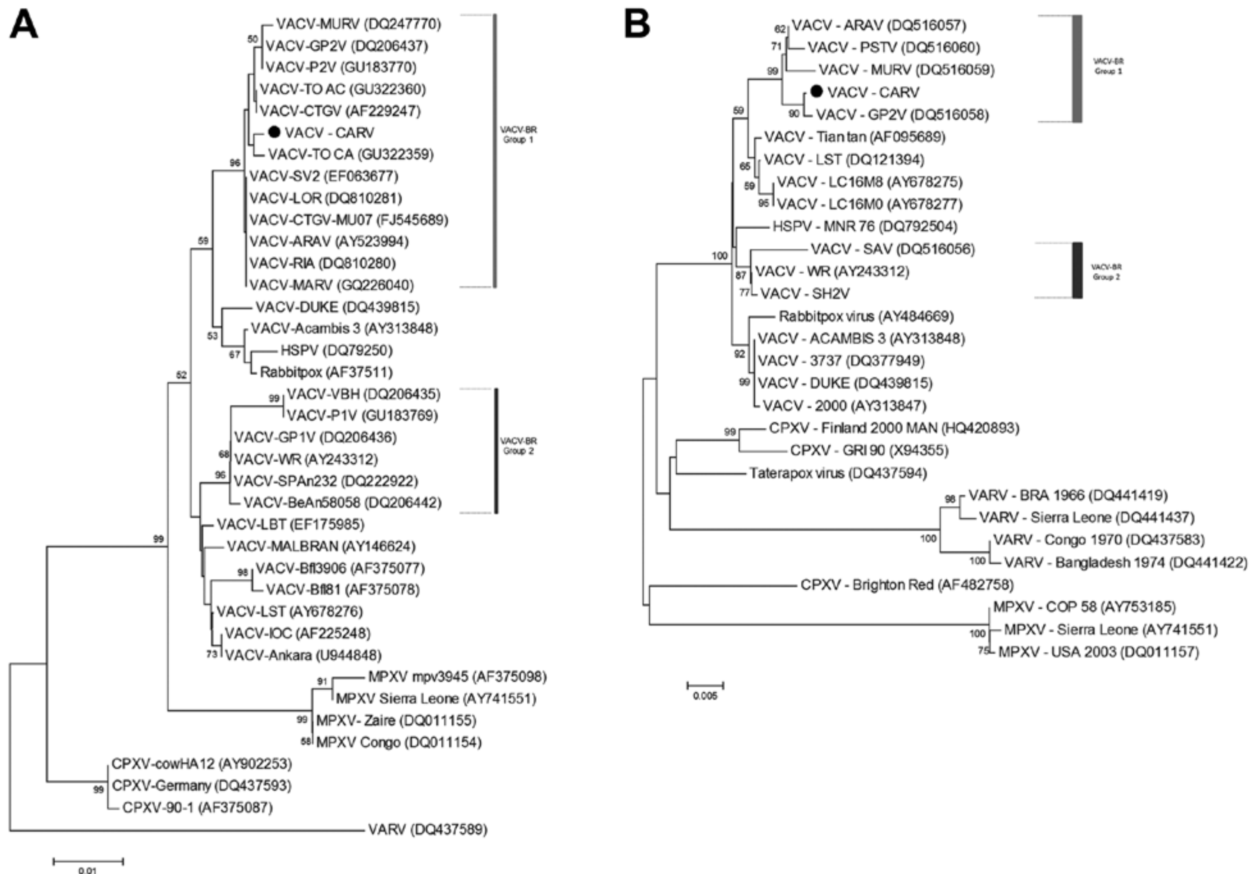

Figure. Phylogenetic trees based on orthopoxvirus nucleotide sequences, including vaccinia virus (VACV) from Brazil (VACV-BR). Phylogenetic analysis was performed for A56R (A) and A26L (B) gene sequences and grouped VACV-BR strains into 2 branches: group 1 and 2. The Carangola virus (CARV) isolate is indicated by the black dots. Both trees show grouping of CARV into VACV-BR cluster composed of Guarani P2 virus (GP2V), Cantagalo virus (CTGV), and other viruses. Trees were constructed by using the neighbor-joining method, the Tamura-Nei model of nucleotide substitutions, and bootstrap values of 1,000 replicates in MEGA version 4.0 (Arizona State University, Phoenix, AZ, USA). GenBank accession numbers are indicated in parentheses. Values along the branches indicate bootstrap values. Scale bars indicate nucleotide substitutions per site. MURV, Muriaé virus; MARV, Mariana virus; HSPV, horsepox virus; MPXV, monkeypox virus; ARAV, Araçatuba virus; PSTV, Passatempo virus; VARV, variola virus.

1000 Sequencer; GE Healthcare, Little Chalfont, UK). Sequences were aligned with published OPV sequences in GenBank by using the ClustalW (www.clustal.org/) method and manually aligned by using MEGA version 4.0 (Arizona State University, Phoenix, AZ, USA). VACV molecular signatures of 18-nt and 12-nt deletions were observed in the A56R and A26L genes, respectively. Phylogenetic trees (Figure), which were constructed by using the neighbor-joining method, the Tamura-Nei model of nucleotide substitutions, and 1,000 bootstrap replicates in MEGA 4.0, demonstrated that this isolate clustered with other group 1 VACV isolates from Brazil. We named this isolate Carangola virus.

We isolated VACV from an indoor environment during a bovine vaccinia outbreak. VACV infections have been frequently associated with occupational activities, primarily direct contact with sick animals (*1,3*). However, in some cases, the source of the infection is unknown, especially in patients who did not participate in milking activities.

Human-to-human transmission has been suggested to have occurred in some bovine vaccinia outbreaks in Brazil, and nosocomial infection has been reported Asia (*2,8*). Household transmission of VACV has also been described in the United States after contact with lesions of a smallpox vaccinee in the military (*9*). VACV from Brazil shows long-lasting stability under environmental conditions, especially when associated with organic matter (*10*). Although the wife of patient 2 did not exhibit any typical clinical symptoms of VAVC infection, we believe that relatives sharing household environments with patients with lesions may be at risk for VACV infection. Isolation of VACV from a household environment raises new questions about nonoccupational risk factors related to bovine vaccinia transmission.

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Porcine Epidemic Diarrhea Virus Variants with High Pathogenicity, China

To the Editor: Porcine epidemic diarrhea (PED), a serious and highly contagious swine disease, is characterized by severe diarrhea and dehydration in suckling piglets. The etiologic agent, porcine epidemic diarrhea virus (PEDV), is an enveloped, single-stranded RNA virus (family *Coronaviridae*, order Nidovirales). The viral disease was discovered in the United Kingdom in 1971 and subsequently reported in many swine-producing countries in Europe and Asia (*1*,*2*). Although most sow herds previously had received CV777-based inactivated vaccine, a large-scale outbreak of PED has been associated with high rates of illness and death in suckling piglets in China since late 2010, resulting in substantial economic losses.

We collected 217 piglets (126 alive, 91 dead) with diarrhea from 42 farms in Shandong Province, China, during November 2010–April 2012. To determine the etiologic agent of the outbreak, we analyzed samples of intestine and its contents. A total of 175 (80.6%) samples were PEDV positive, indicating that PEDV was the dominant pathogen for this diarrheal outbreak. Other pathogens also were identified: porcine transmissible gastroenteritis virus (8.3%), rotavirus (3.7%), and *Escherichia coli* (3.2%). Furthermore, 8.1% of pigs with diarrhea were co-infected by 2 pathogens.

Three PEDV field strains (ZB, YS, and SH) were isolated from different farms where 100% of sucking piglets had diarrhea. The virus isolates were plaque-purified once in Vero cells. The spike protein (S), encoded by S gene of PEDV, plays a pivotal role in cell adsorption, membrane fusion, and induction of neutralizing antibodies (*3*,*4*). The full-length S genes of 3 isolates were amplified by reverse transcription PCR with 2 pairs of primers (*5*) and sequenced to identify the genetic variation of the isolates.

Sequences for the S gene were submitted to GenBank (accession nos. for YS: JQ771753; SH: JQ771751; and ZB: JQ771752). The S gene nucleotide sequences and deduced amino acid sequences of the 3 new isolates were aligned with the sequences of published isolates by using MEGA 4.0 (www.megasoftware.net). S protein identity among the 3 new isolates was 99.4%–99.6% and shared 93.6%–93.7% identity with classical CV777 strain. We identified numerous sequence variations in S protein of the 3 isolates (online Technical Appendix Table, wwwnc.cdc.gov/EID/article/19/12/12-1088-Techapp1.pdf). Two separate insertions were discovered: a 1-aa (N) insertion at position 140 and a 4-aa (QGVN) insertion at positions 59–62. A 2-aa (NI) deletion was identified at positions 163–164. A total of 34 separate substitutions were identified, and the number(s) of replaced amino acids ranged from 1 through 5. These sequence variations were similar to those in CH/FJND-3/2011, CH1, CH8, and CHGD-01 isolates recently reported in China (*6*).

To determine the virulence of the PEDV isolates, we experimentally infected fifteen 4-day-old Duroc crossbred piglets with the YS and ZB isolates. The piglets were randomly allotted to 3 groups, each group consisting of 5 pigs. These piglets were shown by serologic analysis to be negative for antibodies against PEDV, porcine reproductive and respiratory syndrome virus, porcine transmissible gastroenteritis virus, and pseudorabies virus.

In the piglets inoculated orally with 1.5 mL of YS isolate ($10^{3.0}$ 50% tissue culture infectious doses/mL), diarrhea was observed at 3–6 days postinfection (dpi). One piglet died at 5 dpi, and 4 piglets died at 6 dpi. The dead piglets showed hemorrhage and shedding in the gastric mucosa, swelling and congestion in the mesenteric lymph nodes, and hemorrhage in the intestinal wall (Figure, panel A). Histopathologic changes included epithelial cell shedding; intrinsic layer hemorrhage and excessive infiltration of lymphocytes in the stomach; and congestion, edema, and epithelial cell shedding in the intestinal mucosa (Figure, panel B). PEDV was recovered from the dead piglets, and the full-length S gene was amplified by reverse transcription PCR and sequenced. In the S genes of YS and ZB isolates, 3 and 2 single nucleotides were replaced, respectively, but no mutated amino acid was introduced between the inoculated and recovered viruses.

For the piglets orally infected with 1.5 mL of ZB isolate ($10^{3.1}$ 50% tissue culture infectious doses/mL), diarrhea was observed at 3–5 dpi, and all 5 piglets

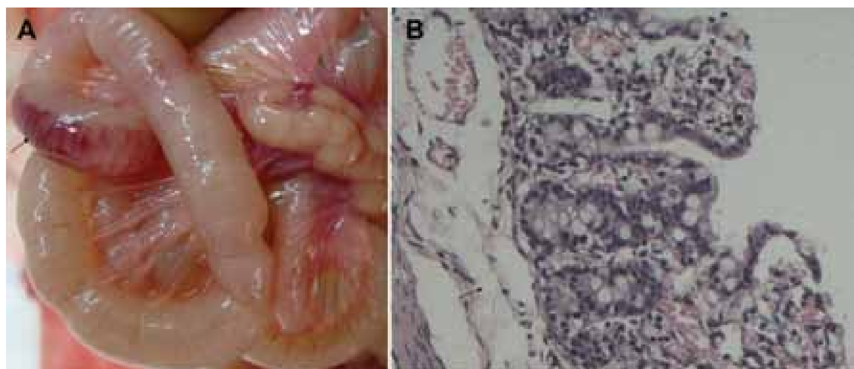

Figure. Signs of porcine epidemic diarrhea virus in piglets, China, November 2010–April 2012. A) Hemorrhage in the intestinal wall. B) Congestion, edema, and epithelial cell shedding in the intestinal mucosa. Hematoxylin and eosin stain; original magnification ×200.

died at 5 dpi. The dead piglets showed similar lesions to those of the piglets infected with YS isolate in the stomach, intestines, and mesenteric lymph nodes. Piglets in the control group orally inoculated with Dulbecco minimal essential medium remained healthy during the experiment, and no obvious pathologic changes were observed.

