EMERGING INFECTIOUS DISEASES®

Emerging Viruses

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EMERGING INFECTIOUS DISEASES

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On the Cover

Egon Schiele (1890–1918)

Self-Portrait with Physalis (1912)

Oil and opaque color on wood (32.2 cm × 39.8 cm)

Leopold Museum, Vienna
www.leopoldmuseum.org

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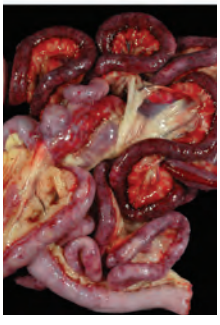

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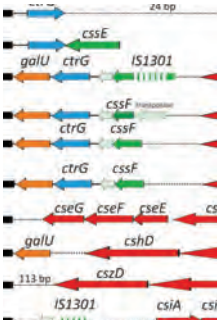

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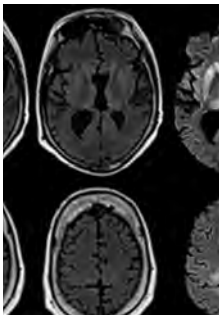

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Etymologia

Discrepancies in Data Reporting for Rabies, Africa

Louis H. Nel

Human rabies is an ancient disease but in modern times has primarily been associated with dog rabies–endemic countries of Asia and Africa. From an African perspective, the inevitable and tragic consequences of rabies require serious reflection of the factors that continue to drive its neglect. Established as a major disease only after multiple introductions during the colonial era, rabies continues to spread into new reservoirs and territories in Africa. However, analysis of reported data identified major discrepancies that are indicators of poor surveillance, reporting, and cooperation among national, international, and global authorities. Ultimately, the absence of reliable and sustained data compromises the priority given to the control of rabies. Appropriate actions and changes, in accordance to the One Health philosophy and including aspects such as synchronized, shared, and unified global rabies data reporting, will not only be necessary, but also should be feasible.

Rabies, despite its high case-fatality rate and preventability (through efficacious preexposure and postexposure prophylaxis), has in recent years progressively become established as a neglected disease, and most human cases are associated with dog rabies endemic to countries in Africa and Asia. However, there are numerous other serious infectious diseases that, like rabies, are underreported and linked with poverty in the developing world. How then should these diseases be prioritized?

This report presents considerations that influence the priority status of rabies, as well as issues that could differentiate rabies and should play a role in establishing the relative role of this disease in Africa and other parts of the developing world. It also discusses 1 key area that needs to be addressed before a bona fide demonstration of rabies incidence and progress toward effective dog rabies control will be feasible. This area is the need for true cooperation and synergy between global organizations and national authorities with respect to responsibilities related to effective and thorough surveillance with synchronized and responsible data reporting. Analyses of examples from Africa indicate that the above aspects are seriously compromised.

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Factors Leading to Complacency and Neglect of Rabies

Rabies virus, a classical zoonotic pathogen, has an extensive host range and can probably infect all terrestrial mammals. Although vampire bat rabies has a major effect with regard to livestock losses in Latin America (*1*), rabies is generally not associated with agricultural animals because the main terrestrial reservoirs are domestic dog populations of the developing world and wildlife carnivores elsewhere. Thus, rabies is often handled unconnectedly by health and veterinary authorities, and there is regular confusion as to who is responsible for controlling this disease. It is also likely that public demand for effective control measures would have been far greater if rabies had been a major disease of economically vital animals with a corresponding effect on livelihood. For Africa, a case in point is rinderpest, a viral disease for which there had been, figuratively speaking, as many eradication campaigns as pandemics. These campaigns were typically pan-African and driven by considerable international support and cooperation (*2*).

The history of rabies in Africa is not well recorded, but it is well accepted that the disease must have been present in northern Africa for hundreds of years, particularly as an urban disease of dogs and also associated with cycles in the Middle East. Rabies became epizootic in many countries of sub-Saharan Africa only during the nineteenth and twentieth centuries; in this region, the disease became well-established in dogs and involved wildlife species over large areas (*3*).

Today, no regions of or countries in mainland Africa are known to be free of rabies (*4*). In addition, Africa harbors several rabies-related viruses. Historically, the isolation and epidemiologic analyses of these viruses largely correlated with specific surveillance studies or diagnostic competencies (*5*). This scenario is also true for the most recent discoveries of 2 novel African lyssaviruses (*6*,*7*), and all indications are

that other as yet unknown lyssaviruses remain to be found. Without more comprehensive and routine surveillance and laboratory-based diagnosis, the epidemiology and the potential role of these viruses or emergence of these viruses remain speculative. Unfortunately, a lack of consistent and sustained or routine laboratory-based diagnosis for rabies (and no capacity to distinguish rabies-related viruses) may well be the status quo in many countries in Africa.

In humans, rabies often develops with a wide variety of nonspecific clinical symptoms, and symptoms believed to be typical of rabies (e.g., foaming at the mouth, hydrophobia, and extreme aggressiveness) are frequently not observed. Approximately 30% of human rabies cases develop in the paralytic or dumb form (*8*), and the overlap of symptoms with those of other infections often leads to misdiagnosis (*9*–*12*). Apart from misdiagnosis, rabies exposures are often ignored or deemed as minimal in dog rabies–endemic areas of the world. Because dogs are common as companion animals in most cultures, the exposure risk is actually greater than for many other zoonotic diseases. However, postexposure prophylaxis is unlikely to be sought after lick-associated exposures to dogs. Some cultures are known to believe that the lick from a dog is useful for wound treatment (*13*). In conjunction with this belief, some cultures believe that the aggressive and uncharacteristic behavior of persons or animals with symptoms of rabies is caused by sorcery or demon possession. Far too often rabies patients end up at tribal or traditional healers whose treatments include exorcism, administration of toxic herbs, and other such undesirable interventions (*14*,*15*).

Rabies is one of the oldest recognized diseases in human history, and there is anecdotal evidence of its presence in Mesopotamia and elsewhere in the Mediterranean basin since antiquity (*16*). Because this evidence has been known for many years, an unfortunate consequence has been a loss of newsworthiness, which has compromised awareness and priority in public and professional practice. A contrasting example could be witnessed with the newly emerged influenza A(H1N1)pdm09 virus, which caused widespread panic and has received much attention over the 3 years since its emergence. In the 17 months from April 2009 through August 2010, a total of 18,000 deaths caused by swine-origin influenza (mostly associated with other primary risk factors) were recorded worldwide (*17*). In contrast, rabies is conservatively estimated to cause ≈50,000 deaths per year, mostly in children, and is not associated with any other health risk factors (*18*).

Global and Regional Structures and Reporting of Rabies Cases

The World Health Organization (WHO) has reported that rabies has the highest case-fatality rate of all infectious diseases of humans (*19*), and most human exposures occur in children <15 years of age. For this reason, WHO has deemed rabies a reportable disease. Although WHO recognizes rabies as a reportable disease, many countries (e.g., India) (*20*) do not.

To attempt an assessment of the incidence of this disease globally, WHO has collected rabies data since 1959 and in the late 1990s created and administered Rabnet, a rabies-dedicated Web platform, to which countries have been requested to submit annual rabies statistics (*21*). These figures were published under various categories, including human cases, animal cases, presence or absence of rabies, national rabies vaccine production and importation, and rabies vaccine administration. In addition, there were several subsections, which enabled specification of certain criteria. For instance, under animal rabies, one could choose total number of dog cases and further specify whether one would want to observe dog rabies positive, dog rabies negative, or both. Figures obtained from Rabnet were frequently used in publications and in country reports. Despite having been a worthwhile undertaking, the Rabnet website has been closed indefinitely (until further notice) since late 2011, given the realization of incorrect reporting and to avoid subsequent misrepresentation.

The World Organisation for Animal Health (OIE) also regards rabies as a reportable disease, and OIE statistics for such diseases are published on the World Animal Health Information Database (*22*). The structure of this database includes 1) immediate notifications and follow-up reports submitted by member countries in response to exceptional disease events occurring in these countries and follow-up reports about these events; 2) reports every 6 months describing OIE-listed disease situations in each country; and 3) annual reports providing further background information about animal health, and laboratory and vaccine-production facilities. Thus, information for specific diseases is available on the website, and one can compare multiple countries with each another. Reports are submitted biannually by each country, and a final report is issued at the end of each year. The information for rabies gives detailed monthly case reports of rabies in animals for every month of each year and, in some cases, for each district or province of the country.

The Southern and Eastern African Rabies Group (SEARG), founded in 1992, focuses on control of dog rabies in countries in Africa. Official meetings are held approximately every 2 years, at which representatives from member countries gather and present data regarding rabies in their country in standardized country reports. The information from these reports is published on an open-access website (www.searg.info). The country reports focus on human and animal rabies (domestic and wildlife) and request information regarding vaccine purchases and/or production and vaccination strategies.

Discrepancies and Deficiencies in Rabies Reporting

Lack of reporting on rabies data by most developing countries is disconcerting. Examination of data on WHO and OIE web sites showed that information was frequently missing for an entire year or more in several countries. Data from selected countries from the southern African region for which SEARG, WHO, and OIE data reports were available are shown in Figure 1. Data submitted to the 3 authorities varied considerably in all examples. The only data from any of the countries that showed some correlation was that of Swaziland, where data submitted to SEARG and WHO were the same, but data submitted to OIE were different. In addition, when the ratio of human to animal rabies cases was analyzed, a vast range of ratios was typically found for data for 2010 (Figure 2). Data for individual countries ranged from high ratios of human rabies cases to animal rabies cases to (the more rational) low ratios of human cases to animal cases. Some countries reported only human rabies cases (clinical diagnoses for most countries in Africa). Such inconsistent data can be considered a further indicator of poor surveillance practices.

Inconsistencies in reporting of rabies epidemiologic information to WHO and OIE did not apply only to developing countries. Although inconsistencies were not as great as those observed in developing countries, which was likely caused by the fact that rabies cases are less frequent because of adequate control measures, they were still evident from the industrialized world. Also, for various countries, data for specific years were submitted only to OIE, only to WHO, or to neither organization.

Discrepancies for rabies epidemiologic data between various authorities could be interpreted as symptomatic of a larger problem, which should be addressed on a global scale. One likely reason for the lack of consistency of rabies data is the different focus areas of WHO and OIE. WHO focuses mainly on human disease, and will most likely receive rabies data from medical health authorities. In contrast, the animal disease focus of OIE suggests that veterinary services will submit rabies data to this body. Inconsistencies described in this report suggest a need for improved collaborative effort and effective communication between all relevant authorities with regard to diseases that simultaneously effect human and animal health. The imminent neglect of any zoonosis of which the main reservoir host is not an economically vital animal species is predictable unless addressed by unconditional execution and instruction of the One Health paradigm on global, regional, and country levels.

Conclusions

Rabies remains endemic throughout Africa, and for all the reasons discussed, loses visibility in Africa because it typically oscillates disconnectedly between authorities concerned with either human or animal health. Poor epidemiologic surveillance and inconsistent reporting, including that to responsible global authoritative bodies, has created a lack of rabies awareness and appreciation of its effect on humans in Africa. The absence of reliable and sustained rabies data compromises the priority that the control of rabies should be given, considering that a lack of laboratory-based proof of disease incidence will innately counteract attempts to justify (on national levels or to global funding agencies) the need for extensive and expensive rabies elimination programs.

OIE has recently released its Fifth Strategic Plan, which includes the One Health approach and is committed to improved cooperation with human–animal–environment interfaces (*23*). Despite this fact, the forms required by each of the organizations to be completed (e.g., for rabies) are different. This difference creates additional work for the authority responsible for submission of data and can lead to inconsistencies. If submission forms are standardized, then the same form can be sent to the various organizations.

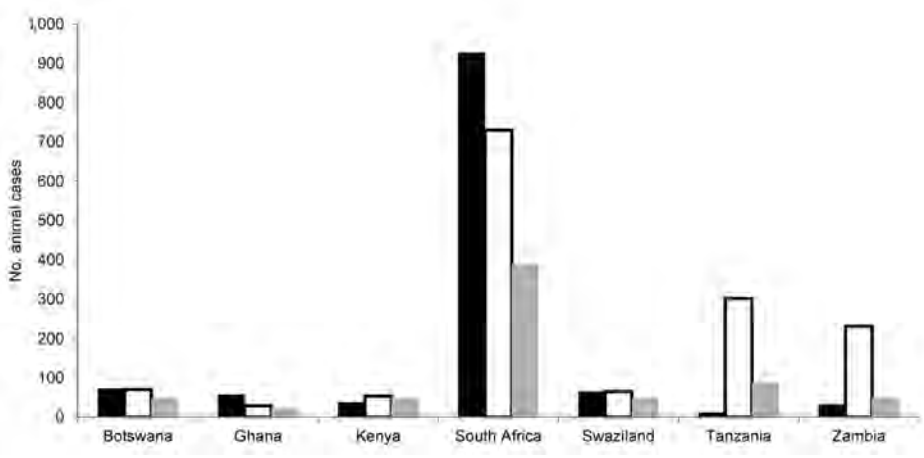

Figure 1. Number of rabies cases in animals reported in 2007 from countries in Africa classified as developing countries. Data were obtained from Southern and Eastern African Rabies Group reports (black bars), the World Health Organization (Rabnet) (21) (white bars), and the World Organisation for Animal Health World Animal Health Information Database (gray bars).