Our investigation indicated that the recent diarrhea outbreaks were mainly caused by PEDV variants with novel genetic markers that distinguish them from classical strains. The YS and ZB isolates were highly virulent in piglets. Unlike CV777, the PEDV variants remained almost unchanged in the epitope at positions 499–638; however, a 2-aa deletion, a 1-aa insertion, and 18 separate substitutions were identified in the epitope at positions 83–276 (*7*,*8*). These variations of amino acid sequences probably changed the immunogenicity of S protein and led to immunization failure of current commercial vaccines made from classical PEDV strains. However, how PEDV has evolved and varies in pig herds are not clear. Further studies, including extensive genomic sequence analyses and serologic cross-neutralization tests, should be conducted.

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New Delhi Metallo-β-Lactamase-1 in Carbapenem-Resistant *Salmonella* Strain, China

To the Editor: Carbapenem resistance in Enterobacteriaceae can occur through the production of carbapenem-hydrolyzing enzymes such as New Delhi metallo-β-lactamase-1 (NDM-1) (*1*). In recent years, plasmid-mediated NDM-1 has spread rapidly worldwide and into multiple Enterobacteriaceae species, such as *Klebsiella pneumoniae* and *Escherichia coli* (*2*).

NDM-1 has been reported in 2 strains of *Salmonella* spp., which were isolated from feces and urine specimens during screening for multidrug-resistant bacteria in patients from India (*3*,*4*). We report the isolation of 1 community-acquired NDM-1–bearing *Salmonella* strain isolated from a child with acute diarrhea.

The *Salmonella* strain was isolated from the feces of an 11-month-old girl at Lishui Central Hospital, Zhejiang Province, China, on July 25, 2012. Six days before admission, a fever ≤40°C, accompanied by a cough, developed in the patient. Four days before admission, physical examination showed fine rales in both lungs. The leukocyte count was 8,900 cells/µL, with 80% neutrophils. No obvious abnormalities were found on a chest radiograph.

The patient was given a diagnosis of acute bronchitis, and the condition was treated with parenteral cefoxitin for 3 days and parenteral piperacillin/tazobactam for 1 day, but fever persisted. Two days before admission, diarrhea (4–5 times/day with loose feces containing mucus and blood) developed. On admission day, fecal analysis showed 3–4 leukocytes and 1–3 erythrocytes per high-power field.

A *Salmonella* sp. was isolated from feces obtained at admission and identified as *S. enterica* subsp. *enterica* serovar Stanley by serotyping by the local Centers for Disease Control and Prevention.

The patient was then given a diagnosis of bacterial enteritis and received intravenous azithromycin and latamoxef. Fever and diarrhea resolved over the next 3 days. On the fifth day of hospitalization, a fecal culture was negative for *Salmonella* spp. and the patient was discharged. At a follow-up visit 3 months later, *Salmonella* spp. or other carbapenem-resistant bacteria were not isolated from feces samples from the patient or her grandmother and brother, who lived with her.

The patient and her family had not traveled to any country during the year, including countries with a high prevalence of NDM-1 producers. The patient was living in a small rural village in southern China and did not have a special diet. She was healthy before hospitalization for fever. She was born by cesarean section and did not have contacts with hospitalized patients.

MICs of antimicrobial drugs were determined by agar dilution and interpreted by using revised Clinical and Laboratory Standards Institute breakpoints (*5*). The *Salmonella* Stanley strain was resistant to all β-lactam antimicrobial drugs tested, including cephalosporins and carbapenems, but susceptible to chloramphenicol, ciprofloxacin, tetracycline, and fosfomycin, and had azithromycin MICs of 4 μg/mL (Table).

A modified Hodge test result for *Salmonella* strain Stanley was weakly positive. Production of metallo-β-lactamase was detected by using an imipenem-EDTA double-disk synergy test. Carbapenamase-encoding genes, including bla_{KPC-2}, bla_{VIM}, bla_{IMP}, bla_{SPM-1}, bla_{GIM-1}, and bla_{SIM-1}, were detected by using PCR as described (*2*). Only the bla_{NDM-1} gene was detected (with primers 5′-GGCGGAATGGCTCATCACGA-3′ and 5′-CGCAACACAGCCTGACTTTC-3′). The PCR product sequence was consistent with that of NDM-1 (GenBank accession no. FN396876).

Conjugation experiments were conducted as described (*6*). Carbapenem resistance could be transferred from *Salmonella* strain Stanley to *E. coli* C600 Rif^r and *K. pneumoniae* 13883 Rif^r at frequencies of 1 transconjugant per ≈1.0×10^4 and 4.0×10^7 bacterial cells, respectively, after exposure for 15 min.

Plasmid DNA was extracted by using a Plasmid Midi Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Electrophoresis showed that donor and transconjugant strains had the same plasmid profile; both contained an ≈140-kb plasmid. A PCR-based method for plasmid replicon typing (*7*) indicated that the plasmid belonged to incompatibility group IncA/C.

Experiments of bla_{NDM-1} stability in *Salmonella* spp., *E. coli* transconjugants, and *K. pneumoniae* transconjugants were conducted by using the method of Wang et al. (*6*). Twenty colonies were randomly selected every day during days 2–15. Only 2 colonies of the *Salmonella* Stanley strain lost carbapenem resistance; these colonies were collected on the second and ninth days of passage, respectively. Plasmids containing bla_{NDM-1} were not present in these 2 colonies. No transconjugants lost carbapenem resistance during 14 days of passage.

Although *Salmonella* spp. have shown increased resistance to cephalosporins and quinolones, resistance to carbapenems is rare (*3,4,8,9*). Because of emerging resistance to traditionally recommended antimicrobial agents, azithromycin is increasingly used for treatment of invasive *Salmonella* spp. infections in children (*10*). The patient with carbapenem-resistant *Salmonella* infection and acute diarrhea was cured by treatment with azithromycin.

This report indicates ongoing spread of NDM-1–bearing *Salmonella* strains. If one considers the high conjugation frequency and stability of the IncA/C plasmid containing NDM-1 in *Salmonella* spp., one

Table. Antimicrobial drug susceptibility of *Salmonella* strain Stanley and transconjugants containing New Delhi metallo-β-lactamase-1, China

	MIC, μg/mL				
Drug	*Salmonella* strain Stanley	*Escherichia coli* C600	*E. coli* C600 transconjugant	*Klebsiella pneumoniae* 13883	*K. pneumoniae* 13883 transconjugant
Piperacillin//tazobactam	>128/4	2/4	128/4	1/4	128/4
Ceftazidime	>128	0.25	>128	0.25	>128
Cefotaxime	>128	<0.06	128	<0.06	>128
Latamoxef	64	0.25	32	<0.06	32
Cefepime	16	<0.06	16	<0.06	16
Imipenem	8	<0.06	8	<0.06	16
Meropenem	4	<0.06	4	<0.06	8
Fosfomycin	<0.06	<0.06	<0.06	<0.06	<0.06
Minocycline	2	0.5	0.5	1	0.5
Ciprofloxacin	<0.06	<0.06	<0.06	<0.06	<0.06
Chloramphenicol	2	4	4	4	4
Azithromycin	4	1	1	2	1
Trimethoprim/sulfamethoxazole	>152/8	0.3/0.015	>152/8	1.2/0.06	>152/8

would conclude that it might increase spread of bacterial drug resistance. Prompt recognition of carbapenem-resistant *Salmonella* spp. and initiation of appropriate infection control measures are essential to avoid spread of these organisms.

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Contagious Caprine Pleuropneumonia in Endangered Tibetan Antelope, China, 2012

To the Editor: Contagious caprine pleuropneumonia is a severe respiratory disease of goats caused by *Mycoplasma capricolum* subsp. *capripneumoniae* (Mccp), a member of the *M. mycoides* cluster (*1*). Mccp infection is associated with a 60% mortality rate and 90% illness rate, and the disease can cause substantial losses of livestock (*1*,*2*). We report a 2012 outbreak of contagious caprine pleuropneumonia in endangered Tibetan antelope (*Pantholops hodgsonii*) in China.

In 2000, the International Union of Conservation of Nature first listed the Tibetan antelope as an endangered species (*3*), and in 2004, the number of these antelope was estimated at 150,000 (*4*). Most Tibetan antelope live on China's Qinghai–Tibet Plateau at an altitude of 3,700–5,500 m (*3*).

During September–December 2012, ≈2,400 endangered Tibetan antelope were found dead in the Naqu area of Tibet; the dead animals represented 16% of the 15,000 Tibetan antelope thought to live in the area. Necropsy was performed on 13 of the antelope at sites within the Shenzha, Shuanghu, and Nima localities of the Naqu area (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/20/1/13-0067-Techapp1.pdf). Gross pathologic lesions were localized exclusively to the lung, where severe pleuropneumonia with partial hepatization was observed (Figure, panel A). The lungs of some affected antelope displayed a thickening of the interlobular septa, pleuritis, and an accumulation of straw-colored pleural fluid. The pleural exudate solidified to form a gelatinous covering on the lung (Figure, panel B).

Samples of lung tissue from 5 of the antelope were selected for histologic examination. Four of the samples showed fibrinous pneumonia with serofibrinous fluid and an inflammatory cell infiltrate consisting mainly of lymphocytes in the alveoli (Figure, panel C) and bronchioles (Figure, panel D). One sample showed pulmonary edema with a protein-rich fluid effusion in alveoli.