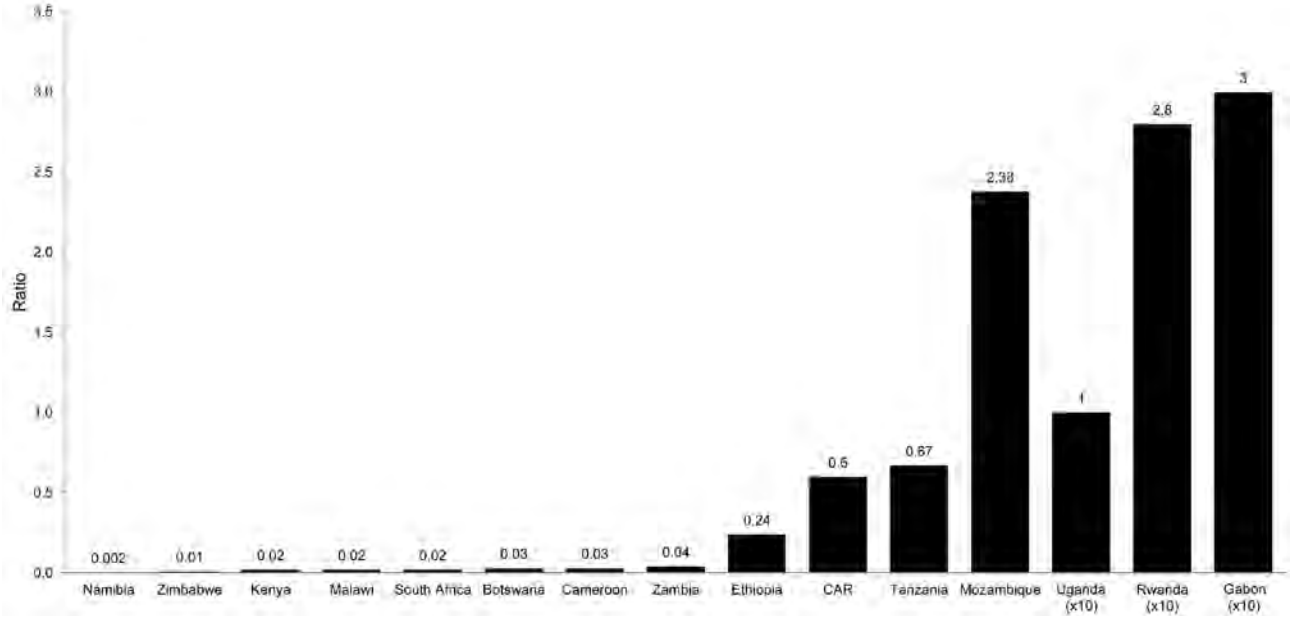

Figure 2. Ratio of human to animal cases of rabies reported in 2010 from Southern and Eastern African Rabies Group countries. Ratios are indicated above the bars. CAR, Central African Republic.

Another alternative would be to have 1 body to which to report epidemiologic data to, and from this body the major organizations can use and publish the appropriate data. This uniformity will prevent submission of inconsistent data but will require true collaboration between medical and veterinary sectors (the One Health approach). Because of the need for consistent and transparent data, appropriate actions and changes, in accordance to the One Health philosophy, will be necessary and feasible.

On a continental level, the One Health approach has already been shown to be beneficial toward rabies control in at least 1 part of the developing world, when implemented by the Pan American Health Organization in Latin America (*24*). In contrast, there is no pan-African approach to rabies control, although small regional efforts present hope. The rabies control program in Kwa-Zulu Natal in South Africa (*25*), which is supported by the Bill and Melinda Gates Foundation, celebrated a year free of human rabies on June 24, 2011. That occasion constituted the first time in 20 years that Kwa-Zulu Natal has not recorded a single human death caused by rabies in a year. However, this is a small victory in the face of the continent-wide challenge. Although dog rabies is rapidly decreasing in Kwa-Zulu Natal, it is still present, and until eliminated from dogs, the likelihood of future human cases remains a stark reality.

I suggest that the road to rabies control in Africa requires a pan-African approach toward establishing sound surveillance and reporting structures that would enable proper demonstration of the expanding effect of this disease in animal and human populations in Africa. The success of such a venture is certain to be conditional to the synchronized cooperative support of OIE, WHO, and other global partners. In this regard, it is encouraging that during a high-level technical meeting in Mexico at the end of 2011 (*26*), the Food and Agricultural Organization of the United Nations together with OIE and WHO have affirmed their commitment to alignment and honing of their respective coordination mechanisms to defend against emerging diseases at the animal–human–ecosystems interfaces.

Acknowledgments

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Dr Nel is a professor of virology and head of the lyssavirus research program at the University of Pretoria. His research interests are rabies and rabies-related viruses and the epidemiology and control of zoonotic diseases, such as rabies.

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Circovirus in Tissues of Dogs with Vasculitis and Hemorrhage

Linlin Li, Sabrina McGraw, Kevin Zhu, Christian M. Leutenegger, Stanley L. Marks, Steven Kubiski, Patricia Gaffney, Florante N. Dela Cruz Jr, Chunlin Wang, Eric Delwart, and Patricia A. Pesavento

We characterized the complete genome of a novel dog circovirus (DogCV) from the liver of a dog with severe hemorrhagic gastroenteritis, vasculitis, and granulomatous lymphadenitis. DogCV was detected by PCR in fecal samples from 19/168 (11.3%) dogs with diarrhea and 14/204 (6.9%) healthy dogs and in blood from 19/409 (3.3%) of dogs with thrombocytopenia and neutropenia, fever of unknown origin, or past tick bite. Co-infection with other canine pathogens was detected for 13/19 (68%) DogCV-positive dogs with diarrhea. DogCV capsid proteins from different dogs varied by up to 8%. In situ hybridization and transmission electron microscopy detected DogCV in the lymph nodes and spleens of 4 dogs with vascular compromise and histiocytic inflammation. The detection of a circovirus in tissues of dogs expands the known tropism of these viruses to a second mammalian host. Our results indicate that circovirus, alone or in co-infection with other pathogens, might contribute to illness and death in dogs.

Circoviruses are nonenveloped, spherical viruses with a single-stranded circular DNA genome of ≈2 kb; they group as a genus within the family *Circoviridae*, together with the proposed genus *Cyclovirus* and the phylogenetically more distinct genus *Gyrovirus* (*1*). Most of the known species in the genus *Circovirus* infect birds and cause signs including malformations and necrosis of the integument, lymphoid depletion, and immunosuppression (*2*).

Before 2012, the only circoviruses reported to infect mammals were the 2 closely related porcine circoviruses (PCVs) (*3*). PCV2 is the primary pathogen associated with a spectrum of swine diseases called porcine circovirus–associated diseases that have been described in pigs worldwide. PCV2 infection causes severe economic losses because of increased mortality and reduced production, making it one of the most economically important viruses in the global swine industry. Among lesions that have been attributed to PCV2 infection are pneumonia, enteritis, lymphadenitis, vasculitis, nephritis, and reproductive disease (*4*). In cases for which PCV2 is considered causative, immunohistochemical and in situ hybridization (ISH) analyses demonstrate large amounts of PCV2 antigen or nucleic acids in the cytoplasm of macrophages and dendritic cells in the depleted follicles in lymphoid tissues (*4*,*5*). Naturally occurring porcine circovirus–associated diseases is often accelerated or exacerbated by concurrent viral or bacterial infections, and secondary infections often occur as a result of immunosuppression (*6*).

Random nucleic acid amplification with or without prior enrichment for viral particle–associated nucleic acids (*7*,*8*), followed by deep sequencing and in silico similarity searches for sequences related to those of known viruses, have been highly productive in the field of animal virus discovery (*9*–*11*). We used this technique to identify virus sequences in affected tissues from companion animals with diseases of unknown cause. We identified a canine circovirus in the liver of a dog that had necrotizing vasculitis and granulomatous lymphadenitis, both of which are described in PCV2-infected pigs (*4*). We named this virus dog circovirus (DogCV) rather than canine circovirus to avoid confusion with the CaCV notation used for canary circovirus (*12*,*13*), canine calicivirus (*14*,*15*), and *Capsicum chlorosis* virus (*16*). A closely related variant of DogCV was sequenced independently in canine serum samples and was published recently (*17*); however, no disease association was described with the virus. To determine whether DogCV could be associated with canine vascular disease, we identified additional dogs with vascular and granulomatous lesions and examined the distribution of DogCV by ISH analysis.

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Materials and Methods

Animal Sample Collection

A 1-year-old castrated male dog that had been kenneled for 3 weeks was brought to the University of California, Davis (UC Davis), Veterinary Medical Teaching Hospital for evaluation of progressive vomiting and diarrhea with hematochezia. Despite initial supportive therapy at the referring veterinarian, clinical signs worsened; at UC Davis, the dog was treated for hypovolemic shock. Because of suspected disseminated intravascular coagulation and a poor overall prognosis, the owner elected to have the dog euthanized and granted permission for routine necropsy, which was performed at the Anatomic Pathology Service of the UC Davis School of Veterinary Medicine. The clinical and postmortem workups for infectious causes in this case included negative test results for infectious causes of enteric disease, such as canine parvovirus, canine enteric coronavirus, *Salmonella* spp., canine distemper virus, *Campylobacter* spp., *Clostridium perfringens* enterotoxin A gene, *Cryptosporidium* spp., and *Giardia* spp. Histologic results showed extensive fibrinoid vascular necrosis, thrombosis, and hemorrhage throughout the gastrointestinal tract and kidneys, as well as granulomatous lymphadentitis of the mesenteric lymph nodes. Special stains of histologic specimens revealed no detectable bacteria or other infectious agents. Liver tissue samples were collected, stored in whirlpack bags, and frozen at −80°C until further processing.

Sample Preparation and Nucleic Acid Extraction

A liver tissue sample (≈25 mg) were immersed in 1 mL cold Hank's balanced saline solution and disrupted with a tissue homogenizer for 30 sec on ice. The resulting homogenates were placed on dry ice for 5 min and then thawed at room temperature (*18*). Freezing and thawing were repeated twice. Samples were clarified by centrifugation at 10,000 × *g* for 3 min; the supernatants were then filtered and underwent nuclease treatment as described (*19*). Viral nucleic acids were extracted by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) and stored at −80°C.

Library Preparation and Sequencing

Viral nucleic acid libraries were prepared as described (*19*). The library of single-stranded DNA fragments was sequenced by using the Genome Sequencer FLX Instrument (Roche, Indianapolis, IN, USA).

Sequence Data Analysis

The pyrosequencing reads were sorted and trimmed as described (*19*). Trimmed reads from each sample were assembled de novo by using the MIRA assembly program (*20*), with a criterion of >95% identity over 35 bp. The assembled sequences and singlets >100 bp were compared with the GenBank nonredundant nucleotide and protein databases (www.ncbi.nlm.nih.gov/genbank) by using BLASTn and BLASTx, respectively (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Potential viral sequences with significant hits (E-value <0.001) to known virus sequences were identified.

Genome Sequencing and Analyses

PCR and Sanger sequencing were used to confirm the presence of virus genome sequences assembled from deep sequencing reads. Inverse PCR was then used to amplify the genome of target circoviruses with primers based on the sequences obtained by deep sequencing. Virus genome sequences obtained were deposited in GenBank (accession nos. KC241982–KC241984). Putative open reading frames (ORFs) with coding capacity >100 aa were predicted by Vector NTI Advance 11 (Invitrogen, Carlsbad, CA, USA). The stem-loop structure was predicted by using Mfold (*21*).

Phylogenetic Analysis

Phylogenetic analyses based on aligned amino acid sequences from full-length replicate (Rep) proteins were generated by using the neighbor-joining method in MEGA4 (*22*), using amino acid p-distances with 1,000 bootstrap replicates. Other tree-building methods, including maximum parsimony and maximum likelihood, were used to confirm the topology of the neighbor-joining tree.

Prevalence Study of DogCV in Sample Cohorts

Real-time PCR using 2 primers and a conventional hydrolysis probe with a 5-primer 6-FAM and 3-primer TAMRA label and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used to detect DogCV in DNA extracts from 3 dog sample cohorts: 1) fecal samples from 204 healthy dogs; 2) fecal samples from 168 dogs with diarrhea; and 3) blood samples from 480 dogs with thrombocytopenia and neutropenia, fever of unknown origin, or past tick bite. Primer pairs and probes for canine circovirus are given in the Table. Total nucleic acid was extracted by using the Corbett X-Tractor Gene platform (QIAGEN). Real-time PCR was conducted by using the real-time PCR instrument LightCycler 480 (Roche, Indianapolis, IN, USA) under these conditions: 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Synthetic DNA fragments (≈150 bp) of the corresponding regions were used to produce a standard curve and an analytical sensitivity of 10 molecules.

ISH Analysis

A fourth sample cohort consisted of tissue samples from 21 necropsy cases of dogs whose clinical signs or microscopic lesions matched the sentinel animal (i.e.,

Table. Oligonucleotide primer pairs and probe for DogCV sequences*

Region and amplicon	Primers	Sequences, 5′→3′
Replicate gene, 66 bases	DogCV-forward	CTTGCGAGAGCTGCTCCTTATAT
	DogCV-reverse	CTCCACTTCCGTCTTCCAGTTC
	DogCV-probe	TCCGGAGATGACCACGCCCC
Capsid gene, 68 bases	DogCV2-forward	CTGTTGTGAAACTGAAAGAGACGAA
	DogCV2-reverse	TGACGTAGGTCTCCGGATACG
	DogCV2 -probe	AGCCTTGCCGCTGTCGCGTC

*DogCV, dog circovirus.

hemorrhagic diarrhea, vasculitis, and/or granulomatous disease); these samples were selected from the tissue archives of Anatomic Pathology at the UC Davis Veterinary Medical Teaching Hospital. Control tissues were obtained from 5 dogs whose cause of death was unrelated to vascular disease. Tissue sections chosen for analysis were, in part, case dependent because the presence of vasculitis or inflammation among these cases was not limited to a single tissue type. Spleen, lymph node, jejunum, and ileum were examined in all cases; other examined tissues were kidney, brain, adrenal gland, pancreas, duodenum, heart, and lung. Tissues from the sentinel dog were included in this analysis.