Lung tissue from each of the 13 antelope was minced and inoculated into modified Hayflick broth, which has been used extensively to isolate *Mycoplasma* spp. from animals. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 (*5*). The medium was examined daily by comparing inoculated broth with an uninoculated control broth. Moderate turbidity, a color change from pink to yellow, and an appreciable swirl of the culture when rotated were used as indicators of

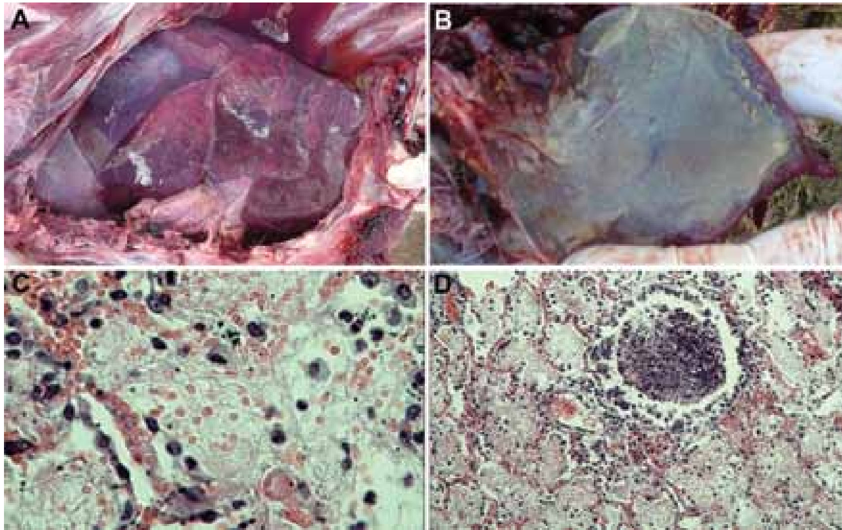

Figure. Pneumonia caused by *Mycoplasma capricolum* subsp. *capripneumoniae* in Tibetan antelope *(Pantholops hodgsonii*), Tibet, 2012. A) Lung of a caprine pleuropneumonia–infected Tibetan antelope (sample SZM2) showing lung hepatization. B) Lung of a caprine pleuropneumonia–infected Tibetan antelope (sample SH3) showing fibrin deposition. C and D) Fibrinous pneumonia with serofibrinous fluid and an inflammatory cell infiltrate, consisting of mainly lymphocytes, in the alveoli (panel C, sample SZM2, hematoxylin and eosin stain; original magnification ×400) and bronchioles (panel D, sample SH3, hematoxylin and eosin stain; original magnification ×100). Refer to online Technical Appendix Table 1 (wwwnc.cdc.gov/EID/article/19/12/13-0067-Techapp1.pdf) for details of the lung samples used to generate images for this figure.

mycoplasma growth. After 2–3 passages in culture, 11 of 13 samples showed growth of mycoplasma. The presence of mycoplasma-like particles in the 11 growth-positive cultures was confirmed by electron microscopy (online Technical Appendix Figure 1). Collectively, these observations implicated mycoplasma as the cause of disease in the affected antelope.

We next screened lung samples from each of the 13 Tibetan antelope by PCR for evidence of *M. mycoides* cluster and *M. bovis*. Eleven samples were positive for Mccp, but no other types of mycoplasma were detected (online Technical Appendix Tables 1, 2). We conducted PCR as described (*6*) on the *arc*D gene of Mccp. In brief, we conducted 35 cycles of 30 s at 94°C, 15 s at 47°C, and 15 s at 72°C. Of note, lung sample SH7, which showed pulmonary edema, was negative for mycoplasma by PCR and culture. Lung samples from the 13 dead Tibetan antelope were also tested for an additional 16 potential pathogens (online Technical Appendix Tables 1, 2) by PCR or reverse transcription PCR. No pathogens other than Mccp were detected.

To assess the relationship of the Mccp strain isolated from infected Tibetan antelope with previously isolated Mccp strains and the closely related *M. capricolum* subsp. *capricolum* (Mcc), we analyzed a 562-bp segment of the H2 gene of Mccp, which was used to distinguish the Mccp and Mcc as reported by Lorenzon et al. (*7*), isolated from an infected Tibetan antelope in Shuanghu county (sample SH3). The partial H2 sequence (GenBank accession no. KC441725) had higher sequence identity with Mccp isolates (99.3%–99.7%) than with Mcc isolates (90.2%–91.2% (online Technical Appendix Figure 2). This phylogenetic analysis demonstrated that the Mccp isolated from infected Tibetan antelope belongs to the same clade as Mccp strains previously isolated in Africa and Asia.

The changing habitat of endangered Tibetan antelope may lead to increased exposure to Mccp, which can cause devastating outbreaks, such as the one reported here. Goats and sheep are herded on grasslands at an altitude of 4,300–5,000 m, the same area where Tibetan antelope reside. Goats are a reservoir for Mccp, and Mccp has been isolated from sheep in mixed herds with goats (*8*). Rail lines traverse the rangelands in this region, limiting the normal migration patterns of the Tibetan antelope population. Interaction among goats, sheep, and Tibetan antelope in this region, combined with the effect of human infringement on their rangeland, may increase the risk for disease emergence and transmission.

Our results show that contagious caprine pleuropneumonia may pose a substantial threat to the survival of endangered Tibetan antelope. Surveillance for Mccp infection among Tibetan antelope populations and domestic and wild goat and sheep populations that have close contact with the Tibetan antelope should be considered.

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Unexpected *Brucella suis* Biovar 2 Infection in a Dairy Cow, Belgium

To the Editor: Belgium was declared free of bovine brucellosis by the European Union in 2003 (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2003:156:0074:0078:EN:PDF). To maintain this status, the Federal Agency for the Safety of the Food Chain implemented a monitoring program, approved by the European Union, that consists of random serologic surveys and mandatory reporting of spontaneous abortion. This reporting enabled the detection of 2 outbreaks of bovine brucellosis in cattle caused by *Brucella abortus* biovar 3, in 2010 and 2012, but the origin of these outbreaks has not been identified.

As part of an epidemiologic survey conducted to prevent the spread of the infection, ELISA testing (Brucellosis Antibody Test Kit; Idexx, Hoofddorp, the Netherlands) was performed on bulk milk samples from 9,013 dairy farms in the country; 75 farms had positive test results and were classified as reactor farms. All cows in milk production on these farms were serologically tested, first by using slow agglutination test with the addition of EDTA to the antigen, and then, if results were positive, by a commercial ELISA. If results of the ELISA were positive, a confirmatory internal ELISA was performed at the national reference laboratory. A total of 41 seropositive cows from 27 farms were identified. All confirmed seropositive cows were slaughtered for bacteriologic investigation; all had negative test results for *B. abortus*.

On March 23, 2012, bulk milk sample testing for a farm in the province of Namur showed positive results. Testing performed in January 2011 on milk collected from the same farm had yielded negative results. The 150 cattle (including 55 dairy cows) on this farm were further serologically tested. One nonpregnant dairy cow had positive test results by slow agglutination test and ELISAs and was slaughtered on April 23, 2012. The cow was >4 years old, born in the farm, last calved in March 2011, and showed no clinical sign of brucellosis.

Bacteriologic examination was conducted on spleen, uterus, lymph nodes, and udder tissue samples; *Brucella* spp. were cultured from the spleen and uterus. Bacterial colonies grew on *Brucella* agar supplemented with 5% horse serum in the presence of basic thionine and safranine O; CO_2 was not required for growth, and H_2S was not produced. The isolates showed catalase, oxidase, and urease activity, a biochemical profile typical of *B. suis* biovar 2; identity was confirmed by real-time PCR on DNA extracted directly from the uterus (*1*).

A stamping out with compensation policy was implemented for this farm by the Federal Agency for the Safety of the Food Chain, according to European Union regulations, and subsequent epidemiologic investigations were performed. The farm owner is not a hunter. The culture-positive cow originated from a group of 10

nonpregnant or dry dairy cows that had been held in the same pasture, distant from the main farm structures, during October 15–November 15, 2011; during the stamping out process, a second dairy cow from this group had a positive test result by ELISA.

Hunting of wild boar (*Sus scrofa*) had been organized during September–December 2011 in the adjacent forest, and wild boar offal was discarded in a corner of the pasture, with no biosecurity precautions. A recent study confirmed the high prevalence of *B. suis* biovar 2 infection in wild boars in this province (*2*). These findings suggest that these animals were naturally infected with *B. suis* biovar 2; because of the period between infection and testing, the results indicate that antibodies can be detected in cattle by ELISA performed on milk or serum >16 weeks after infection.

Blood samples were taken from the farmer, his wife, and their 2 children, all of whom regularly consumed raw milk. No clinical signs or symptoms suggestive of brucellosis were reported, and slow agglutination test results for all family members were negative (titer <160), which suggests they had no exposure to *B. suis* biovar 2 (*3*). A total of 111 cattle carcasses, including that of the second seropositive cow, were sampled at the abattoir, and all other samples were negative for *Brucella* spp.

Our findings indicate that preventive measures against the spread of pathogens such as *Brucella* spp. must be implemented by hunters (i.e., awareness campaigns, biosecurity education, and responsible hunting practices). In addition, biochemical typing of *Brucella* spp. is necessary to trace the source of infections (*4,5*), and epidemiologic inquiry of positive test result(s) should be conducted to identify or exclude bovine brucellosis and to investigate possible *B. suis* biovar 2 infections. Our bacteriologic results (absence of isolation of *B. suis* biovar 2 from all samples collected at the abattoir) suggest that stamping out is not necessary because *B. suis* biovar 2 is not likely to be transmitted between cattle because they are spillover hosts, not preferential hosts for *B. suis* biovar 2, and are thus not likely to sustain the infection. Finally, from a veterinary public health perspective, *B. suis* biovar 2 has a low residual pathogenicity in humans (*5,6*).

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Hepatitis E and Lymphocytic Leukemia in Man, Italy

To the Editor: Hepatitis E is an enterically transmitted infection with worldwide distribution and high prevalence in developing countries. This disease can occur as large water-borne epidemics associated with hepatitis E virus (HEV) genotypes 1 and 2. Hepatitis E is less common in industrialized countries, including Italy (*1*), where sporadic autochthonous cases associated with genotypes 3 and 4 have been reported. Virus strains of these genotypes are widespread in different mammalian species, including wild boar (*2*).

We report a case of hepatitis E in a 60-year-old man born and living in Vicenza, Italy, who was admitted to the Emergency Department of Vicenza Hospital on May 9, 2012 with symptoms of acute icteric hepatitis. He had been given a diagnosis of chronic lymphocytic leukemia and hemolytic anemia in 2003 and underwent 8 treatment cycles of cyclophosphamide and steroids, which were completed 20 days before he came to the Emergency Department.

His liver function test results at admission were the following: alanine aminotransferase 1,804 IU/L, total bilirubin 24.1 mg/dL, and alkaline

phosphatase 137 IU/L. Test results for other causes of viral hepatitis were negative (Figure). A liver biopsy performed on June 1 showed severe acute lobular hepatitis with necrosis and cholestasis. Serum obtained at admission was positive for IgM and IgG against HEV (Dia.Pro Test, Milan, Italy).

HEV RNA was detected by reverse transcription PCR and open reading frame 2 (*3*) was detected in serum and feces samples on May 9. Phylogenetic analysis of sequences identified HEV genotype 3 subtype h in serum and fecal samples (GenBank accession nos. KC782933 and KC782934).

Three months after admission, the patient had viremia, and results of liver function tests were abnormal. Recent data suggest that immunosuppressed persons who are viremic 3 months after HEV infection do not spontaneously clear HEV (*4*). Therefore, the patient was given antiviral therapy to achieve viral clearance. Ribavirin, 1,000 mg/day in 2 doses (400 and 600 mg), was administered during August 2–November 2, 2012. This drug was well tolerated, although the patient experienced mild anemia (hemoglobin level 10.5 mg/dL), which did not require any treatment.

Liver function test results returned to reference levels on day 14 of treatment. HEV RNA was detected in blood and feces on day 18 of treatment (August 20). Viral clearance (HEV absent from feces and serum) was achieved on day 54 of treatment (September 27) and was sustained over a 6-month period after the end of therapy.

The source of the HEV infection was uncertain. The patient had never traveled outside Italy. However, he had butchered a wild boar that he had hunted in Barberino del Mugello (Tuscany) in March 2012. The patient's wife, who also butchered the animal, was positive for IgG against HEV but negative for IgM against HEV and for HEV RNA in February 2013. No boar meat was available for HEV testing, which indicated that this route of transmission was likely, but not confirmed.