Tissue sections were mounted on 3-aminopropyltriethoxylane–coated slides (Fisher Scientific, Pittsburgh, PA, USA). A 25-nt oligomer (CTCAGACAGAGACACCGTTGCTATG) complementary to a segment of ORF2 (capsid) was 3′-end labeled by the addition of a single digoxigenin-II-dideoxy undine (Eurofins MWG Operon, Huntsville, AL, USA). A manual capillary-action work station (Fisher Scientific) was used to perform colorimetric DNA in situ hybridization. Tissue sections were deparaffinized and digested by incubation at 37°C for 10 min in 0.25% pepsin in 1× Tris-buffered saline (pH 2.0); pepsin activity was stopped by a 5-min incubation at 105°C. Nucleic acid was denatured by 5-min incubation at 105°C in 100% formamide. Tissue sections were then incubated at 37°C in 10 μmol of the digoxigenin-labeled probe in hybridization buffer (22.5% deionized formamide, 7.5% chondroitin sulfate, 5× saline sodium citrate, 0.25% blocking reagent, and 50 mmol phosphate buffer). Sections were incubated in an antidigoxigenin antibody solution (500:1 dilution) containing 2.5 mL buffer 1 with 5 μL of antidigoxigenin Fab fragments conjugated with alkaline phosphatase (750 U/mL) (Roche). Sections were then washed and developed according to manufacturer instructions before counterstain with 1% fast-green FCF for 5 min. Slides were mounted with ImmunoHistoMount (Immunobioscience, Mukilteo, WA, USA) and coverslipped with SHUR/Mount (Triangle Biomedical Sciences, Durham, NC, USA). No background hybridization was seen when replicate tissue sections were incubated with an unrelated digoxigenin-labeled probe with similar guanine-cytosine content or when matched tissue from unaffected dogs were incubated with the DogCV-specific probe (*23*–*25*).

Electron Microscopy

Selected pieces of formalin-fixed lymph node tissue from the sentinel dog were post fixed in 2.0% glutaraldehyde and then routinely processed and embedded in epoxy resin (Eponate12 kit; Ted Pella, Inc., Redding, CA, USA). Selected thick sections were stained with toluidine blue, as described (*26*). Ultrathin sections from select areas of the lymph node were examined by using a Zeiss (Göttingen, Germany) 906E transmission electron microscope.

Metagenomic Identification of Canine Circovirus

Of ≈10,000 squesnce reads, 5 contigs and singlets composed of 52 sequence reads from the liver tissue had significant similarity to the Rep protein of circoviruses (E-value $<1^{-10}$). Two bocavirus sequences were also detected. Because circoviruses have a circular genome, the full viral genome was then amplified by inverse nested PCR and the amplicon sequenced by primer walking. The assembled genome was named DogCV strain UCD1 (DogCV-UCD1).

Results

Genome Analysis and Phylogenetic Relationship

The complete circular genome of DogCV-UCD1 was 2,063 nt (GenBank accession no. KC241982). Analysis of the genome sequence showed characteristics typical of circoviruses, including an ambisense organization with 2 major inversely arranged ORFs encoding the putative replication-associated (Rep, 303 aa), and capsid (Cap, 270 aa) proteins. A characteristic stem-loop structure with a conserved nonanucleotide motif (5′-TAGTATTAC-3′, similar to the consensus of bird and pig circoviruses) was also found in the 5′-intergenic region (135 nt, between the start codons of the 2 major ORFs). The 3′-intergenic region of DogCV-UCD1 between the stop codons of the 2 major ORFs was 203 nt (Figure 1, panel A) (*27*).

The complete genome of another DogCV strain (DogCV-UCD2, GenBank accession no. KC241984) was amplified and sequenced from a fecal sample from a shelter-housed dog that had vomiting and diarrhea. This strain shared 95% overall genome nucleotide identity with DogCV-UCD1, and both strains showed 96%–97% nucleotide identity to the recently reported canine circovirus isolate 214 from blood (*17*).

The putative Rep proteins of DogCV-UCD1 showed 42%–54% amino acid identity to the Rep proteins of porcine and avian circoviruses, with the closest identity to PCV1. The DogCV-UCD1 capsid showed <30% amino acid identity to known circovirus capsids. Sequence alignment of the putative Rep protein of DogCV-UCD1 with those of known species in the genus *Circovirus* identified several highly conserved amino acid motifs, including WWDGY, DDFYGW, and DRYP. Motifs associated with rolling circle replication (FTLNN, TPHLQG, and CSK) and the dNTP binding (GXGKS) were also identified. The N terminal region of the Cap protein was highly basic and arginine-rich, as is typical for circoviruses. A phylogenetic analysis of the complete Rep protein of DogCV strains and all known circoviruses was performed, with chicken anemia virus (genus *Gyrovirus*) as the outgroup (Figure 1, panel B). The phylogenetic tree showed that DogCV strains grouped with PCV1 and PCV2, forming a distinct clade of mammalian circovirus, whereas circoviruses affecting birds clustered separately.

Prevalence Study

DogCV was detected by real-time PCR in fecal samples from 14/204 healthy dogs and 19/168 dogs with diarrhea; the difference in prevalence was not significant (11.3% vs. 6.9%; $p>0.05$ by χ^2 test). Of the 19 dogs with diarrhea who had DogCV detected in fecal samples, 13 (68%) were co-infected with ≥1 other pathogens, including canine enteric coronavirus, *Cryptosporidium* spp., *C. perfringens* α toxin, *Giardia* spp., *Salmonella* spp., *Campylobacter jejuni*, and *Campylobacter coli* (tested by PCR).

The prevalence of DogCV in blood samples from the cohort of dogs with thrombocytopenia and neutropenia, fever of unknown origin, or past tick bite was 3.3% (16/480), similar to that reported for canine serum samples (2.9%, 6/205) (*17*). The partial Rep and/or Cap protein regions (≈350 bp) were amplified from 11/16 samples. All showed >96% nucleotide identity, except 1 amplicon, which had <90% nucleotide distance to DogCV-UCD1 and -UCD2. We sequenced the complete genome of this virus (DogCV-UCD3; GenBank accession no. KC241983); it showed 91%–92% amino acid identity of the complete Rep and Cap proteins to DogCV-UCD1 and -UCD2 and to the published canine circovirus (CaCV-1 strain NY214; GenBank accession no. JQ821392) (*17*).

ISH Analysis and Pathologic Findings in Positive Cases

To establish tissue distribution and investigate whether DogCV contributes to canine disease, we developed and

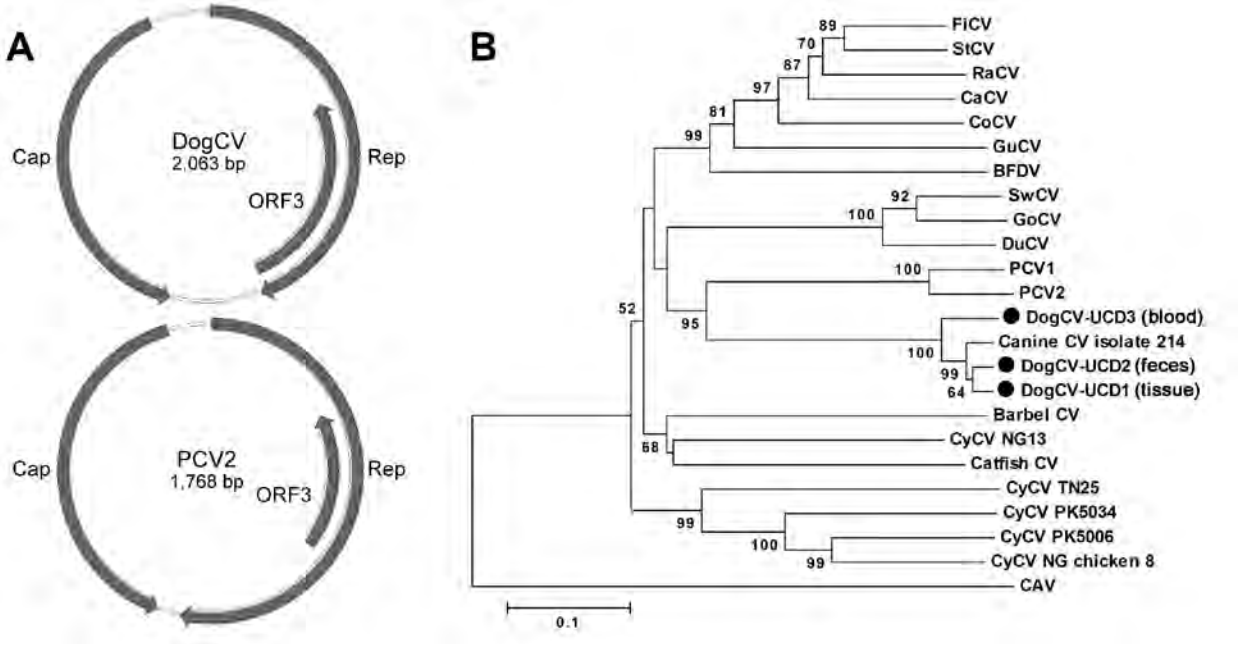

Figure 1. A) Genome organization of dog circovirus (DogCV) and porcine circovirus 2 (PCV2). B) Phylogenetic analysis of DogCV strains (UCD1–3, isolated from tissue, feces, and blood respectively) based on the amino acid sequence of the replicate (Rep) protein. GenBank accession numbers for circoviruses used in the analysis: Finch circovirus (FiCV), DQ845075; Starling circovirus (StCV), DQ172906; Raven circovirus (RaCV), DQ146997; canary circovirus (CaCV), AJ301633); Columbid circovirus (CoCV), AF252610; Gull circovirus (GuCV), DQ845074; beak and feather disease virus (BFDV), AF071878; Cygnus olor circovirus (SwCV), EU056310; Goose circovirus (GoCV), AJ304456; Duck circovirus (DuCV), DQ100076; Porcine circovirus 1 (PCV1), AY660574; PCV2, AY424401; canine CV, JQ821392; Barbel CV, GU799606; Cyclovirus (CyCV) NG13, GQ404856; Silurus glanis circovirus (Catfish CV), JQ011378; CyCV TN25, GQ404857; CyCV PK5034, GQ404845; CyCV PK5006, GQ404844; CyCV NG Chicken8, HQ738643); and chicken anemia virus (CAV), M55918.

validated an ISH oligomeric probe and examined the sentinel dog and dogs from 21 suspected, retrospective cases that included ≥2 of these 3 signs: vasculitis, hemorrhage, or granulomatous disease. A wide spectrum of affected tissues was represented in this group; matching tissues were also examined from 5 control dogs in which these signs were not present. Samples from the sentinel dog (dog 1) and 3 other dogs (dogs 2–4) were positive for DogCV by ISH analysis. All other tissue samples from control dogs were negative by ISH analysis. Clinical signs, gross and histologic findings, and distribution of virus DNA as determined by ISH were used to examine a possible causal role for DogCV.

Dog 1 was a male beagle who had acute onset of vomiting and hemorrhagic diarrhea. Dog 2 was a 5-year-old, female, spayed Boston terrier who had vomiting and diarrhea. Dog 3 was a 1-year-old, female, spayed boxer with a 5-day history of lameness and progressive tetraparesis. Dog 4 was a 2-year-old Greyhound found dead with bicavitary hemorrhage; blood smear and PCR showed this dog was co-infected with *Babesia conradae*.

Gross examination revealed consistent lesions among these 4 dogs, including lymphadenopathy and hemorrhage. In dogs 1 and 2, the hemorrhage was associated with the gastrointestinal tract (Figure 2, panels A, C); dog 2 had additional multifocal to coalescing regions of hemorrhage in the kidneys (Figure 2, panel B). Gross evidence of hemorrhage in dog 3 was restricted to the ventral surface of the brain along the basilar artery overlying the medulla; dog 4 had bicavitary hemorrhage. The common histologic lesion in all dogs was fibrinonecrotizing vasculitis, although the distribution of affected vessels and the amount of associated hemorrhage varied. In dog 1, segments of inflamed or necrotic vessels were seen in the intestine (multiple segments), urinary bladder, liver, spleen, and lungs. In dog 2, vasculitis was limited to the intestine and spleen, and in dog 3, vasculitis was in kidneys, intestine (Figure 2, panel G), heart, liver, spleen, and meninges. In dog 4, only a few vessels were affected, at the corticomedullary junction in the kidneys. For all dogs, histiocytic drainage or granulomatous lymphadenitis were seen in Peyer's patches (Figure 2, panels C, E) and in ≥1 lymph node. In addition, in dogs 2 and 3, multiple lymph nodes were severely necrotic. All dogs had microscopic lesions in the kidneys, but intensity and character varied widely. Dogs 1 and 2 had tubular necrosis with little inflammation, dog 3 had a severe granulomatous interstitial nephritis, and dog 4 had multifocal hemorrhage and minimal lymphocytic, plasmacytic nephritis. Dogs 1 and 3 had multifocal pancreatitis and adrenalitis. No multinucleate giant cells, common in pigs infected with PCV2, were seen.

By ISH analysis, abundant cytoplasmic viral nucleic acid was detected in macrophages within germinal centers and subcapsular and medullary sinuses of the mesenteric

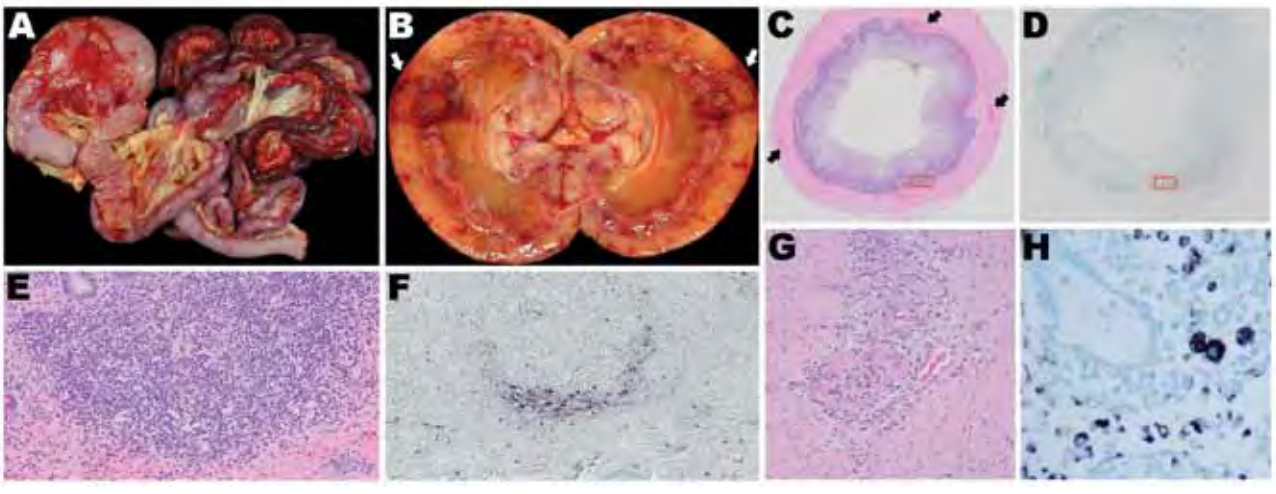

Figure 2. Organ tissues from sentinel dog (dog 1) and 2 other dogs (dogs 2 and 3) that were positive by in situ hybridization (ISH) analysis for dog circovirus (DogCV). A) Gross view of the gastrointestinal system from dog 1. Multifocal to coalescing hemorrhages are shown in the stomach and intestinal serosa. B) Gross view of the kidney from dog 2. Segmental regions of hemorrhage and necrosis (infarction) can be seen within the cortex and radiating from the medullary papilla to the capsule. C) Hemolyxin and eosin (H&E) stain of the ileum from dog 1. Peyer's patches are moderately depleted, and multifocal regions of hemorrhage are present within the muscular intestinal wall. D) ISH of the ileum from dog 1. Peyer's patches circumferentially contain abundant DogCV DNA. E) H&E stain of a Peyer's patch in the ileum from dog 1, showing moderate depletion of lymphocytes and an increased population of macrophages within the germinal center and the peripheral base of the follicle. F) ISH of a Peyer's patch in the ileum from dog 1. DogCV DNA is rich within the cytoplasm of abundant cells at the periphery of the follicle, and individual cells are scattered within the germinal center, lymphatic channels, and submucosa. The positive cells have the morphologic appearance of macrophages. G) H&E stain of the jejunum from dog 3, showing segmental, circumferential, fibrinoid necrosis of the artery. H) ISH of a mesenteric lymph node from dog 2. Macrophages in the medullary sinus and lymphatic cords contain abundant DogCV DNA.

lymph nodes from all 4 dogs (Figure 2, panel H), mandibular lymph nodes from 2 dogs, ileal Peyer's patches from all 4 dogs (Figures 2, panels D, F), ellipsoids (terminal arterioles) of the spleen for 1 dog, and germinal centers in splenic white pulp for 2 dogs. Although the reticular network was abundantly positive in dog 1 (Figure 2, panel H), the common pattern was localization to the centers of lymphoid or Peyer's patch follicles (Figure 2, panels D, F), corresponding to the morphology and distribution of dendritic cells. Rare nuclei of elongate cells (presumed endothelium) lining small vessels of the adrenal cortex or intestinal lamina propria suprajacent to the Peyer's patches were positive in 2 dogs. No nucleic acid was detected by ISH analysis in other tissues, regardless of the character or intensity of the inflammation. None of the 5 control dogs showed DogCV DNA by ISH.

Transmission Electron Microscopy Analysis

Ultrastructural analysis of the mesenteric lymph node from dog 1 revealed macrophages laden with large numbers of intracytoplasmic inclusions (Figure 3, panel A). Inclusions were round, oblong, or irregular; were <0.5 μm; and often clustered (up to 25 per cell) within the cytoplasm, (Figure 3, panel B). Most inclusions were granular and electron-dense and had a distinct periphery but were nondelineated by membrane. Some inclusions contained paracrystalline arrays of icosahedral virions that were 9–11 μm in diameter (Figure 3, panel C).

Discussion

We identified a novel circovirus, DogCV, in the liver of a dog that had necrotizing vasculitis and granulomatous lymphadenitis. We characterized the genome of multiple DogCV strains, determined DogCV prevalence in dog fecal and plasma samples and tissue distribution in infected animals, and detected paracrystalline arrays in inclusion bodies in macrophages. Real-time PCR analysis showed a prevalence of 11.3% and 6.9% in fecal samples from dogs with diarrhea and healthy dogs, respectively. DogCV DNA was also found in 3.3% of blood samples from dogs with thrombocytopenia and neutropenia, fever of unknown origin, and past tick bite, which is approximately the same percentage as previously reported (*17*).

ISH analysis of the sentinel dog and 21 additional dogs selected retrospectively from past necropsies detected viral nucleic acid in 4 dogs, including the sentinel dog, and the histopathologic features and distribution of virus in tissue samples from these dogs were evaluated. The organs affected varied even in this small set of animals, but all dogs had necrotizing vasculitis and hemorrhage, and all but 1 had lymphadenitis and granulomatous disease. Because the tested retrospective animals were chosen to match the sentinel case, our sampling is biased, and the spectrum of diseases associated with this virus might be broader than we detected. Among dogs positive by ISH, disease signs varied, and clinical, gross, and microscopic features in some of the disease syndromes were similar to those associated with PCV2 infection (*4*,*5*). In particular, porcine dermatitis and nephropathy syndrome shares many of the histologic features seen in DogCV-positive dogs (*28*,*29*), and PCV2 has been reported to cause necrotizing lymphadenitis, vasculitis, or neurologic disease (*4*,*30*,*31*). Virus distribution, as assessed by ISH analysis, is also similar between DogCV and PCV2.

Viral DNA was consistently detected in the cytoplasm of macrophages and monocytes in lymphoid tissues of infected dogs. Virus distribution in pigs infected with PCV2 is most consistently within lymphoid tissue, with sporadic

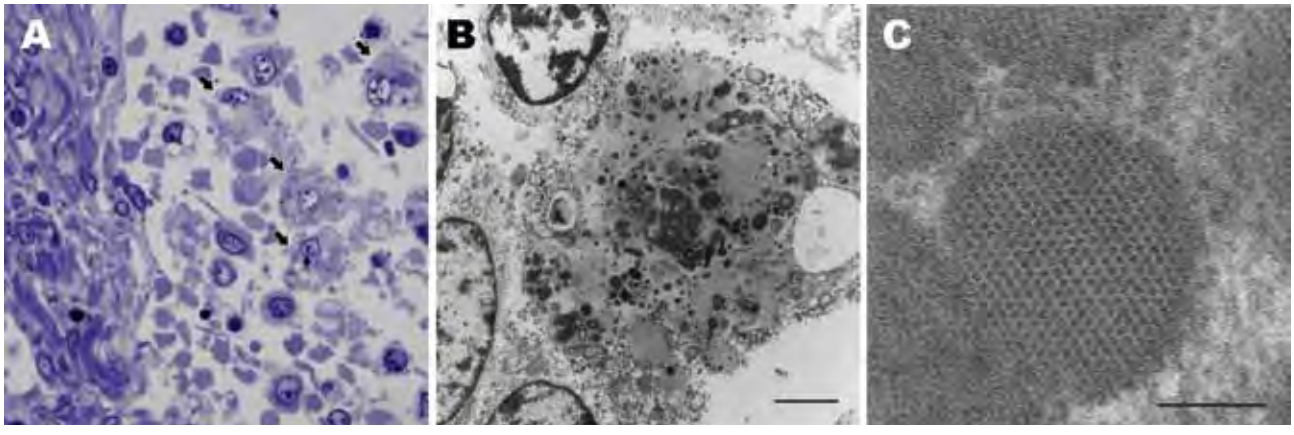

Figure 3. Lymph node from sentinel dog from which dog circovirus was identified. A) Toluidine blue stain shows multiple macrophages within the medullary sinus contain vacuoles and discrete, oblong to round, variably stained cytoplasmic bodies (arrows). B) A single macrophage adjacent to a lymphocyte (upper left) and partial profiles of other cells. Intracytoplasmic inclusion bodies are distributed throughout the macrophage cytoplasm, along with mitochondria and vacuoles. Scale bar indicates 2 μm. C) Intracytoplasmic inclusion bodies contain granular content and sometimes paracrystalline to herringbone arrays of 10–11 nm diameter viral-like particles. Scale bar indicates 100 nm.

reports of virus in other tissues (*4*,*23*,*32*,*33*). For example, in pigs with dermatitis and nephropathy syndrome, granulomatous inflammation of the kidneys is commonly reported; however, virus is detected in renal tissue in only a few cases by any method. In our study, DogCV DNA was detected in lymphoid tissues, including Peyer's patches, even for dogs 3 and 4, where no clinical or histologic enteric lesions were detectable. DogCV DNA was also found in small, end-capillary endothelial channels of the intestinal lamina propria and the adrenal cortex; however, confirmation of these cells as endothelial will require further investigation.

Two distinct histologic features of PCV2 infection are viral inclusions and multinucleate giant cell formation (*34*), neither of which was detected by routine histology in the dogs infected with DogCV. By electron microscopy, however, macrophages within the lymph node contained abundant cytoplasmic viral inclusions composed of dense granular or paracrystalline arranged virus. Affected cells were found in both the sinus and medullary cords of the mesenteric lymph node examined. Ultrastructural inclusions were similar to a subset of cytoplasmic inclusions that have been described in PCV2 infected tissues (*35*,*36*).

Numerous experimental and natural disease studies have indicated that PCV2 infection most often leads to clinical diseases in the presence of co-infection with other swine pathogens. PCV2 enhances viral (e.g., porcine parvovirus, porcine reproductive and respiratory syndrome virus), bacterial, protozoal, metazoal, and fungal infections in pigs (*6*). Among the dogs with diarrhea in our study, most (68%) of those positive for DogCV had co-infection with ≥1 enteric pathogens. Also, for the small set of cases in which we identified virus in situ, 1 dog was co-infected with a canine bocavirus and 1 with *Babesia conradae*. The role of co-infection in the pathogenesis of disease in these cases is unclear.

In summary, DogCV should be considered in cases of unexplained vasculitis in dogs, although further studies will be required to ascertain whether and when DogCV causes disease. DogCV also could be a complicating factor in other canine infectious diseases, as is the case with PCV2, which is most dangerous in pigs co-infected with other pathogens. Future research into the contribution of DogCV to disease should carefully consider these potential viral and host factors.

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Cost-effectiveness of Novel System of Mosquito Surveillance and Control, Brazil

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Of all countries in the Western Hemisphere, Brazil has the highest economic losses caused by dengue fever. We evaluated the cost-effectiveness of a novel system of vector surveillance and control, Monitoramento Inteligente da Dengue (Intelligent Dengue Monitoring System [MID]), which was implemented in 21 cities in Minas Gerais, Brazil. Traps for adult female mosquitoes were spaced at 300-m intervals throughout each city. In cities that used MID, vector control was conducted specifically at high-risk sites (indicated through daily updates by MID). In control cities, vector control proceeded according to guidelines of the Brazilian government. We estimated that MID prevented 27,191 cases of dengue fever and saved an average of $227 (median $58) per case prevented, which saved approximately $364,517 in direct costs (health care and vector control) and $7,138,940 in lost wages (societal effect) annually. MID was more effective in cities with stronger economies and more cost-effective in cities with higher levels of mosquito infestation.

Dengue viruses cause ≈50 million infections annually worldwide, and ≈1% of these infections require hospitalization because of dengue hemorrhagic fever (*1*). Brazil accounts for ≈75% of all dengue cases in the Western Hemisphere (*2*), and during 2000–2005, Brazil reported more cases than any other country in the world (*3*). Since the reemergence of dengue in Brazil in 1982, there has been an epidemiologic shift to hyperendemicity (*4*,*5*) and more severe disease (*5*,*6*). Moreover, of all countries in the Western Hemisphere, Brazil has the highest economic losses caused by dengue ($1.35 billion) annually for direct medical and nonmedical costs and indirect costs from loss of work (*7*). This high economic cost of the disease occurs even after Brazil spent $1 billion annually on the dengue vector control program. Cost-effective methods of vector control are needed to decrease the huge economic effects of this disease in Brazil.

The most accurate method of assessing dengue risk by vector surveillance is one that specifically counts dengue vectors that are actively in search of a blood meal: adult female *Aedes aegypti* and occasionally *Ae. albopictus* mosquitoes. Traditional methods of vector monitoring in Brazil, which include surveys of larvae and pupae (*8*,*9*) and capture of adult mosquitoes by aspiration (*10*), are less specific and labor-intensive. Surveys of larvae target both vector sexes and can only predict the number of mosquitoes that will survive to adulthood, rather than directly measure adults. Capturing adults by aspiration does not specifically target female mosquitoes, is labor-intensive, and requires access to premises.