Autochthonous hepatitis E in industrialized countries is usually an acute, self-limiting disease, but chronic disease can occur in immunocompromised hosts (*5*). These hosts include transplant recipients, persons infected with HIV, and patients with hematologic malignancies. Chronic infection with HEV has only been documented with genotype 3 strains and has been observed in many countries in Europe, However, to our knowledge, no cases of chronic infection with HEV have been reported in Italy. Our results indicate that chronic infection with HEV genotype 3 occurs in Italy.

Acute and chronic hepatitis E have been reported in patients with hematologic malignancies. An autochthonous case of acute hepatitis E was recently described in Germany in a patient with chronic lymphocytic leukemia that had been treated with chemotherapy, a bone marrow transplantation, and hemodialysis (*6*). He did not receive any specific treatment for hepatitis E and died of acute liver failure 39 days after diagnosis. Reactivation of hepatitis E in a patient with acute lymphoblastic leukemia was reported after allogeneic stem cell transplantation (*7*). HEV can also be transmitted directly from an infected transplanted organ.

Ribavirin monotherapy is an effective treatment for most patients with chronic HEV infection (*8*). It has also been used successfully to treat acute severe infection by genotype 1 of HEV in developing countries and by genotype 3 in industrialized countries (*9*), and is used to treat hepatitis C,

Figure. Clinical and laboratory data for a 60-year-old man with hepatitis E and lymphocytic leukemia, Italy, 2012. Start of ribavirin treatment and virologic response are indicated. A differential diagnosis was obtained by using abdominal ultrasound, which showed an enlarged hypoechogenic liver and thickening of the gallbladder wall (5 mm) with gallstones in the lumen, but a regular biliary tree. An enlarged spleen (bipolar diameter 15.07 cm) and lymph nodes were attributed to chronic lymphocytic leukemia. Test results for the following markers were negative: viral hepatitis A, B, and C; hepatitis B virus DNA (nested PCR; Roche, Switzerland); hepatitis C virus (nested PCR; Roche); cytomegalovirus virus DNA; Epstein-Barr virus; Q fever; agglutination for *Leptospira* spp. (Galton's test); enteric fever; *Borrelia* spp.; *Bartonella* spp.; autoantibodies (anti-nuclear, anti–liver kidney microsomal, anti-mitochondrial); blood and feces cultures; and ova and parasites in feces. HEV, hepatitis E virus; NA, not available; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Reference ranges were 8–48 IU/L for AST, 7–55 IU/L for ALT, and 0.1–1.0 mg/dL for total bilirubin.

although its mechanism of action against HCV and HEV is uncertain. Data are limited on the use of ribavirin in patients with chronic hepatitis E and hematologic malignancies (*10*). The outcome for our patient suggests that ribavirin might be useful for treating hepatitis E in such patients.

In conclusion, all patients with hepatitis of unknown origin should be tested for HEV, in particular, immunocompromised patients, because they are at risk of acquiring chronic hepatitis and having an adverse outcome. Ribavirin appears to be efficacious in treating hepatitis E and should be considered for any immunocompromised person who has viremia 3 months after acute infection.

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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.

Q Fever Surveillance in Ruminants, Thailand, 2012

To the Editor: Two cases of fatal endocarditis in Khon Kaen Province in northeastern Thailand were found to be caused by *Coxiella burnetii* (*1*). Although *C. burnetii* is known to be present in many countries, including in Thailand (*2*), human infection is more commonly associated with sheep and goats, possibly because these animals shed the organism more frequently in vaginal secretions and feces than do large ruminants (*3*).

Surveillance for Q fever, which is caused by *C. burnetii*, in livestock is currently based primarily on serologic or PCR testing of milk (*4*). However, problems in estimating prevalence include serologic assay insensitivity (*5*,*6*) or unavailability of milk from nondairy animals.

For diagnosis of Q fever, the placenta of the animal is commonly tested, but testing is usually conducted only when abortions occur, which is only likely when uninfected animals first encounter *C. burnetii*. Therefore, this approach might underestimate true organism distribution in a disease-endemic area (*7*). In addition, nearly all abortion storms have occurred in sheep or goats, which are rare in Thailand. Ruminant abortion is rarely reported to veterinary authorities in Thailand.

Comparison of paired colostrum and placental samples from sheep showed that *C. burnetii* was found more frequently in placental samples (*8*), which suggested that the placenta is a better sample than milk for surveillance purposes. Also, a placenta may be more useful because it is more likely to contaminate the farm environment. Milk is an unlikely source of Q fever in adult persons because it is seldom consumed by adults in Thailand.

The ideal surveillance strategy would include all relevant samples (serum, milk, and products of conception, both normal and abnormal). However, in practice, cost and logistical limitations dictate refinement of sampling. *C. burnetii* is frequently detected in normal ruminant placentas, but offspring are apparently not affected (*9*). We report that surveillance of normal placentas can provide useful surveillance data.

To test this hypothesis, in 2012 we asked local veterinarians in selected subdistricts in Thailand to contact farmers at their convenience to request that the veterinarians be alerted when a ruminant gave birth. Only grossly normal placentae from normal births of apparently healthy offspring were sampled. Cotyledonary (preferred) or intercotyledonary chorioallantoic tissue was obtained, chilled, and shipped cold to the National Institute for Animal health (Bangkok, Thailand) for analysis. Tissue was ground, extracted, and analyzed by PCR for IS*1111* of *C. burnetii* in a Light Cycler 2.0 Apparatus (Roche, Basel, Switzerland) as described (*10*). To minimize false-positive results, we repeated the PCR with a separate portion of tissue from the original sample. Samples were considered positive if the PCR had a cycle threshold <35 for each assay, or suspected of being positive if this occurred in 1 of 2 separate assays.

Results indicate a high frequency of *C. burnetii* infections in some provinces (Table), which roughly match locations where fatal human cases of endocarditis have occurred (Figure, Appendix, wwwnc.cdc.gov/EID/article/19/12/13-0624-F1.htm). It is common practice among the agrarian population in Thailand to consume ruminant placenta. Although this tissue is reportedly cooked before consumption, the preparation process may result in environmental contamination sufficient to expose persons who were not in close contact with the infected animal.

This study demonstrates that sampling and PCR of grossly normal ruminant placenta is a viable stand-alone approach for surveillance of *C. burnetii* that might enable the generation, at a minimal cost, of a highly detailed map showing areas where humans and animals are at risk for Q fever. The results indicate that *C. burnetii* is highly endemic in the study region. However, in light of the extreme rarity of serious complications in human infections and lack of any indication of a serious effect on animal production, these results do not indicate a need for veterinary control measures. Nonetheless, food safety practices should be addressed. It is essential that physicians monitoring patients with underlying heart valve conditions encourage such patients to seek diagnosis of any febrile illness so that appropriate treatment may be initiated to minimize risk for complications.

We report a novel approach to Q fever surveillance, which is potentially useful for countries such as Thailand, where subclinical ruminant infections are common. Our results also provide an initial indication of risk factors associated with recent cases of fatal Q fever endocarditis in Thailand. Follow-up research should include broader reservoir species surveillance, environmental surveillance, and comparison of genotypes of organisms found in ruminant placenta with those found in persons with endocarditis. These further efforts will result in clearer understanding of Q fever ecology and potential routes of human exposure.

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Table. PCR results for Q fever surveillance in ruminants, Thailand, 2012

Province (no. sites)	Animal	No. cases		
		Positive	Negative	Suspected
Chaiyapum (13)	Beef cattle	3	10	0
Chaiyapum (3)	Dairy cattle	1	0	2
Chaiyapum (1)	Goat	0	0	2
Chiang Mai (1)	Buffalo	0	2	2
Kalasin (1)	Goat	1	0	0
Khon Kaen (8)	Beef cattle	8	0	0
Khon Kaen (2)	Buffalo	2	0	0
Maha Sarakham (1)	Goat	2	6	0
Nakon Pathom (9)	Goat	1	9	0
Nakon Ratchasima (1)	Beef cattle	1	1	0
Nakon Ratchasima (17)	Dairy cattle	20	30	0
Nakon Ratchasima (3)	Goat	15	13	0
Prachuap Kiri Khan (9)	Dairy cattle	3	1	6
Ratchaburi (2)	Goat	1	9	0

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Treponemal Infection in Nonhuman Primates as Possible Reservoir for Human Yaws

To the Editor: In 2012, the World Health Organization launched plans for a second campaign to eradicate the neglected tropical disease, yaws (*1*). The first campaign, conducted during the mid-20th century, was tremendously successful in terms of treatment and reduced the number of cases by 95%. However, it failed to eradicate the disease, and when local efforts to prevent new cases proved insufficient, yaws resurged in some areas. Comments on the new yaws eradication campaign have emphasized the need for sustained support and resources. Here we draw attention to an additional concern that could impede yaws eradication efforts.

The success of any eradication campaign depends on the absence of a nonhuman reservoir. Smallpox had no known animal reservoir, and polio and dracunculiasis (guinea worm disease), which are currently the focus of the World Health Organization eradication campaigns, also have none. By contrast, compelling evidence suggests that yaws exists in wild nonhuman primate populations residing in regions where humans are also infected (Figure).

The subspecies of the bacterium *Treponema pallidum* that cause the non–sexually transmitted diseases yaws (subsp. *pertenue* infection) and endemic syphilis (subsp. *endemicum* infection) and the sexually transmitted infection syphilis (subsp. *pallidum*) are close relatives. The 3 diseases cannot be distinguished serologically. Instead, the diseases they cause are usually differentiated by clinical characteristics and geographic distribution. Whereas syphilis is a venereal disease with a worldwide distribution, yaws primarily affects children in hot and humid areas of Africa and Asia, and endemic syphilis occurs in arid regions. Because methods available to differentiate between the *T. pallidum* subspecies were unavailable in the past, prevalence data for yaws were sometimes vague and inaccurate. Recently, molecular tests capable of distinguishing between the subspecies by using single nucleotide polymorphisms have been developed (*2*,*3*). These tests have enabled us to learn more about the *T. pallidum* strains that infect wild nonhuman primates.

During the 1960s, researchers reported that many baboons in West Africa were seropositive for treponemal infection (*4*). Since then, high levels of infection have been documented in other monkey species in West Africa and in great apes (*5*). Recently, we documented *T. pallidum* infection in olive baboons (*Papio anubis*) at Lake Manyara National Park in Tanzania (*6*). In West Africa, clinical signs of infection in nonhuman primates are usually mild, if present at all, consisting of small lesions around the muzzle, eyelids, and armpits (*4*). A recent survey in 2013 at Parc National du Niokolo-Koba, Senegal, revealed *T. pallidum* antibodies in Guinea baboons (*P. papio*) with no signs of infection (S. Knauf et al, unpub. data). By contrast, severe manifestations resembling tertiary-stage yaws have been reported in wild gorillas (*5*). In terms of genetic distance, studies thus far indicate that the organisms infecting baboons in West and East Africa closely resemble *T. pallidum* subsp. *pertenue,* the agent responsible for yaws in humans (*2*,*7*). In fact, the genome sequence of a *T. pallidum* strain collected from a baboon in Guinea indicates that it should be considered a *T. pallidum* subsp. *pertenue* strain (*8*). Infection has been confirmed by serologic tests in a variety of nonhuman primate species in the yaws belt of Africa and by PCR in baboons from East and West Africa (Figure).