Fixed-position traps designed to capture gravid mosquitoes (e.g., MosquiTRAPs) (Ecovec SA, Belo Horizonte, Brazil) have been developed to reduce personnel costs and directly measure adult female mosquito abundance in Brazil (*11*,*12*). MosquiTRAPs have been implemented in the form of a large-scale mosquito surveillance system, Monitoramento Inteligente da Dengue (Intelligent Dengue Monitoring System [MID]; Ecovec SA), which is used to count mosquitoes in real time. MID involves weekly monitoring of MosquiTRAP (placed in a 300 m × 300 m grid format) counts and trapped-mosquito infection status with automated database updating (in situ mosquito data entry by cell phones directly to a Web-based database). The mosquito data are managed by a spin-off company (Ecovec SA), which provides daily updates to control personnel so they can specifically target highly infested areas. Preliminary results from 3 cities (Tres Lagoas in Mato Grosso do Sul State, and Presidente Epitacio and Bastos in Sao Paulo State) during 1 season of MID

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implementation showed that this system is effective in decreasing dengue cases (*13*). However, an estimate of cost-effectiveness for more cities over a longer period is needed for deciding whether MID should be maintained.

We evaluated the cost-effectiveness of supplementing vector control methods with MID in 21 cities in Minas Gerais State, Brazil, after use during 2 dengue seasons. We also identified factors that affected efficacy and cost-effectiveness of MID. We reported direct savings for health care costs and vector control activities separately from indirect savings for lost wages so that results are relevant to public health budgets and societal concerns.

Methods

Case Data

Monthly dengue cases during January 2007–June 2011 were obtained from each municipality in Minas Gerais, Brazil, by using Sinan Net (Information System for Notification of Grievances), a publicly available database of the Health Ministry of Brazil. Dengue cases were expressed as incidence per 100,000 inhabitants on the basis of the Brazilian Institute of Geography and Statistics (Rio de Janeiro, Brazil) 2010 population census.

MID Mosquito Surveillance System

MID was implemented in 21 cities in Minas Gerais during April 2009–June 2011. These cities are dispersed throughout the state in areas that included a range of population sizes and incidences (Figure 1). Cities that had the highest dengue incidence in the state were chosen by the Minas Gerais State Department of Health to receive MID. These cities were Aguas Formosas, Araguari, Bom Despacho, Caratinga, Conselheiro Lafaiete, Coronel Fabriciano, Curvelo, Governador Valadares, Ipatinga, Itabira, Joao Monlevade, Lavras, Malacacheta, Manhuaçu, Padre Paraiso, Paracatu, Pirapora, Ponte Nova, Sete Lagoas, Teofilo Otoni, and Visconde do Rio Branco. The only difference in vector-control activities between cities that used MID and those that did not use MID was that vector control in MID cities targeted sites that MID identified as highly infested with gravid adult mosquitoes. Details of the structure and function of MID and control efforts are shown in online Technical Appendix 1 (wwwnc.cdc.gov/EID/article/19/4/12-0117-Techapp1.pdf).

Data Analysis

Dengue incidence was strongly seasonal, and outbreak probability varied substantially between cities (Figure 2, panel A), which did not follow any common statistical probability distribution in the exponential family. Thus, we adopted a nonparametric approach to data analysis. On the basis of potential differences in dengue transmission caused by population size (*14*) and demographics (*15*), the 21 treatment cities were divided into 5 groups by population size: 18,000–21,000, 35,000–60,000, 70,000–90,000, 100,000–140,000 and 150,000–300,000 for comparison with control cities (Figure 2, panel B). Cities within Minas Gerais that did not implement MID were referred to as control cities. There were 147 control cities that could be

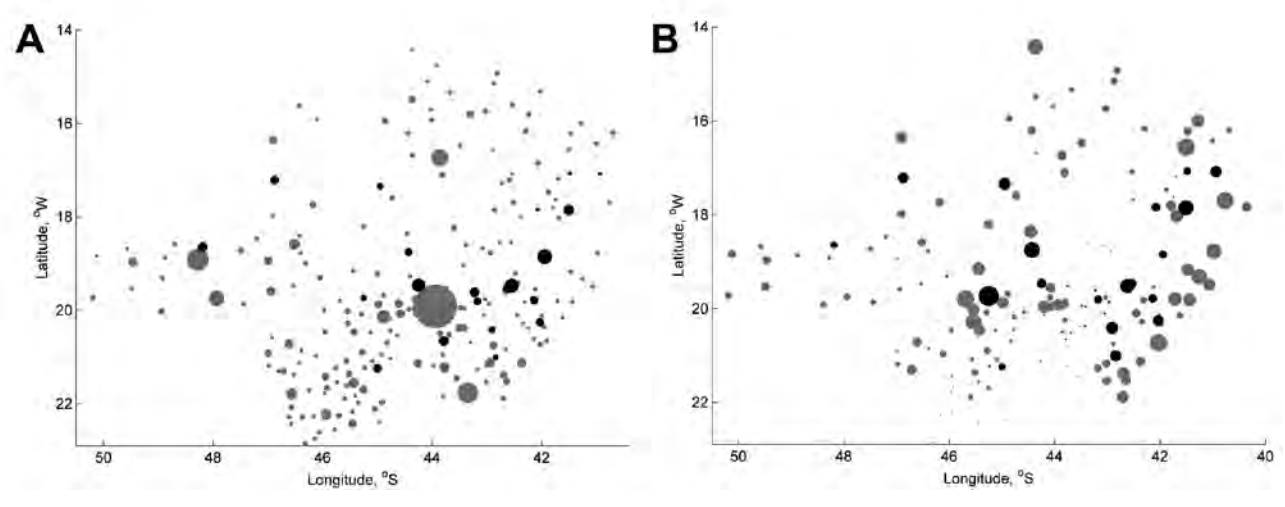

Figure 1. Spatial distribution of 21 cities tested with Monitoramento Inteligente da Dengue (Intelligent Dengue Monitoring System [MID]), Minas Gerais, Brazil, 2009–2011. A). Size of city centroids (n = 218) (circles) is proportional to population size. B) Size of city centroids (n = 147) (circles) is proportional to total dengue fever incidence during 2007–2011. Gray circles indicate cities that never implemented MID, and black circles indicate cities that implemented MID during mid-2009–June 2011. Areas of higher and lower total incidence are positively clustered with each other (Moran's I, $p<0.0001$). Cities that implemented MID and those that had not implemented MID are distributed throughout areas of high and low incidence. Only cities with populations >15,000 are shown. Incidence data were not available for all cities.

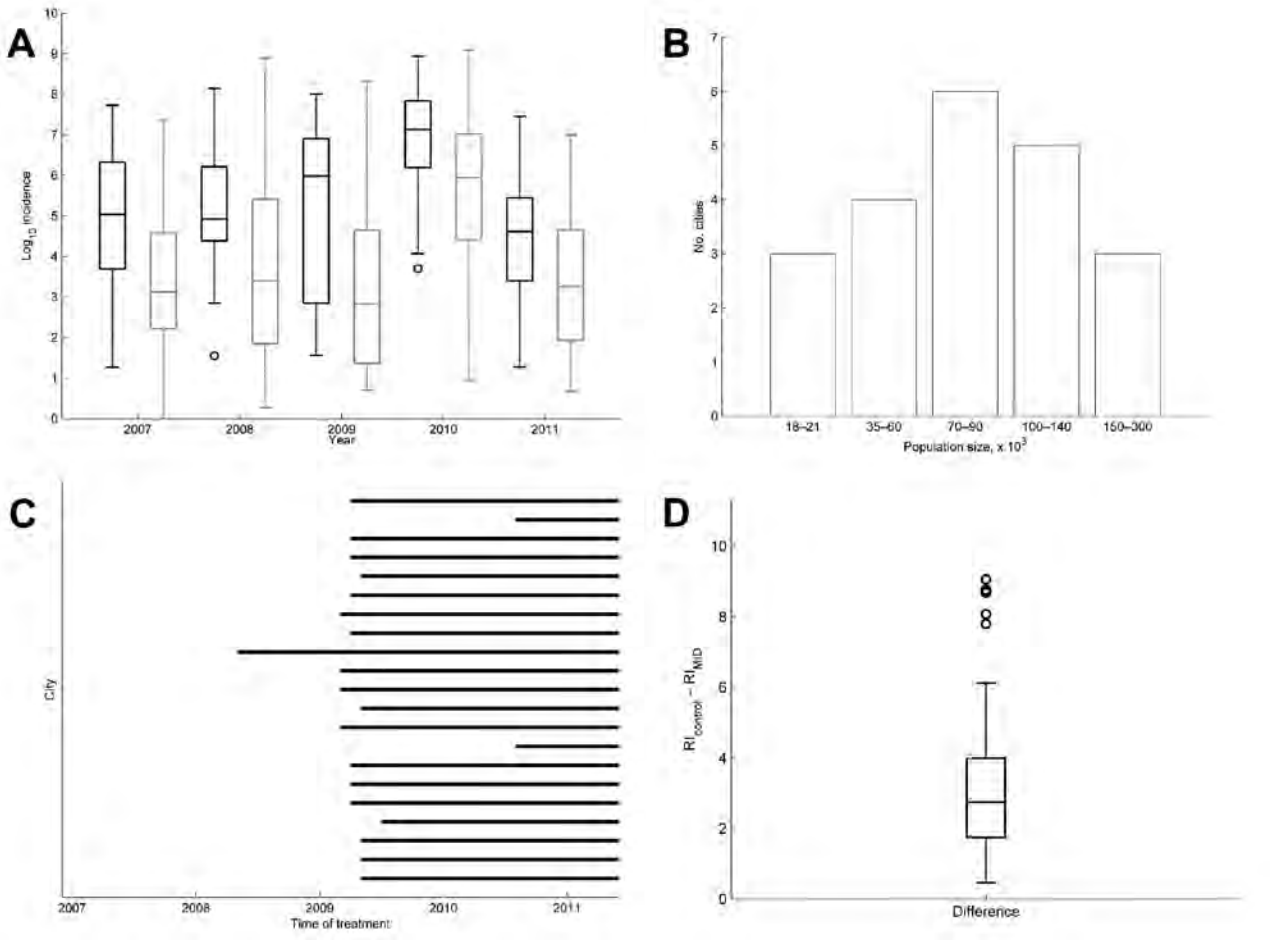

Figure 2. Changes in incidence of dengue fever in 21 cities that implemented Monitoramento Inteligente da Dengue (Intelligent Dengue Monitoring System [MID]), Minas Gerais, Brazil, mid-January 2007–June 2011. A) Annual incidence in 21 cities that implemented MID (bars outlined in black) and 147 cities that had not implemented MID (bars outlined in gray). Horizontal lines in boxplots indicate medians of 1,000 medians. Whiskers indicate ± 2.7 SD. Circles indicate points that fall outside ± 2.7 SD. B) Distribution of population sizes in cities that implemented MID. C) Time that MID was implemented in each city. D) Median relative increase (RI) in incidence for cities that implemented MID versus cities that had not implemented MID. RI was calculated as the sum of monthly incidence after MID was implemented divided by the sum of monthly incidence before MID was implemented for the same number of months. For cities that implemented MID, the median is a single value for the 21 cities. For cities that had not implemented MID, 21 cities with the same distribution of population sizes as MID cities were selected at random 1,000 times and their median relative differences during the same set of time frames were calculated. Horizontal line in the boxplot indicates median of 1,000 medians. Whiskers indicate ± 2.7 SD. Circles indicate points that fall outside ± 2.7 SD.

grouped into a distribution of population sizes of treatment cities.

To compare this large sample size in a case–control format to only 21 MID cities, we generated 1,000 random sets of 21 control cities with the same population distribution as the MID cities. Next, we calculated the relative difference in incidence (RI) for the same period before and after the start of surveillance for each treatment city (i.e., incidence for x time before MID/incidence for x time after MID). Likewise, for each set of the control cities, we calculated RI using the distribution of time frames in each group of treatment cities (Figure 2, panel C) matched to the corresponding group in control cities. Lastly, we calculated the median RI for each of the 1,000 sets of control cities and the set of treatment cities and calculated the difference ($d = RI_{\text{control}} - RI_{\text{MID}}$). Under the null hypothesis that MID had no effect at decreasing RI, the median of the 1,000 differences would be 0. We tested this hypothesis using a sign test. The alternative hypothesis was that the median of the 1,000 differences would be significantly >0 if MID decreased the RI of treatment cities.

We identified factors that affected the effectiveness and cost-effectiveness of MID by using a generalized linear model (γ distribution, log link) with either RI or US dollars/prevented case as response variables. Factors considered were population size (PS); distance to 3 large populations (D3L); distance to 3 high-incidence populations; a

ranking system for the effectiveness of using MID (PED); a measure of average mosquito infestation during the dengue season in 2011 (IMFA); population density; income per capita; and an index between 0 and 1 that included employment, income, education, and health, all with equal weight. Distances were the sum of Euclidian distance to 3 cities with population size (D3L) or density in the 90th percentile. We fit each variable individually and fit all possible linear combinations of the 8 variables.

We chose between competing models by using delta Akaike Information Criterion (ΔAIC = AIC of intercept only model – AIC of target model). A higher ΔAIC indicates a better model of the data. When comparing nested models that differed by only 1 factor, 2 AIC points is considered a significant difference ($\alpha = 0.05$). Statistics for all single-variable models, model selection results, and fits of the best multivariable and full models are shown in online Technical Appendix 2 in Tables 1, 2, and 3 (wwwnc.cdc.gov/EID/article/19/4/12-0117-Techapp2.xlsx).

We estimated the number of cases prevented by MID by predicting the number of cases that would have occurred in the absence of MID and taking the difference between those and the number of observed cases (i.e., cases prevented/year = predicted cases in the absence of MID [*E*] – observed annual cases [*O*]). We calculated *E* by using a logistic model according to the equation $E_i = d_i O_i(1 - O_i/K_i)$, where *K* is the maximum number of possible cases in city *i*. The logic is that the number of cases prevented depends on the estimated growth coefficient ($d = RI_{control} - RI_{MID}$) and the observed cases (*O*) but is capped by a theoretical maximum on the number of possible new cases (*K*). In the main text, we assumed that *K* was equal to 30% of the population in city *i*, which has been observed (*16*). However, we also considered higher and lower values of *K* (5%, 10%, 20%, and 50%) (Figure 3).

Cost Data

All costs were in US dollars. Costs per dengue case were taken from the report of Sheppard et al. (*7*). They calculated direct and indirect costs for ambulatory ($69 and $317, respectively) and hospitalized ($428 and $460, respectively) case-patients. We considered dengue fever case-patients to be ambulatory and dengue hemorrhagic fever case-patients, dengue shock syndrome case-patients, and case-patients who died to be hospitalized. We did not distinguish deaths (0.045% of case-patients) from severe cases (0.38% of case-patients) because we could not obtain the age distribution of deaths and gross domestic product estimates from each city. Indirect costs assumed an average of 4.5 days of lost work for ambulatory case-patients and 14 days for hospitalized case-patients (*7*). Estimates of indirect costs per case were adjusted to account for case-patients who did not miss work by using the age distribution of case-patients in Brazil (*7*).

Total costs for MID in the 21 cities were measured directly by Ecovec SA (Table). MID costs in individual cities varied from $25,566 to $163,944 (online Technical Appendix 2 Table 4). Cost-effectiveness per city was calculated as the measured cost of MID in a given city divided by its number of cases prevented, as estimated from the model. In Minas Gerais, vector control activities are conducted

Table. Total costs of MID in 21 cities, Brazil, 2007–2011*

Product or service	Cost in US dollars (%)
Royalties to UFMG and FAPEMIG	
MID†	29,918.96 (2.0)
MI-Virus†	38,894.65 (2.6)
Consumables (licensing)	
MosquiTRAP†	77,533.50 (5.2)
Sticky card	112,776.00 (7.5)
AtrAedes†	131,572.00 (8.8)
Web software	21,000.00 (1.4)
Mobile software	96,041.00 (6.4)
Services	
MI-Vírus kit and analyses	58,485.00 (3.9)
MID	61,303.96 (4.1)
Shipping and freight	
MI-Virus and traps	11,056.45 (0.7)
Stationary and materials	668.40 (0.0)
Technical and supervision (employees and taxes)	
Technical support at Ecovec SA, 12 h/d	115,499.00 (7.7)
Technical support at cities visited	80,208.00 (5.4)
Full-time biologist	372,520.54 (24.9)
Technical visits on site	19,200.00 (1.3)
Taxes	269,270.66 (18.0)
Total	1,495,948.13 (100.0)

*MID, Monitoramento Inteligente da Dengue (Intelligent Dengue Monitoring System); UFMG, Universidade Federal de Minas Gerais; FAPEMIG, Fundação de Amparo a Pesquisa do Estado de Minas Gerais; MI-Virus, Intelligent Virus Monitoring System; MosquiTRAP, fixed-position trap designed to capture gravid mosquitoes; AtrAedes, synthetic ovipostion attractant.
†Manufactured by Ecovec SA (Belo Horizonte, Brazil).

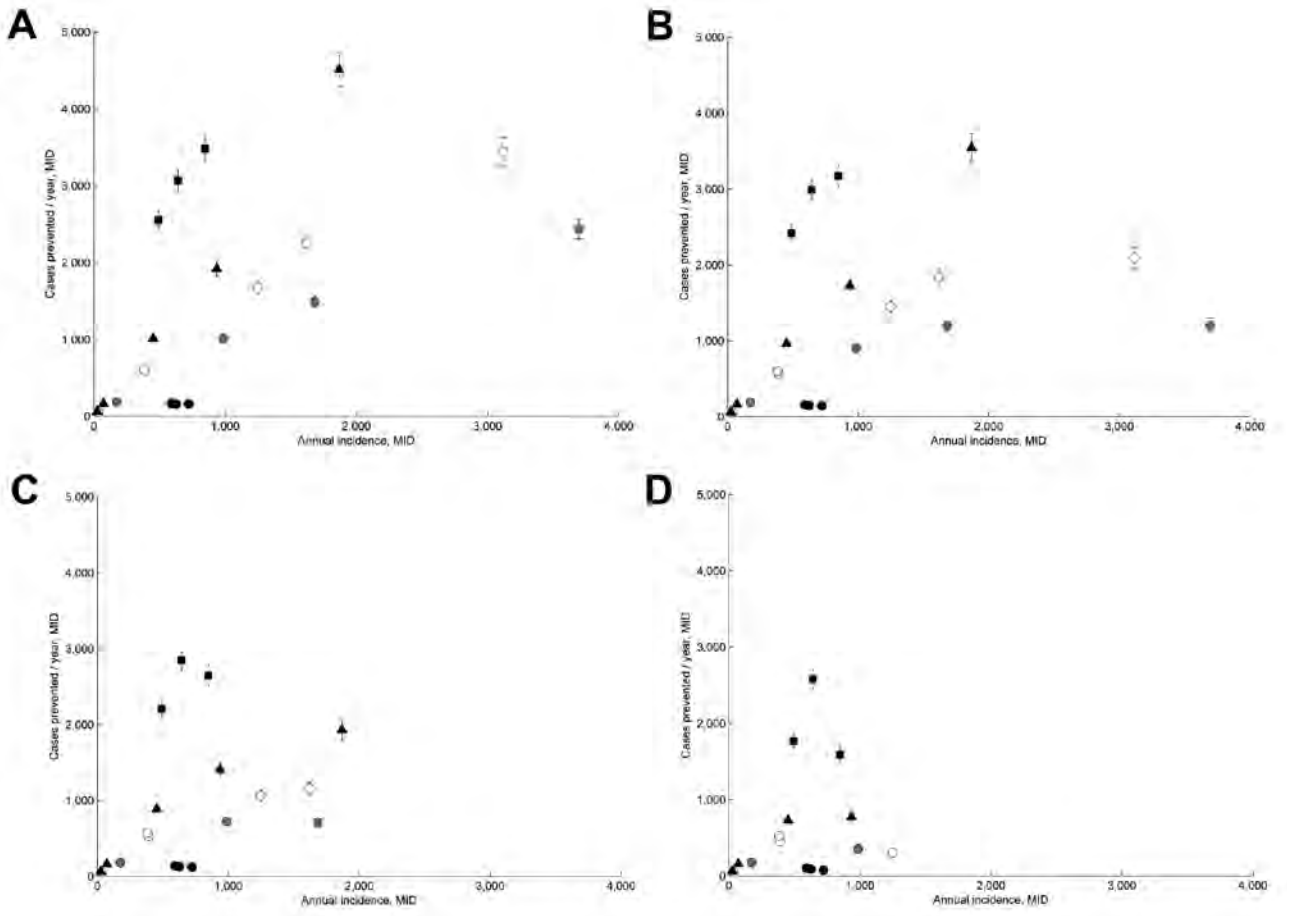

Figure 3. Effectiveness of Monitoramento Inteligente da Dengue (Intelligent Dengue Monitoring System [MID]), Minais Gerais, Brazil, mid-2009–mid 2011. Predicted number of dengue fever cases prevented per year during the time of MID are plotted against the annual incidence of dengue fever in each city during the same time. K is a percentage value of the population size in a city. Error bars indicate 2 SE. A) 29,533 cases were prevented when K = 50%. B) 24,263 cases were prevented when K = 20%. C) 16,578 cases were prevented when K = 10%. D) 9,219 cases were prevented when K = 5%. Shaded symbols distinguish population size classes as follows: black circles indicate 18,000–21,000; gray circles indicate 35,000–60,000; white circles indicate 70,000–90,000; triangles indicate 100,000–140,000; squares indicate 150,000–300,000.

according to guidelines of the National Program for Dengue Control (*17*) and the state department of health in Minas Gerais. Government resources are apportioned to cities on the basis of their population size and history of dengue incidence. Thus, we assumed that the per capita cost of control was similar in each treatment city. To estimate the cost of mosquito control activities in each city, we took the per capita cost ($1.11) from a study in Sao Paulo, Brazil, in 2005 (*18*) and multiplied this cost by the population size in each city. The previous study measured 3 components of dengue control costs: vector control activities (larval survey, insecticide spraying); laboratory activities (entomology and serologic analysis); and public education and database maintenance. Labor comprised ≈60% of costs, and materials needed for conducting the work comprised 31% of costs (*18*).

For each treatment city, we calculated the direct, indirect, and total costs of dengue. Direct costs comprised medical and nonmedical direct costs, as well as vector control and MID. Indirect costs comprised costs for lost wages and MID costs in treatment cities. Costs for MID cities were calculated from the number of observed cases (divided into ambulatory and hospitalized case-patients). Similarly, the estimated costs of dengue in the absence of MID were divided into ambulatory and hospitalized case-patients by multiplying the sum of the number of observed cases plus the number of prevented cases by the proportion of observed cases in persons who were ambulatory or hospitalized. The dollars saved annually were calculated by subtracting the cost of dengue in MID cities from the predicted cost of dengue if MID were not implemented. Underreporting was not accounted for because we had no city-specific data to inform estimates. Costs were not discounted because we considered all cases to be nonfatal and our study period was only 2.5 years.

Results

The annual incidence of dengue in control cities varied more widely than in treatment cities, and the median annual incidence in treatment cities was generally higher (Figure 2, panel A). However, there was a trend of decreased difference in incidence between annual incidence in treatment cities relative to control cities during the years (2010–2011) in which MID was used during the peak dengue season (January–May or June) (Figure 2, panel A). This trend was confirmed by the finding that the RI before and after the time frame of MID was 2.7× higher (a decrease from 4.0 in control cities to 1.3 in treatment cities; 68%), in control cities relative to those that used MID ($z = -31.59$, $p<0.0001$) (Figure 2, panel D). The RI for treatment cities for each population group is shown in Figure 4.

The most parsimonious generalized linear model of RI in MID cities included PS and IDFM (online Technical Appendix 2 Table 2). PS and IDFM showed a negative correlation with RI, although the correlation of PS was marginally not significant ($p = 0.083$ for PS and $p = 0.0023$ for IDFM) (online Technical Appendix 2 Table 3). This finding indicates that MID effectiveness was higher in cities with stronger economies and that there is a trend of higher effectiveness in larger populations. In contrast, the most parsimonious model of cost-effectiveness included IMFA and D3L (online Technical Appendix 2 Table 2). IMFA and D3L showed a negative correlation with cost-effectiveness ($p = 0.0086$ and $p = 0.032$, respectively) (online Technical Appendix 2 Table 3). Thus, cost-effectiveness was higher in cities with higher mosquito infestation levels and cities that were farther from cities with large populations.

Under the assumption that dengue could affect 30% of a population, we estimated that the number of cases prevented by MID annually in the largest cities (>130,000 inhabitants) was 2,300–3,900 (Figure 5). In the smallest cities (<40,000 inhabitants), these estimates decreased to 143–182, and the total number in all 21 cities was 27,191. However, these numbers depend on the assumed number of potentially susceptible persons (Figure 3). The average cost-effectiveness was $227/case prevented, which was driven mainly by a few larger values (Figure 6, panel A). The median value was $58, indicating that the average value was higher than the cost-effectiveness value in most cities. The number of cases prevented translated to net total savings of $8,999,406 annually. Savings in health care and vector control costs was $364,517, and savings in lost wages was $7,138,940 (Figure 6, panel B; online Technical Appendix 2 Table 4).

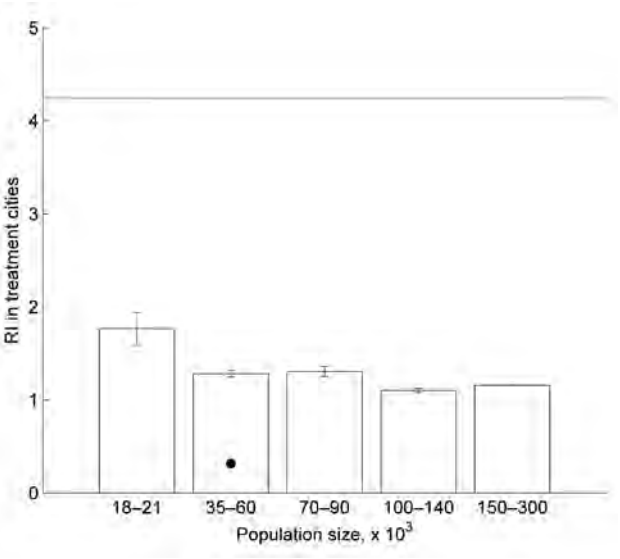

Figure 4. Mean relative difference in incidence (RI) of dengue fever cases for treatment cities grouped by population size using Monitoramento Inteligente da Dengue (Intelligent Dengue Monitoring System), Minas Gerais, Brazil, mid-2009–mid 2011. Horizontal line indicates mean RI for the 1,000 median RI of control city sets. Error bars indicate 2 SD. Error bars for the largest population size group are too small to be shown. The black dot is an outlier that was excluded from the general linear model results.