The high prevalence of nonhuman primate infection in areas of tropical Africa where yaws is common in humans (Figure) suggests that cross-species infection may occur. Decades ago, researchers reported that the Fribourg-Blanc simian strain, collected in Guinea, can cause sustained infection in humans after inoculation (*9*). Such experiments are ethically questionable and the details given are scant, but this work suggests that simian strains have zoonotic potential. Additional research is needed to determine whether interspecies transmission of *T. pallidum* occurs under natural conditions. Bush meat preparation is common in many African countries and a major source of zoonotic infection. It involves frequent skin-to-skin contact, which is the preferred mode of transmission for yaws. Insects also have been proposed to be vectors of infection, although this has not been documented (*10*). If evidence of interspecies yaws transmission, either direct or by vector, is discovered, then nonhuman primates may be a major reservoir of infection for humans.

Additional studies comparing human and simian strains may show whether zoonotic transmission of *T. pallidum* occurs frequently, an important consideration with regard to disease eradication and the conservation of great apes and other endangered nonhuman primates. To eradicate yaws, all host species and any possible reservoirs need to be taken into account. We, like the rest of the world, want the second yaws eradication campaign to succeed and hope that nonhuman primate infection will be evaluated as a factor in disease transmission.

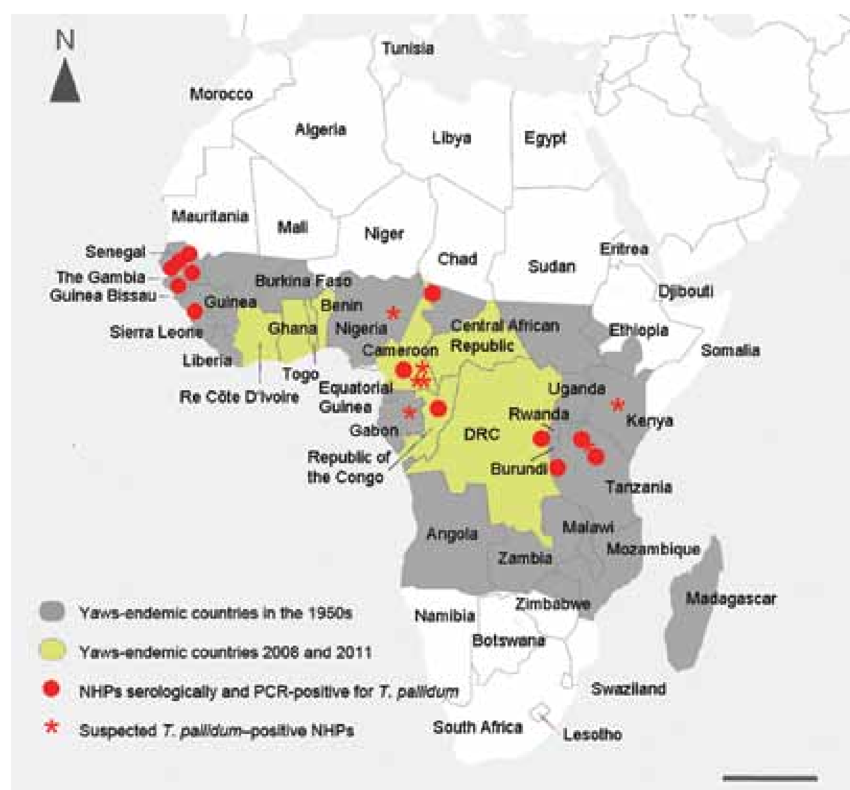

Figure. Geographic proximity between human yaws and endemic syphilis, as estimated by the World Health Organization, and locations in which treponemal infection has been identified in nonhuman primates (NHPs), Africa, 1990s. Dots indicate infection in NHPs confirmed by sensitive and specific treponemal serologic tests (TPI/FTA-ABS/MHA-TP [Treponema-pallidum-immobilization reaction/fluorescence-Treponema-antibody-absorption test/Treponema pallidum microhemagglutination assay]) and, in some cases, PCR. Stars indicate suspected infection (i.e., sightings of NHPs with lesions consistent with infection). Sources include the following: 1) Cameroon: *Gorilla gorilla*, observation (W. Karesh, pers. comm.); *Pan troglodytes*, *G. gorilla*, and *Papio* sp., skeletal analysis and serology (*4*;*11* in online Technical Appendix, wwwnc.cdc.gov/EID/articlepdfs/19/12/13-0863-Techapp1.pdf). 2) Chad: *Erythrocebus patas*, serology (*4*). 3) Democratic Republic of Congo (DRC): *Pan troglodytes*, serology (*4*). 4) Gabon: *G. gorilla*, observation (W. Karesh, pers. comm.). 5) Guinea: *Papio* sp., serology and PCR (*4,8*). 6) Kenya: *Papio anubis* and *Chlorocebus* sp., observation and serology (J. Fischer, pers. comm.); *12* in online Technical Appendix). 7) Nigeria, *Papio anubis* (J. Wallis, pers. comm.). 8) Republic of Congo: *G. gorilla*, serology and observation (W. Karesh, unpub. data; *5*). 9) Tanzania: *P. anubis*; observation, serology, PCR (*6,7*; *13* in online Technical Appendix; S. Knauf, unpub. data). 10) Senegal: *Papio* sp., *Chlorocebus* sp., colobus monkeys, and *Erythrocebus patas*; serology (S. Knauf, unpub. data; *4*; *14* in online Technical Appendix). Scale bar = 1,000 km.

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Porcine Hokovirus in Domestic Pigs, Cameroon

To the Editor: Since 2005, new parvoviruses forming a novel genus of the proposed name *Partetravirus*, within the subfamily *Parvovirinae*, have been described (*1*). Human parvovirus 4 (PARV4) with 3 different genotypes globally infects humans (*2*). A related porcine virus, hokovirus (HoV or porcine partetravirus), was found in wild boar and domestic pig populations in Germany, Romania, China, and the United States, with prevalences of 12%–47%, forming 1 common genotype (*3*–*6*). Prevalence figures from sub-Saharan Africa are not available. Furthermore, no information about possibly region-associated genotypes is available for porcine HoV, although it is for human PARV4 from the same genus. We therefore used samples (collected during February–March 2012) from a study investigating hepatitis E virus (HEV) in pigs from Cameroon (*7*) to analyze the occurrence of porcine HoV in pigs in Africa and to determine the respective genotype.

Viral DNA was extracted from liver samples by using the RTP DNA/RNA Virus Mini Kit II (STRATEC-Molecular, Berlin, Germany) according to the manufacturer's instructions. DNA samples were pooled, with each pool containing 3 different samples. A total of 94 pooled samples from 282 animals originating from 3 districts in Cameroon (Doula, Yaoundé, and Bamenda) were investigated by using quantitative real-time PCR (*3*,*7*). Samples from pools that tested positive were analyzed individually.

We detected HoV in 65 (69%) of the 94 pooled samples: 2 (15%) of 13 from Bamenda, 39 (70%) of 56 from Douala, and 24 (96%) of 25 from Yaoundé. We used an online tool to estimate the individual prevalence from pooled samples for fixed pool size and perfect test with exact 5% upper and lower CIs (http://epitools.ausvet.com.au/content.php?page = PooledPrevalence). A pool size of 3 with a total of 94 pooled samples and 65 positive samples resulted in an estimated general prevalence of 32.4% (95% CI 27%–39%). For Bamenda, the estimated prevalence was 5.4% (95% CI 1%–16%); for Douala, 32.8% (95% CI 25%–41%); and for Yaoundé, 65.8% (95% CI 44%–87%).

From 94 positive pools, a total of 184 samples were available for individual testing: 6 from Bamenda, 110 from Douala, and 68 from Yaoundé; 12 were missing. Using the results from the negative tested pools and the individual testing, we found an estimated general prevalence of 47% (128/270). The regional prevalence was 10% (4/39) for Bamenda, 41% (65/160) for Douala, and 83% (59/71) for Yaoundé.

These prevalences are higher than the estimates, but lie within the regional estimates within the range of the CI determined with the online tool. The discrepancy in the total prevalence might be due to the missing samples for the individual testing. Our results show that pooled sample testing can yield a good approximation of the actual prevalence, at least for settings in Africa. The varying prevalence and inhomogeneous regional distribution of porcine HoV correspond to previous findings from Europe, China, and the United States in wild boar and domestic pigs (*3*,*5*,*6*). Overall, no general defined pig-breeding program is in place in Cameroon. Douala and Yaoundé are the main markets for pig trade. Yaoundé, the main town for pig purchase and slaughter, gets live pigs from northwestern (Bamenda), western, and northern Cameroon, and Douala receives pigs from northwestern (Bamenda), western, and southwestern Cameroon. To fully understand the observed regional prevalences, the presence of HoV needs to be investigated in detail in the southwest, west, and north, where intensive farming systems are in place and pig farming is of economic importance.

Near full-length genome data were generated from 3 positive samples, and partial sequence information was retrieved for 8 additional samples (Figure) as described (*3*). The phylogenetic analysis showed a very close relation, with 98%–99% homology between the porcine HoV isolates from Cameroon, Europe, the

GQ869540 p. Hokovirus EU
GQ869539 p. Hokovirus EU
JN990266 p. Partetravirus CH
JN990269 p. Partetravirus CH
EU200677 p. Hokovirus CH
EU200673 p. Hokovirus CH
JF738368 p. Hokovirus EU
FJ982253 p. Parvovirus 4 EU
▲ **KF225545 Kam66**
▲ **KF225547 Kam74**
▲ **KF225544 Kam53**
▲ **KF225541 Kam30**
▲ **KF225542 Kam49**
▲ **KF225546 Kam67**
JQ425257 p. Partetravirus US
FJ982248 p. Parvovirus 4 EU
JQ425259 p. Partetravirus US
▲ **KF225548 Kam79**
▲ **KF225550 Kam87**
▲ **KF225549 Kam82**
▲ **KF225543 Kam52**
▲ **KF225540 Kam29**
porcine Hokovirus
JF504699 o. Hokovirus
EU200669 b. Hokovirus Gt1
JF504698 b. Hokovirus Gt2
JN798208 PARV4-like (Piliocolobus badius)
JN798211 PARV4-like (Colobus polykomos)
JN798203 PARV4-like (Pan troglodytes verus)
HQ113143 PARV4-like (Pan troglodytes troglodytes)
EU200667 PARV4 Gt1
DQ873390 PARV4 Gt2
JN798193 PARV4 Gt3
JQ037753 Eidolon helvum parvovirus 1
86 89 99 88 75 75 99 99 99 98
0,2

Figure. Phylogenetic tree of near full-length (4.7 kbp) and partial sequences (open reading frame 2, 0.4–1.9 kbp) of porcine hokovirus (HoV)/partetraviruses (PtV) created by using MEGA5.1 (www.megasoftware.net) with the maximum-likelihood method (GTR+G+I) and bootstrap analysis of 500 resamplings. New sequences from Cameroon are shown in **boldface**. EU, Europe; CH, China; US, United States; Gt, genotype; PARV4, parvovirus 4. Scale bar indicates nucleotide substitutions per site.