Discussion

Accurate estimates of dengue incidence and its economic effects are more limited (*16*,*19*) than are estimates of other infectious diseases that pose similarly serious public health threats. This finding is caused mainly by high variability in clinical disease, high underreporting rates, and lack of studies that directly measure the efficacy of controls. Consequently, only a few studies have demonstrated the cost-effectiveness of vector control activities (*19*–*21*). In one of these studies, targeted source reduction was more effective than nontargeted vector control, reducing vector abundance by 52%–82% depending on the country (*21*). Another study found that targeted vector control reduced the dengue case load by 53% (*20*). Our estimate of a 68% reduction in incidence caused by targeted control efforts by MID was higher than that in the study by Suaya et al. (*20*). One reason may be geographic differences in the effects of source reduction methods (the previous study was conducted in Cambodia). Alternatively, our higher estimate may be caused by implementation of MID at a fine spatial scale over a broader area, which produced higher intervention efficacy.

The trend of increased effectiveness in larger populations might not be significant in the multivariable model (which includes IDFM) because PS and IDFM showed a positive correlation ($r = 0.53$). The single-variable model results suggest that MID may be more effective in larger populations (online Technical Appendix 2 Table 1). The fact that MID was more cost-effective in cities with higher mosquito infestation levels emphasizes the power of targeting vector control practices to areas in which gravid

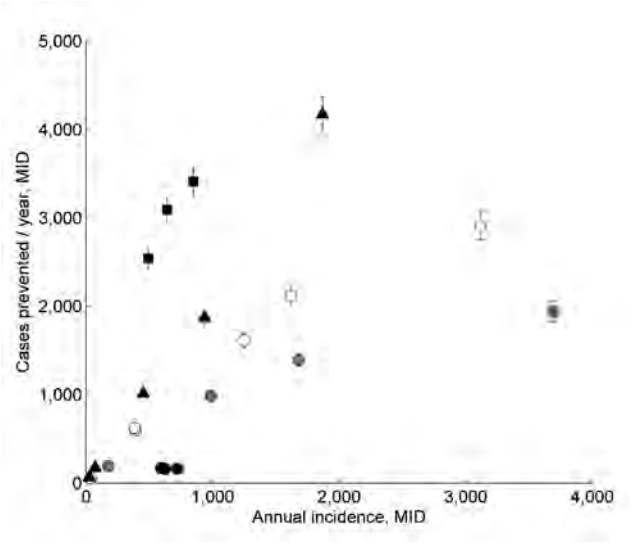

Figure 5. Effectiveness of Monitoramento Inteligente da Dengue (Intelligent Dengue Monitoring System [MID]), Minas Gerais, Brazil, mid-2009–mid-2011. Predicted number of dengue fever cases prevented per year during the time of MID are plotted against the annual incidence of dengue fever cases in each city during the same time. A total of 27,191 cases were prevented. Cases prevented/year = predicted cases in the absence of MID (*E*) – observed annual cases (*O*), where $E_i = d_i O_i(1 - O_i/K_i)$, *d* is the difference between median relative difference (RI) in incidence in control cities (mean 1,000 datasets) minus the RI in each treatment city, and K is 30% of the population size in city *i*. Error bars indicate 2 SE of the number of predicted cases that were prevented (points without bars are shown because the SEs are smaller than the size of the point). Shaded symbols distinguish population size classes as follows: black circles indicate 18,000–21,000; gray circles indicate 35,000–60,000; white circles indicate 70,000–90,000; triangles indicate 100,000–140,000; squares indicate 150,000–300,000.

mosquitoes are most abundant. A possible reason for higher cost-effectiveness in cities that were farther from large cities could be that proximity to larger cities may enable a higher proportion of cases that were contracted elsewhere (i.e., during travel or commuting to large metropolitan areas) (*22*,*23*).

PED, a measure of MID quality, was not correlated with effectiveness or cost-effectiveness of MID. This result suggests that variation in the force of infection between cities overwhelmed differences in PED, the current measure of PED is inaccurate, or both. The relationship between mosquito infestation and human incidence is highly variable in space and time (*24*–*26*). Studies of dengue virus serotype circulation in Brazil have found dominance of a single serotype during any given year, and different genotypes within the serotypic groups have caused large, severe outbreaks because of reduced population immunity (*5*,*27*–*29*). Thus, variations between cities in novel genotype dynamics might have affected variation in RI because of PED. In addition, PED pertains only to MID activities and does not evaluate control activities. It is likely that the quality with which cities conduct prescribed control practices varies, which could also explain the lack of relationship between PED and RI. Furthermore, PED is assessed by a yes or no checklist for MID activities, rather than by an evaluation of the quality of each activity. If only minor activities constitute most of the variation in PED, then little meaningful variation in MID quality between cities might be observed. Regardless, the lack of relationship between PED and efficacy of MID suggests that an additional method for assessing MID quality, perhaps through collaboration with city control personnel, might be useful for maintaining, standardizing, and improving quality.

One caveat to our method of estimating cost-effectiveness is that it was necessary to estimate the number of susceptible hosts in the absence of MID (we used 30%) to predict the number of cases that would be prevented. Although 30% is not unreasonable based on previous studies (*16*), the maximum incidence observed in a given city in Minas Gerais during 2007–2010 was only 8.8%. This discrepancy was partly caused by underreporting, which was not accounted for in our study. Nevertheless, we also provided predictions for lower values of *K* to understand how it could affect our estimates. When fewer hosts are susceptible, the number of cases prevented is also lower, which decreases the cost-effectiveness of MID. Thus, previous large outbreaks with the same serotype and vaccination programs would be expected to decrease the cost-effectiveness of MID. Another caveat to our assumption is that *K* varies by city because of historical disease patterns and other factors. Collection of longitudinal serologic data would be useful for more accurate, city-specific predictions of *K*. Last, our study used previously estimated costs for control activities, health care, and lost wages. These costs were per capita estimates that we assumed could be extrapolated to each city equally. The accuracy of our results could be improved through microcosting analyses within each city.

Although MID showed an average cost-effectiveness value of $227 (median $58) per case prevented in Minas Gerais, the average value increased to $616 in 6 moderately sized cities (population 73,000–117,000) that did not show any savings in direct costs (online Technical Appendix 2 Table 4). Three of the cities saved on indirect costs and total costs, but the 3 other cities (Joao Monlevade, Itabira, and Conselheiro Lafeite) had a net loss of up to $81,042 in direct costs and $66,246 in indirect costs because of incorporating MID into their budgets. These 3 cities had relatively low annual dengue incidence in the 2 years before MID implementation (12, 18, and 72 cases per 100,000 population relative to a range of 104–2,014 cases in the other 18 cities except for Paracatu, which had 4 cases). Thus, in general, cities with annual incidences of

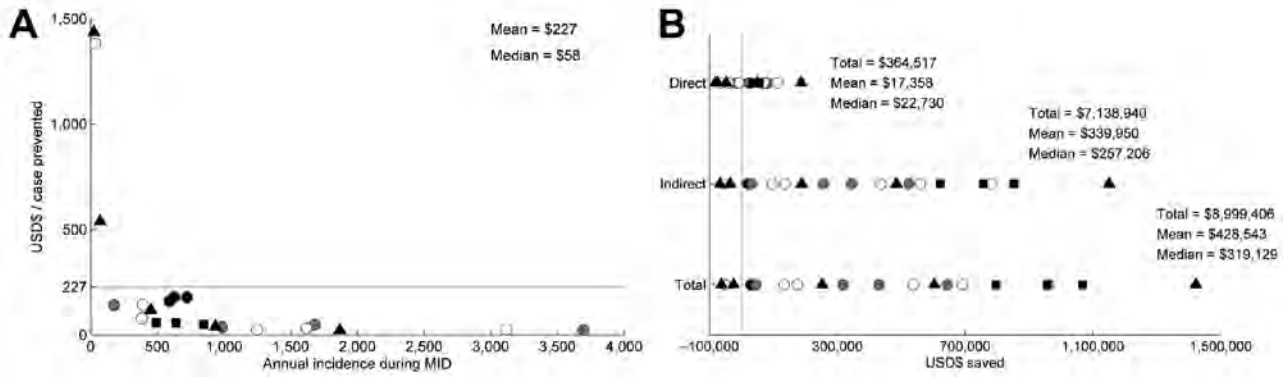

Figure 6. Cost-effectiveness of and savings from Monitoramento Inteligente da Dengue (Intelligent Dengue Monitoring System [MID]), Minas Gerais, Brazil, mid-2009–mid-2011. A) For cost-effectiveness, the number of US dollars (USD$) spent per dengue fever case prevented is plotted against the annual incidence of dengue fever cases during MID for the city. Each point represents cost-effectiveness for a city. Points are coded by population size classes. Horizontal line indicates average cost-effectiveness ($227) per case prevented. B) Savings for each cost component from the benefits of MID. Direct savings include only health care, nonmedical direct savings, and vector control savings. Indirect savings include only savings in the work force. Total savings include direct and indirect savings. Negative values indicate dollars lost because of implementing MID. Vertical line indicates 0. Shaded symbols distinguish population size classes as follows: black circles indicate 18,000–21,000; gray circles indicate 35,000–60,000; white circles indicate 70,000–90,000; triangles indicate 100,000–140,000; squares indicate 150,000–300,000.

>72 cases per year were more likely to have higher MID cost-effectiveness.

Furthermore, cities in which MID was implemented had historically high dengue incidences relative to control cities (mean ± SD 2007 and 2008 were 549.1 ± 592 in MID cities and 240.4 ± 567.6 in control cities). Thus, average estimates of cost-effectiveness may be high in cities in which MID was implemented. However, factors determining incidence patterns in a given city, such as population immunity, infrastructure, or human behavior, may not be static over time because high population immunity is not protective against novel serotypes (or genotypes with high forces of infection) and human behavior and infrastructure are continually changing. A predictive model of serotype dynamics across cities formulated on the basis of serologic data would be useful for decisions on which cities should implement MID so that the most cost-effective strategy can be achieved statewide.

Our study showed that MID is generally effective for decreasing case loads and suggested that an MID strategy is theoretically better than other strategies. Although MID cost-effectiveness varied between cities, implementation of MID saved hundreds of thousands of dollars on health care and ≈7 million dollars in lost wages statewide, and half the cities had cost-effectiveness values <$58. Furthermore, these numbers are underestimates because our study did not account for underreporting or additional costs from deaths. Investing more effort into integrating MID strategies and costs with vector control operations, and standardizing the MID-based control system across cities, should help to increase MID cost-effectiveness.

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Serotype IV and Invasive Group B *Streptococcus* Disease in Neonates, Minnesota, USA, 2000–2010[1]

Patricia Ferrieri, Ruth Lynfield, Roberta Creti, and Aurea E. Flores

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Learning Objectives

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

- Describe the classification of infant group B *Streptococcus* (GBS) disease and the impact of maternal screening on this illness
- Analyze the epidemiology of infant GBS disease in the current study
- Distinguish the most common GBS genotypes among infants infected in the current study
- Evaluate the significance of GBS genotype IV

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Disclosures: ***Patricia Ferrieri, MD; Ruth Lynfield, MD; Roberta Creti, PhD;*** *and* ***Aurea Flores PhD*** *have disclosed no relevant financial relationships.*

Group B *Streptococcus* (GBS) is a major cause of invasive disease in neonates in the United States. Surveillance of invasive GBS disease in Minnesota, USA, during 2000–2010 yielded 449 isolates from 449 infants; 257 had early-onset (EO) disease (by age 6 days) and 192 late-onset (LO) disease (180 at age 7–89 days, 12 at age 90–180 days). Isolates were characterized by capsular polysaccharide serotype and surface-protein profile; types III and Ia predominated. However, because previously uncommon serotype IV constituted 5/31 EO isolates in 2010, twelve type IV isolates collected during 2000–2010 were studied further. By pulsed-field gel electrophoresis, they were classified into 3 profiles; by multilocus sequence typing, representative isolates included new sequence type 468. Resistance to clindamycin or erythromycin was detected in 4/5 serotype

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IV isolates. Emergence of serotype IV GBS in Minnesota highlights the need for serotype prevalence monitoring to detect trends that could affect prevention strategies.

Streptococcus agalactiae, or group B *Streptococcus* (GBS), is one of the leading causes of invasive bacterial diseases, such as bacteremia, pneumonia, and meningitis, in newborns and infants in the first months of life in the United States (*1*,*2*) and in other parts of the world (*3*–*6*). In newborns birth through 6 days of age, invasive GBS disease is designated as early-onset (EO) and late-onset (LO) in infants 7 days to 3 months of age; some investigators report an ultra-late period extending well beyond 3 months of age (*3*).

During the past 20 years, prevention of EO and LO invasive GBS disease in the United States has been an area of interest for clinicians and public health officials. In the early 1990s, the overall rate of EO disease in the United States was 1.7/1,000 live-born infants (*7*,*8*) but differed from one part of the country to another: for example, 0.56 for Minneapolis/St. Paul, 1.3 for Houston (*9*), and 1.3 for Maryland (*10*). This high rate prompted the Centers for Disease Control and Prevention (CDC) to issue guidelines in 1996 for preventing EO disease by screening pregnant women at 35–37 weeks' gestation for GBS colonization and administering antimicrobial drug prophylaxis to women at risk of transmitting the organism to the child (*11*). Although implementation of the prescribed measures reduced the rate of EO disease in the United States to 0.47/1,000 live births by 1999–2001, problems remained (e.g., risk-based vs. culture-based approaches to prevention, laboratory detection of colonized mothers, use of antimicrobial drugs in women with allergies to penicillin, use of secondary prevention among infants) (*12*); these factors required revision of the guidelines in 2002 (*7*) and again in 2010 (*13*). However, measures designed to prevent EO disease have had little effect on the rate of LO disease, which remained 0.4/1,000 live births throughout the 1990s, varying only slightly from year to year (*8*,*12*).