United States, and China. In contrast to bovine HoV or PARV4 in humans and chimpanzees, only 1 porcine genotype has been observed worldwide, which might point to 1 common ancestor (*8*,*9*). Because the liver samples were taken from European landrace or cross-bred pigs, the common ancestor of HoV might have originated from Europe. Pigs chronically infected with HoV could have been imported from Europe to Cameroon during the extensive global industrial farming and pig trade in the last century. The detection of the European HEV genotype (gt) 3 instead of the "traditional" African gt1 and gt2 in these pigs supports this hypothesis (*7*,*10*). Nevertheless, the high grade of homology of porcine HoV found worldwide suggests a highly species-specific virus with a low mutation frequency. To study the circulating genotypes in Africa and to generate hypotheses on the influence of trade, researchers need to college data from other African countries. However, data on HoV in wild animals, such as warthogs and pot-bellied pigs, are limited and need to be addressed to elucidate the genomic intravariability and intervariability of this new family of parvoviruses.

Generated sequences were submitted to GenBank under accession nos. KF225540–KF225550.

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Evaluation of 3 Electronic Methods Used to Detect Influenza Diagnoses during 2009 Pandemic

To the Editor: Conducting influenza surveillance in hospitals is imperative to detect outbreaks, inform infection control policy, and allocate resources (*1*). Hospital administrative data could be harnessed for this purpose (*2*,*3*) but are not currently used for infection surveillance because of data lag times. Influenza cases could be identified by using International Classification of Diseases, Tenth Revision, Clinical Modification (ICD-10-CM), codes within the discharge abstract, pharmacy, and microbiology laboratory information systems. Although these approaches are assumed to accurately identify influenza cases, this assumption has not been widely tested, especially during a pandemic. In this retrospective cohort study, we aimed to identify and evaluate 3 electronic methods of influenza case detection during 1 peak of influenza A(H1N1)pdm09.

With ethics board approval, we used the Ottawa Hospital Data Warehouse (OHDW) (Ottawa, ON, Canada) to identify 398 adult inpatients at the Ottawa Hospital during October–December 2009 who had cardiac, infectious, or respiratory disease diagnoses (ICD-10-CM codes: all J codes, A15–19, A37, A40, A41, A49, I26, I28, I50, I51.4, R57). OHDW is a relational database containing pharmacy, laboratory, and discharge diagnosis information for inpatients at Ottawa Hospital. We detected influenza in the following ways: influenza diagnosis in the discharge abstract database (DAD) (ICD-10-CM codes J09–J11); prescription for an antiviral drug (oseltamivir, zanamivir) in the pharmacy system; and a positive laboratory test during the hospital encounter (without specifying test type or specimen) in the laboratory system.

We assessed these case definitions against a criterion standard of influenza diagnosis on the hospital chart, determined by a physician reviewer blinded to the electronic values for the case definitions. We constructed 2 × 2 contingency tables for each classification method and calculated sensitivity, specificity, positive predictive value (PPV), and likelihood ratios using standard equations.

Influenza prevalence in this cohort was 13.6% (54/398) by our criterion standard. The proportion of male and female patients was equal, with a median age of 69 years (interquartile range 53–81 years). Median length of hospital stay was 6 days (interquartile range 1–12 days). A total of 77 (19.3%) patients were admitted to the intensive care unit, and 51 (12.8%) patients died in hospital. Two (0.5%) patients died with a primary diagnosis of influenza. The Table shows the performance characteristics of each influenza classification method against the criterion standard. The DAD-based influenza diagnosis algorithm was most accurate, with sensitivity of 90.7% (95% CI 79.7%–96.9%), specificity of 96.5% (95% CI 94%–98.2%), and PPV of 80.3%.

Our results demonstrate adequate correlation between ICD-10-CM coding for influenza in adults during a 3-month peak of the pandemic season within a single institution. Coding or interpretative errors were the probable cause of the 10% false-negative and 3% false-positive rates of ICD-10 coding for influenza on the DAD.

Classifying influenza by antiviral prescription was sensitive but less specific than clinical diagnosis. This finding could be explained by empiric antiviral prescriptions for

Table. Performance characteristics of electronic influenza classification methods compared to criterion standard chart review, Ottawa Hospital, Ottawa, Ontario, Canada, October–December 2009*

Method	No.				% (95% CI)		%		% (95% CI)	
	TP	FP	TN	FN	Sensitivity	Specificity	PPV	NPV	PLR	NLR
DAD flu diagnosis	49	12	332	5	90.7 (79.7–96.9)	96.5 (94–98.2)	80.3	98.5	26 (14.9–45.7)	0.10 (0.04–0.22)
Positive laboratory result	43	7	337	11	79.7 (66.5–89.4)	98 (95.9–99.2)	86.0	96.8	39.1 (18.6–82.5)	0.21 (0.12–0.35)
Antiviral drug prescribed	51	83	261	3	94.4 (84.6–98.8)	75.9 (71–80.3)	38.0	98.8	3.9 (3.2–4.8)	0.07 (0.02–0.22)

*TP, true positive; FP, false positive; TN, true negative: FN, false negative; PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio; DAD Flu Diagnosis, International Classification of Diseases, Tenth Revision, Clinical Modification, diagnosis code for influenza on the discharge abstract database stored in the Ottawa Hospital Data Warehouse.

infectious respiratory symptoms being written before confirmatory testing (*4*). Influenza classification by positive laboratory tests was specific but less sensitive in this analysis, probably because of nonuniform laboratory testing among inpatients from lack of specific criteria to guide testing and lack of testing in those with less severe illness. Not all patients who have influenza are tested for it, and these diagnoses would be classed as false negatives, influencing the sensitivity downward. Furthermore, laboratory testing would be likely to miss patients with influenza-triggered exacerbations of congestive heart failure and chronic obstructive pulmonary disease (*5*), which would underestimate influenza cases.

Our study correlated ICD-10-CM–specific codes for influenza in hospitalized adults during 1 peak of the 2009 influenza pandemic. A previous study in the United States in 2006 evaluated ICD-9-CM admission and discharge influenza codes in hospitalized children (*6*). The authors found that of 715 laboratory-confirmed influenza cases, ICD-9-CM codes were only 65% sensitive, suggesting that use of these codes for surveillance would underestimate influenza hospitalizations by 35% (*6*). This work was undertaken in 3 consecutive nonpandemic influenza seasons during 2001–2004.

Our findings must be generalized with caution because our study evaluated ICD-10-CM coding accuracy over 3 months of a pandemic influenza season in adults at 1 academic hospital. With lower influenza prevalence, the PPV would drop, suggesting that the coded diagnosis would overestimate influenza hospitalizations. Furthermore, sensitivity and specificity of codes might not be static measures because the diagnosis of influenza on the chart might be influenced by the prevalence of influenza in communities (*7*).

Given these limitations, further work is needed to fully validate ICD-10 codes for influenza during seasons of low prevalence and in other populations including children. Despite this, our results have implications for future research using administrative data to develop timely surveillance systems, track costs, and monitor resource use.

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Bicolored White-toothed Shrews as Reservoir for Borna Disease Virus, Bavaria, Germany

To the Editor: Borna disease (BD) is a fatal neurologic disorder in horses and sheep. The etiologic agent, Borna disease virus (BDV), belongs to the order Mononegavirales, which is composed of many reservoir-bound, highly pathogenic, and zoonotic viruses.

To investigate whether small mammals, especially bicolored white-toothed shrews (*Crocidura leucodon*), which act as BDV reservoirs in Switzerland (*1,2*), harbor BDV in disease-endemic areas in Bavaria, Germany, we screened 120 small mammals (53 from the family Cricetidae, 41 from the family Muridae, and 26 from the family Soricidae) (Table). We also determined whether BDV infections in small mammals might have different disease courses and whether shrew-to-horse virus transmission occurs.

The small mammals were captured during pest control efforts in stables in Upper Bavaria and Swabia that had a history of acute equine BD during 1997–2012. These stables also had a high probability for presence of *C. leucodon* shrews as documented by a recent distribution model (*3*).

BDV-specific serum antibodies were identified by using an indirect immunofluorescence test and blood samples or thoracic or abdominal effusions as described (*4*). Antibodies against BDV were found in 8/105 specimens (Table) at serum dilutions ranging from 1:40 for *Mus musculus* mouse #1008, 1:80 for *M. musculus* mouse #1014, 1:2,560 for *C. leucodon* shrew #5063, 1:10,240 for *C. leucodon* shrew #2001, and 1:20,480 for *C. leucodon* shrew #5017.

Amplification of viral RNA was conducted by using real-time reverse transcription PCR (RT-PCR) (*5*) or nested RT-PCR (*6*) on 119/120 brain samples. In 2/4 BDV-seropositive *C. leucodon* shrews (#2001 and #5017) BDV RNA was amplified from the brain. The remaining 117 mice and insectivores were negative for BDV RNA, including 6/8 BDV-seropositive animals (Table).

Histologic and immunohistochemical (IHC) analyses for detection of BDV antigen were performed for

Table. Small mammals from 7 stables tested for Borna disease virus infection, Bavaria, Germany, 1997–2012*

Stable, species	Common name	No. tested	No. positive for antibodies against BDV	No. positive for BDV RNA by RT-PCR
A				
Sorex araneus	Common shrew	2	0/2	0/2
Mus musculus	House mouse	17	2/13†	0/17
Apodemus sylvaticus	Wood mouse	1	0/1	0/1
Microtus sp.	Vole	1	0/1	0/1
B				
Crocidura leucodon	Bicolored white-toothed shrew	1	1/1	1/1
Mus musculus	House mouse	2	0/2	0/2
C				
Micromys minutus	Harvest mouse	1	1/1	0/1
Mus musculus	House mouse	3	0/2†	0/3
Myodes glareolus	Bank vole	1	0/1	0/1
Microtus sp.	Vole	1	0/1	0/1
D				
Microtus sp.	Vole	2	0/2	0/2
E				
Crocidura leucodon	Bicolored white-toothed shrew	19	3/13†	1/19
Crocidura russula	Greater white-toothed shrew	1	0/1	0/1
Sorex araneus	Common shrew	3	1/3	0/3
Micromys minutus	Harvest mouse	1	0/1†	0/1
Mus musculus	House mouse	6	0/6	0/6
Apodemus sylvaticus	Wood mouse	5	0/5	0/5
Apodemus flavicollis	Yellow-necked mouse	1	0/1	0/1
Arvicola terrestris	European water vole	1	0/1	0/1
Microtus sp.	Vole	34	0/32†	0/34
F				
Apodemus sylvaticus	Wood mouse	1	0/1	0/1
Myodes glareolus	Bank vole	1	0/1	0/1
Arvicola terrestris	European water vole	7	0/6†	0/7
Microtus sp.	Vole	2	0/1†	0/1†
G				
Apodemus sylvaticus	Wood mouse	3	0/3	0/3
Apodemus flavicollis	Yellow-necked mouse	1	0/1	0/1
Arvicola terrestris	European water vole	1	0/1	0/1
Microtus sp.	Vole	1	0/1	0/1

*BDV, Borna disease virus; RT-PCR, reverse transcription PCR.
†No blood or brain samples were available.

small mammals that had antibodies against BDV or BDV RNA. In addition, histologic and IHC analyses were used to test 36/112 small mammals negative for BDV (by indirect immunofluorescence test and RT-PCR), including 15/16 *C. leucodon* shrews from 3 stables (B, C, and E), in which BDV-positive mammals were captured. None of the small mammals showed obvious gross or histologic lesions, even in the brain.

IHC analysis was performed by using monoclonal antibody Bo18 against BDV nucleoprotein (BDV-N) as described (*7*). The 2/2 *C. leucodon* shrews (#2001 and #5017) harboring viral RNA had BDV antigen in the central and peripheral nervous system (brain, spinal cord, spinal trigeminal ganglia, and peripheral nerves). Immunostaining of the skin showed evidence of BDV infection, mainly in epidermal keratinocytes and sebaceous glands, as well as in squamous epithelium and connective tissue of the esophagus. In shrew #5017, renal tubuli and glomeruli, as well as nuclei of bronchiolar epithelial cells, had BDV-N. No evidence for viral antigen was found in the other 42/44 small mammals tested.

In situ hybridization was performed by using established protocols (*8*). Viral genomic RNA and mRNA encoding for the BDV-N gene were found in the brain, spinal cord, ganglia, parotid gland, and sebaceous glands of the skin of 2 shrews positive for BDV by RT-PCR. Thus, BDV antigen and RNA were found in nervous tissue and peripheral organs of 2 *C. leucodon* shrews, as reported for shrews in Switzerland (*1,2*).

Viral dissemination into peripheral organs represents a prerequisite for successful viral excretion and transmission to other susceptible species. Simultaneous detection of viral genomic RNA and mRNA can indicate viral replication and transcription in peripheral organs. RNA from brains of the 2 BDV-positive *C. leucodon* shrews (#2001 and #5017) and from 1 horse that had BD and lived in the same stable as shrew #5017 was sequenced as described (*2*). Comparison of BDV sequence (GenBank accession no. KF275185) from *C. leucodon* shrew #5017 with sequence (GenBank accession no. KF275184) from the affected horse showed 100% identity in a 2,150-nt region (nt 17–2161 covering the N, X, and P genes and half of the M gene). Moreover, the BDV sequence showed 98% homology with those of the BDV isolates of the Baden-Wurttemberg and Bavaria II group (*9*).

The 2 BDV-positive shrews were trapped in April (#2001) and July (#5017) 2012 in different stables in the feeding area for hay (B for #2001) or in the storage area for feed (stable E for #5017), which probably indicates that this food was contaminated with BDV. Viral shedding in shrews might occur from skin, kidney, or gastrointestinal tract, which is similar to shedding by persistently infected, immunotolerant, neonatal Lewis rats (*10*).

In conclusion, BDV RNA, viral antigen, and serum antibodies against BDV were detected in 2/20 *C. leucodon* shrews, indicating that this shrew is reservoir of BDV in Bavaria. Whether seropositivity without other evidence of BDV infection indicates different courses of infection in small mammals, as known for horses, is not known and warrants further investigation. The absolute homology of shrew and equine BDV suggests successful interspecies virus transmission. Our study provides reliable evidence that *C. leucodon* shrews acts as reservoirs for BDV in disease-endemic areas in Bavaria, Germany, argues for a general role of this shrew as a reservoir for mammalian bornaviruses.

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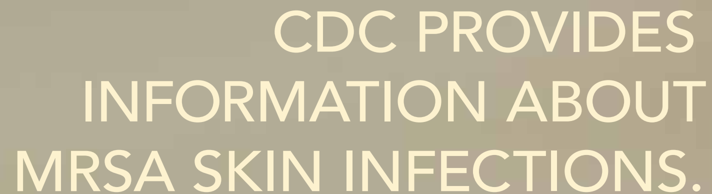

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Foodborne Infections and Intoxications

J. Glenn Morris, Jr., and Morris E. Potter, editors

ISBN: 978-0-12-416041-5

Academic Press, London, UK, 2013
Pages: 541; Price: US $149.95

The global supply of food has led to an increasingly connected planet, not only in terms of food products but also in terms of risks for foodborne diseases. The fourth edition of Morris and Potter's Foodborne Infections and Intoxications delivers an in-depth look at the global effects of foodborne illnesses, provides pathogen-specific information, and describes processes and policies intended to prevent these illnesses. The text is a well-written and well-referenced guide to foodborne illnesses, containing contributions from >70 experts in epidemiology and the basic sciences of foodborne diseases.

The text has been organized into 6 sections. The first section describes the epidemiology of foodborne disease, highlighting the most common illnesses in the United States and abroad with country-specific data as well as the most common outbreak-associated foods and microbial risk assessment. The next 4 sections encompass microbe-specific illnesses; each section comprises chapters on specific pathogens. The sections are organized to include bacterial pathogens, viral pathogens, parasites (along with mycobacteria and prions), and agents involved with food intoxications. Each chapter is specific to a given pathogen and includes a clinical description of the disease, the microbiology of the pathogen, exposure risks, and disease prevention.

Chapters have been written by leading researchers in their respective fields and are filled with up-to-date references on each pathogen. One example, in Chapter 8, includes a description of the 2011 outbreak of *Escherichia coli* O104:H4 infections in northern Germany. This outbreak illustrates the importance of food safety and the critical role of public health officials, clinicians, and scientists in identifying new outbreaks and limiting the spread of newly identified illnesses. The final section of the book outlines prevention of foodborne diseases and discusses food safety and the legal basis for food safety regulation.

This edition of Foodborne Infections and Intoxications updates the third edition, published in 2006, with increased emphasis on global disease prevention and a risk-based approach to food safety. This text is particularly valuable for students and practitioners in the fields of public health and food safety. It can also serve as a useful reference for public health investigators and officials.

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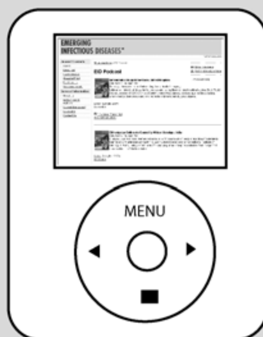

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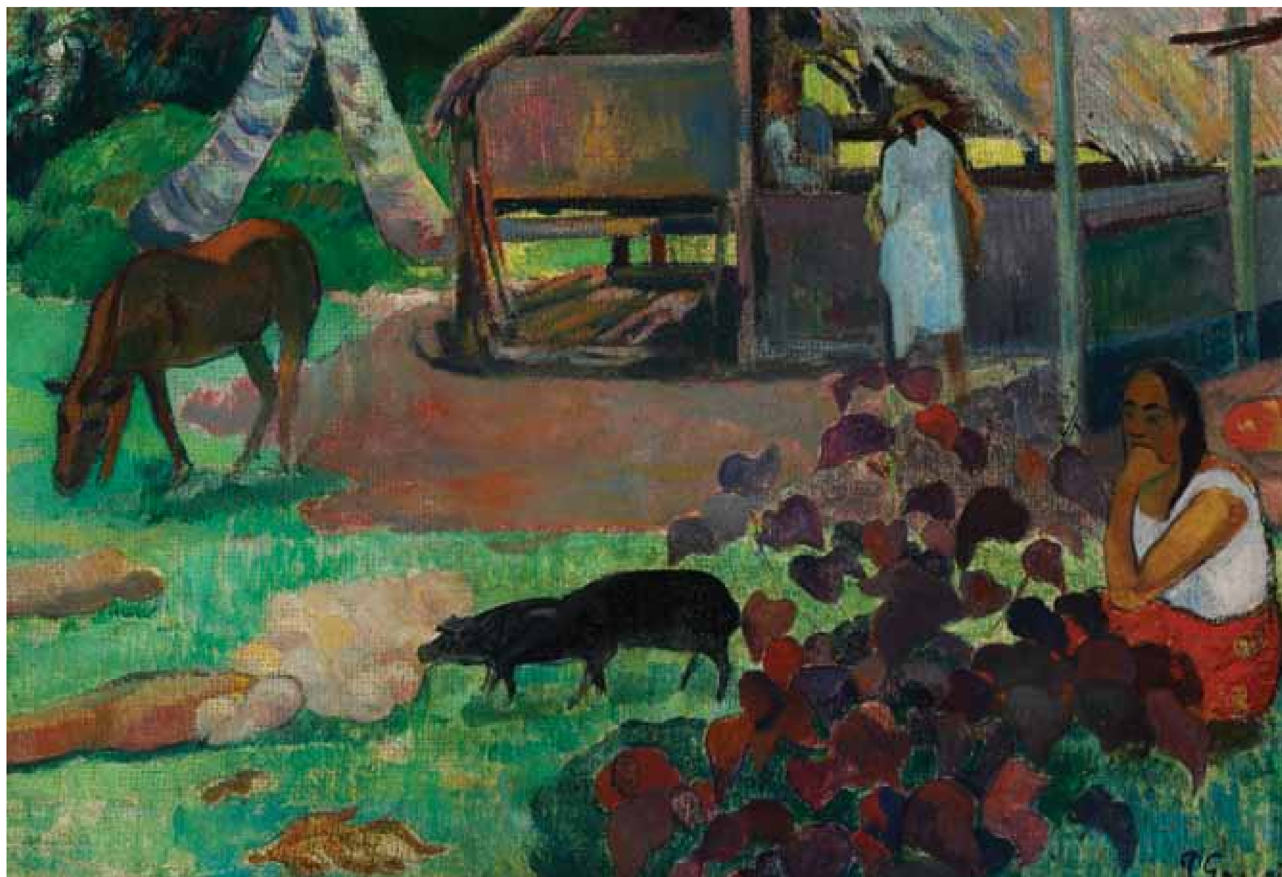

Paul Gauguin (1848–1903) *Black Pigs* (1891) Oil on canvas (91 cm × 72 cm) (detail) Museum of Fine Arts, Budapest, Hungary

Savage Nature and Ecologic Exchange

Polyxeni Potter

"There are neither carnivores nor reptiles in Tahiti," wrote Paul Gauguin in his journal Noa Noa. "The only 'wild game' on the island are the pigs which have escaped into the forest, where they have multiplied and become entirely wild."

The artist's first impression after the long voyage, "sixty-three days of feverish expectancy," was "nothing very extraordinary; nothing, for instance, that could be compared with the magnificent bay of Rio de Janeiro." A native Parisian, Gauguin had spent his childhood in South America, where his family had ties to Spanish nobility in Peru. His mother collected pre-Columbian pottery. These early life experiences he mythologized into a complex persona, often referring to himself as part savage. "You know that I have Indian blood, Inca blood in me, and it's reflected in everything I do…. I try to confront rotten civilization with something more natural, based on savagery."

This desire to escape his own times and the vestiges of a civilization he considered corrupt and dehumanizing guided Gauguin's work. He came to art late in life, first cautiously as a collector; then for pleasure, as friend of Edgar Degas, Paul Cézanne, and Camille Pissarro. He became entirely consumed by art when he worked for a time with Vincent van Gogh. "I am a great artist and I know it." After the stock market crash in 1882, he left Paris to travel and seek the authentic life he believed would lead

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him to greatness. “You must remember,” he wrote to his wife, “that I have a dual nature, [that of] the Indian and [that of] the sensitive man.”

He moved to the French coast. “I love Brittany; I find the wild and the primitive here. When my clogs resonate on this granite ground, I hear the muffled and powerful thud that I’m looking for in painting.” Then he settled in Martinique “to live as a savage,” but soon he left France altogether. “There in Tahiti,” he wrote to his wife, “in the silence of the tropical night, I shall be able to listen to the sweet murmuring music of my heart’s movements in loving harmony with the beings around me. Free at last with no money troubles and able to love, sing, and die.” He painted his best work during his first of two trips to Tahiti. He died of syphilis nearly penniless in the Marquesas.

During his travels to the South Sea Islands, Gauguin found that many of his expectations for a life of wildness filled with savages and untamed nature existed only in myths and fables. “The Tahitian soil is becoming completely French.” Unable to find the world he dreamed about, he sought his own. This preferred world he created in his art. From the exotic islands he extracted magical colors he then placed side by side in unconventional combinations and clearly outlined. Despite his admiration for and friendship with the impressionists, he shunned their work in favor of the flat planes that anticipated modern art.

Black Pigs, on this month’s cover, was painted the first year Gauguin was in Tahiti. Although he had not yet entirely abandoned perspective, spatial relationships are secondary to color and form. Traces of impressionist technique appear in the brushwork of the foliage and thatched roof of the hut. But the human and animal figures, including the namesake pigs, are simplified, almost archaic in their peacefulness, and entirely at ease with the surroundings.

In island culture, the sacred pig was the bond between gods and humans, facilitating their intermarriages or mitigating their disputes. “Food for gods and men” and the backbone of household prosperity, pigs were mentioned in Gauguin’s writings again and again, his cordial interactions with island neighbors sprinkled with “daintily prepared little pig” or “little pigs roasted on hot stones.”

Tahiti “Is the summit of a mountain submerged at the time of one of the ancient deluges,” the artist wrote. “Only the very point rose above the waters. A family fled thither and founded a new race—and then the corals climbed up and along it, surrounding the peak, and in the course of centuries builded a new land. It is still extending, but retains its original character of solitude and isolation, which is only accentuated by the immense expanse of the ocean.” Yet even this isolation that Gauguin sensed so acutely was an illusion. During a period of exotic introductions, local animals were modified along with the political, economic, spiritual, social, and physical landscape.

Animal movement and trade around the globe, which already had altered the island fauna in Gauguin’s diminishing paradise, now have eliminated even the possibility of zoonotic isolation. As animals and the viruses, bacteria, and internal and external parasites that travel with them find themselves evolving independently from their forbearers in new ecologic niches with new pressures and pathogenic tools, genetic changes occur in them. These changes facilitate emergence of new pathogens that can then start the process over again as the pathogens continue to span the globe.

Island people, Gauguin believed, “Had been richly endowed with an instinctive feeling for the harmony necessary between human creations and the animal and plant life which formed the setting and decoration of their existence.” This harmony, which included the savage element he sought and captured in garish tones, he viewed as a main ingredient of great art, along with the unity of humans with nature. This oneness in nature, with its inherent intimacy of all the macroscopic and microscopic players, requires concurrent attention to human health, animal health, and environmental health. For in this cauldron, as full participants and hosts of the wild microbes, which inhabit and kill us, we remain, like Gauguin, part savages.

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To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Epidemiologic Investigations into Outbreaks of Rift Valley Fever in Humans, South Africa, 2008–2011

CME Questions

1. A 28-year-old man presents to your clinic in South Africa with fever and malaise. Your differential diagnosis includes Rift Valley fever (RVF). What should you consider regarding this diagnosis?

A. It is primarily endemic to Asia, but there have been outbreaks in Africa

B. RVF virus is transmitted through direct contact with infected animals only

C. Most infections are self-limited

D. The mortality rate of RVF approaches 40%

2. What does the current study suggest regarding temporal trends and demographics of RVF?

A. There was only 1 outbreak of RVF during a 7-year period

B. Most cases were diagnosed during winter

C. RVF was equally common among men and women

D. Farmers and farm workers constituted the majority of patients with RVF

3. What was the most common mode of transmission of RVF virus in the current study?

A. Mosquito bite

B. Drinking unpasteurized milk

C. Consuming infected meat

D. Direct contact with infected animals

4. What specifically was the greatest risk factor of acquiring RVF among patients in the current study?

A. Handling and disposal of fetal material after abortions

B. Physical contact with animal carcasses

C. Slaughter of animals in commercial abattoirs

D. Slaughter of animals in areas apart from farms

Activity Evaluation

1. The activity supported the learning objectives.

Strongly Disagree				Strongly Agree
1	2	3	4	5

2. The material was organized clearly for learning to occur.

Strongly Disagree				Strongly Agree
1	2	3	4	5

3. The content learned from this activity will impact my practice.

Strongly Disagree				Strongly Agree
1	2	3	4	5

4. The activity was presented objectively and free of commercial bias.

Strongly Disagree				Strongly Agree
1	2	3	4	5

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Potential Role of Deer Tick Virus in Powassan Encephalitis Cases in Lyme Disease–endemic Areas of New York, USA

CME Questions

1. You are seeing a 62-year-old man with several days' history of fever and muscle weakness. You suspect that he might have encephalitis. What should you consider regarding the virology of Powassan virus (POWV)?

A. Both lineages feature tick vectors
B. Both lineages feature deer as the principal reservoir hosts
C. POWV is maintained by *Ixodes scapularis*
D. Deer tick virus (DTV) is maintained by *I. cookei*

2. Which of the following statements regarding the clinical presentation of POWV/DTV encephalitis in the current study is most accurate?

A. Most cases were diagnosed in the late fall
B. Most patients reported a history of tick bite
C. Subjective fever was the most common symptom
D. The incubation from tick bite to the onset of symptoms was 3 to 5 days

3. Which of the following was characteristic of laboratory and imaging data from patients in the current study?

A. Computed tomography of the brain was abnormal in over 90% of patients
B. Most patients had transaminitis
C. DTV was confirmed in 2 of 14 cases and was probably present in even more cases
D. Only 8% of patients experienced a substantial increase in POWV plaque reduction neutralization test titers between acute and convalescent sera

4. Which of the following statements regarding the outcomes of POWV/DTV encephalitis in the current study is most accurate?

A. 50% of patients were hospitalized
B. Most patients admitted to the hospital went to the intensive care unit
C. All deaths occurred in the hospital
D. Corticosteroid therapy was associated with a higher risk of mortality

Activity Evaluation

1. The activity supported the learning objectives.

Strongly Disagree				Strongly Agree
1	2	3	4	5

2. The material was organized clearly for learning to occur.

Strongly Disagree				Strongly Agree
1	2	3	4	5

3. The content learned from this activity will impact my practice.

Strongly Disagree				Strongly Agree
1	2	3	4	5

4. The activity was presented objectively and free of commercial bias.

Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Severe Fever with Thrombocytopenia Syndrome, Shandong Province, China, 2011

Historical Perspective of Influenza A(H1N2) Virus

Cryptococcal Antigen Lateral Flow Assay and Quantification by Laser Thermal Contrast Measurement

Population-based Surveillance for Bacterial Meningitis in China, September 2006–December 2009

Raw Milk Consumption among Patients with Non–Outbreak-related Enteric Infections, Minnesota, USA, 2001–2010

Effects of Drinking Water Filtration on Cryptosporidiosis Seroepidemiology, Scotland

Dynamic Modeling of Cost-effectiveness of Rotavirus Vaccination in Kazakhstan

Use of Electronic Death Certificates for Influenza Death Surveillance

Genetic Analysis of *Vibrio cholerae* during Outbreaks, Bangladesh

Rapidly Fatal Hemorrhagic Pneumonia and Group A *Streptococcus* Serotype M1

Mother-to-Child Transmission of Congenital Chagas Disease, Japan

Contact Tracing for Influenza A(H1N1)pdm09 Virus–Infected Passenger on International Flight

Resolution of Novel Human Papillomavirus Warts on Immunosuppessed Patient after HPV Vaccination

Avian Hepatitis E Virus in Chickens, Taiwan

Co-production of NDM-1 and OXA-232 by *Klebsiella pneumoniae*

Human Salmonellosis and Live-Bird and Animal-Slaughter Markets, United States, 2007–2012

Foodborne Trematodiasis Caused by *Opisthorchis felineus* in Italy

Bat Lyssaviruses, Northern Vietnam

Hepatitis E Virus Genotype 4, Denmark

Endemicity of *Opisthorchis viverrini* Liver Flukes, Vietnam

Tour Leaders' Knowledge of and Attitudes toward Rabies Vaccination, Taiwan

Corynebacterium ulcerans in Ferrets

Complete list of articles in the January issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

November 30–December 4, 2013
ASLM
African Society for Laboratory Medicine Innovation and Integration of Laboratory and Clinical Systems
Cape Town, South Africa
http://www.ASLM2014.org

January 27–29, 2014
ASM Biodefense and Emerging Diseases Research Meeting
Washington Marriott Wardman Park
Washington, DC
http://www.asmbiodefense.org

April 2–5, 2014
16th International Congress on Infectious Diseases
Cape Town, South Africa
http://www.isid.org/icid/

April 9–11, 2014
9th Conference Louis Pasteur
Emerging Infectious Diseases
Paris, France
http://www.clp2014.org/

May 17–20, 2014
ASM 2014
114th General Meeting
American Society for Microbiology
Boston, Massachusetts

June 24–27, 2014
EMBO conference on Microbiology after the genomics revolution – Genomes 2014
Institut Pasteur, Paris
http://www.genomes-2014.org

Announcements

To submit an announcement, send an email message to EID Editor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

EMERGING INFECTIOUS DISEASES®

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- ★ New infections resulting from changes or evolution of existing organisms.
- ★ Known infections spreading to new geographic areas or populations.
- ★ Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- ★ Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - ★ Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - ★ Reports laboratory and epidemiologic findings within a broader public health perspective.
   - ★ Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - ★ Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - ★ Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
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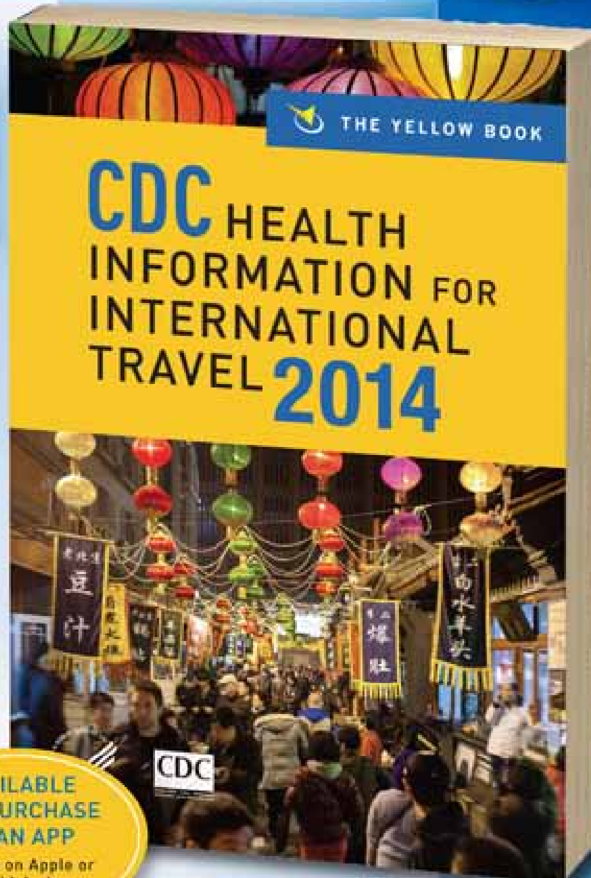

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Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); 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.

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Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. 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.

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