GBS isolates are characterized according to capsular polysaccharide (CPS) serotype, of which 9 are recognized: Ia, Ib, II–VIII (*9, 14–16*), and a recently proposed serotype IX (*17*). Results from earlier studies in various parts of the United States, including Minnesota, indicated that Ia, III, and V were the predominant serotypes in EO disease, whereas serotype III and Ia were predominant in LO disease (*9*,*10*,*12*,*15*).

Since 1995, our laboratory has collaborated with the Minnesota Department of Health (MDH) to serotype isolates from cases of EO and LO disease in Minnesota in conjunction with the CDC Emerging Infections Program (*7*). This collaboration has enabled us to follow for almost 2 decades changes in serotype distribution of GBS isolates that cause invasive disease in Minnesota. Here we report on the epidemiology of EO and LO GBS disease in Minnesota over 11 years, the recent emergence of invasive disease in infants with serotype IV GBS, and an increase in disease caused by this serotype in 2010 compared with our previous findings (*15*). We provide serotyping results of all isolates from EO and LO disease collected during 2000–2010 and present data from molecular characterization by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) of serotype IV isolates from EO and LO disease.

Materials and Methods

Study Design

As part of the CDC Active Bacterial Core Surveillance Program, GBS isolates from 453 infants in Minnesota with invasive GBS disease reported during January 1, 2000–December 31, 2010, were submitted to the MDH Public Health Laboratory, and the case details were reviewed (*7*). Infants were classified according to GBS disease onset and age: 257 (age birth–6 days) with EO disease, 180 (age 7–89 days) with LO disease, and 12 (age 90–180 days) with delayed LO disease. Four infants with first GBS-positive culture at age 6–13 months were classified with ultra-LO disease (ULOD) (*3*) and were not included in our analyses. The total numbers of live births in Minnesota by year were provided by MDH.

Study Isolates and Culture Sites

Of the 449 EO and LO isolates studied and analyzed, 403 were from blood, 42 from cerebrospinal fluid (CSF), and 4 from normally sterile sites (1 bone, 1 joint, and 2 obtained postmortem from liver and lung). One isolate per infant was included in the analysis. Isolates received from MDH without patient identifiers were serotyped and studied by molecular methods at the GBS Molecular Reference Laboratory (University of Minnesota Medical School Twin Cities Campus, Minneapolis, MN, USA). Isolates were tested by using single-colony picks in Todd-Hewitt Broth (THB) (Bacto; Becton, Dickinson and Company, Sparks, MD, USA), supplemented with 2% sheep blood.

Reference Strains and Antiserum for Serotyping

We used GBS prototypes from our laboratory of internationally recognized reference strains of serotypes Ia, Ib, II–VIII, and newly proposed IX; Rabinowitz 3139 was used for serotype IV (*17–19*). The prototype strains for surface-expressed proteins were those used previously (*18*). Monospecific rabbit antisera to serotypes Ia, Ib, II–VIII, proposed IX, and atypical V (serotype V genetic variant); surface proteins C-α and C-β; group B protective surface (BPS) protein; and the R4(Rib), R1, and R1, R4 (Alp3) species of R were produced in our laboratory against international reference strains (*17*,*18*,*20*,*21*). All antisera were tested against all serotypes to ensure no immunologic

cross-reactions. In addition, molecular typing by PCR confirmed all prototype strains used for production of each type-specific antiserum, including serotype IV.

CPS and Detection of Surface-expressed Proteins

Isolates grown in THB were extracted with hot hydrochloric acid and typed by double-diffusion immunoprecipitation in agarose slides with specific antisera to serotypes Ia, Ib, and II–VIII, as described (*18*). An isolate nonreactive with any of these antisera was considered nontypeable and was regrown in supplemented THB to upregulate CPS production; the extract was retested (concentrated 3-fold) with antisera to Ia–VIII, proposed IX, and atypical V. Extracts from nontypeable and serotype IV isolates were also tested with antisera to GBS surface-expressed proteins (*16*,*18*–*20*). Statistical analyses of the typing results (2-sided p value, Fisher exact test) were done by using InStat (GraphPad Software, Inc., San Diego, CA, USA); p values <0.05 were considered significant.

DNA Macrorestriction Profile Analysis

All 12 serotype IV and 2 nontypeable isolates were studied by PFGE according to published methods (*16*,*22*,*23*) by using bacterial DNA digested with *Sma*I (Invitrogen, La Jolla, CA, USA). The PFGE profile of an isolate was determined by comparing its macrorestriction band pattern to those of the prototype isolates in our PFGE library (*16*,*22*), including the prototypes from 4 PFGE profile groups delineated among our serotype IV isolates from recent years.

MLST and Clonal Complex Assignment

At least 1 serotype IV isolate from each PFGE profile was studied by MLST as described (*14*,*16*,*24*). Also studied were 4 serotype IV blood isolates collected from 2 mothers during the peripartum period and from 2 nonpregnant adults. Results from partial sequencing of 7 housekeeping genes were compared with data from the *Streptococcus agalactiae* MLST Database (http://pubmlst.org/sagalactiae/) to arrive at an allelic profile and sequence type (ST) for each isolate, as described (*16*). The clonal complex (CC) assignment of each isolate was determined by using eBURST software (*25*) so that each CC comprised STs that had alleles at 6 of 7 loci in common with ≥1 other member of the complex. The study STs were compared with all STs in the *Streptococcus agalactiae* MLST Database.

Antimicrobial Drug Susceptibility Studies

All 45 isolates from 2010 were tested for susceptibility to clindamycin and erythromycin according to Clinical and Laboratory Standards Institute guidelines (www.microbiolab-bg.com/CLSI.pdf) by a microdilution MIC method or gradient diffusion E-test. For 2000–2009, only the 7 serotype IV isolates were tested. Isolates sensitive to clindamycin but resistant to erythromycin were further tested by using a double-disk diffusion D-zone test to determine whether resistance to clindamycin could be induced (*26*).

Results

During January 2000–December 2010, a total of 257 infants in Minnesota with invasive GBS disease had EO disease, with GBS isolated from cultures taken during the first 6 days of life (Table 1). An additional 192 infants had LO disease, 180 with GBS-positive cultures at 7–89 days and 12 at 90–180 days of age (delayed LO). Four isolates from infants with ULOD were not included in our analyses. Nearly 90% of all isolates were from blood, but the source of the isolates differed significantly for EO versus LO disease: 251 EO isolates were from blood, compared with 152 LO isolates (p<0.0001), but CSF was the culture source for 4 EO isolates compared with 38 LO isolates (p<0.0001).

III and Ia were the predominant serotypes, accounting for approximately two thirds of the 449 GBS isolates from all EO and LO invasive disease (Table 2). Nearly another third consisted of serotypes V, II, and Ib. A few isolates, mostly from infants with EO disease, were serotype IV or VII; 5 (1.1%) were nontypeable. All serotype IV isolates were identified by routine serotyping and did not require supplemental growth or concentration of the extracts; only a few isolates of other serotypes required these extra approaches.

When the distribution of serotypes in EO versus LO disease was compared, Ia was most commonly isolated in the 257 EO cases (79, 30.7%), followed by III (57, 22.2%), II (43, 16.7%), and V (39, 15.2%). In contrast, among the 192 LO isolates, serotypes III (95, 49.5%) and Ia (57,

Table 1. Distribution of GBS invasive disease in infants by isolate source and age of infant at time of culture, Minnesota, USA, 2000–2010*

	No. infants with GBS invasive disease†				
		Late onset			
Culture source	Early onset	7–89	90–180	>181	Total
Blood	251	142	10	3	406
Cerebrospinal fluid	4	37	1	1	43
Other sites‡	2	1	1	0	4
All	257	180	12	4	453

*GBS, group B *Streptococcus*.
†No. isolates studied were 1 per infant (n = 453). Early-onset is birth–6 days. Late-onset categories (age in days at time of culture): classical (7–89); delayed (90–180); ultra (>181; not included in analyses).
‡Other sites, normally sterile: 1 bone, 1 joint, 2 tissues (1 liver, 1 lung).

Table 2. Serotype distribution of invasive GBS isolates for cultures collected from infants, Minnesota, USA, January 2000–December 2010*

CPS type	No. (%) patients†		
	Early onset	Late onset	Total
Ia	79 (30.7)	57 (29.7)	136 (30.3)
Ib	25 (9.7)	12 (6.2)	37 (8.2)
II	43 (16.7)	5 (2.6)	48 (10.7)
III	57 (22.2)	95 (49.5)	152 (33.9)
IV	8 (3.1)	4 (2.1)	12 (2.7)
V	39 (15.2)	18 (9.4)	57 (12.7)
VII	2 (0.8)	0 (0.0)	2 (0.4)
Nontypeable	4 (1.6)	1 (0.5)	5 (1.1)
Total	257 (100.0)	192 (100.0)	449 (100.0)

*GBS, group B *Streptococcus*; CPS, capsular polysaccharide serotype.
†Early-onset, patient age birth–6 days; late-onset, patient age 7–180 days.

29.7%) predominated. Serotypes Ia and IV were distributed similarly among EO and LO isolates (Ia, 30.7% of EO vs. 29.7% of LO; IV, 3.1% of EO vs, 2.1% of LO). Serotypes II and III, however, had significantly different distribution in EO and LO disease; serotype II accounted for 16.7% of EO versus 2.6% of LO isolates ($p<0.0001$), whereas serotype III accounted for 22.2% of EO versus 49.5% of LO isolates ($p<0.0001$). Of the 42 isolates from CSF, serotype III accounted for 50%, whether from infants with EO (2/4) or LO (19/38) disease. The 4 isolates from ULOD were 1 each of serotypes Ia, Ib, II, and V (data not shown).

During the 11-year study period, the number of infants with EO or LO invasive disease varied from year to year; most years had more EO than LO cases (Figure 1). This variation resulted in yearly incidence that ranged from 0.21 to 0.45 (mean 0.33) per 1,000 live births for EO and from 0.14 to 0.34 (mean 0.25) per 1,000 live births for LO disease (Figure 2). Although serotype IV was seen only sporadically in previous years (Figure 1), incidence of this serotype increased in 2010, when it was isolated from 5 (16.1%) of 31 infants with EO disease. These 5 cases were not clustered geographically or temporally.

Among the 12 infants who had serotype IV invasive disease during 2000–2010, 11 survived (Table 3). Eight infants had EO disease, all of which were diagnosed within 2 days of birth; only 4 infants, separated temporally, had LO disease. All infants with EO disease were full term (gestational age 37–42 weeks); for LO infants, all but 1 were not full term (gestational age 23–34 weeks). Overall, the proportion of babies born prematurely (<37 weeks) and infected with any GBS serotype was 23.4% (54/231) for EO disease, 47.0% (77/164) for LO disease, and 53.8% (7/13) for delayed LO or ULOD disease (data not shown).

Blood was the most common culture source for serotype IV isolates (11/12); 1 was from a joint fluid. Isolates from all 7 infants who had serotype IV GBS disease during 2000–2009 were susceptible to clindamycin, but 4/5 (80%) isolates from infants who had EO disease in 2010 were clindamycin resistant.

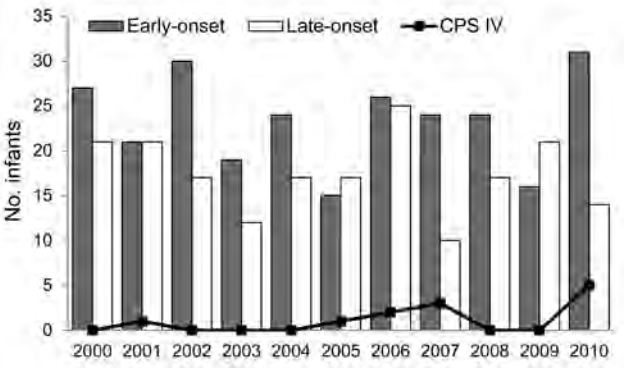

Figure 1. Distribution of early-onset and late-onset invasive group B *Streptococcus* disease in infants, by year, Minnesota, USA, 2000–2010. Bars indicate isolates of all capsular polysaccharide serotypes (CPS); line indicates all serotype IV isolates. A total of 257 infants had early-onset and 192 infants late-onset disease; 12 infants had type IV infection.

Emergence of resistance to clindamycin in serotype IV isolates during 2010 led us to investigate antimicrobial drug resistance for all 45 serotype IV isolates cultured from infants with invasive GBS disease during that year. Antimicrobial drug susceptibility profiles (Table 4) revealed that 14 (31.1%) of these isolates were resistant to clindamycin and erythromycin; clindamycin resistance was inducible for 4 of these 14 isolates. In addition, a higher percentage (≥60%) of isolates of serotypes IV, V, or Ib were clindamycin resistant compared with isolates of the predominant serotypes Ia (8.3%) and III (11.1%). Specifically, the percentage of serotype IV isolates that were clindamycin resistant (4/5, 80%) was significantly higher than for all other serotypes combined (10/40, 25%; $p = 0.027$). In 2010, serotype IV accounted for 28.6% (4/14) of all isolates resistant to clindamycin.

Because of the increase of invasive disease caused by serotype IV GBS, we pursued molecular studies on isolates

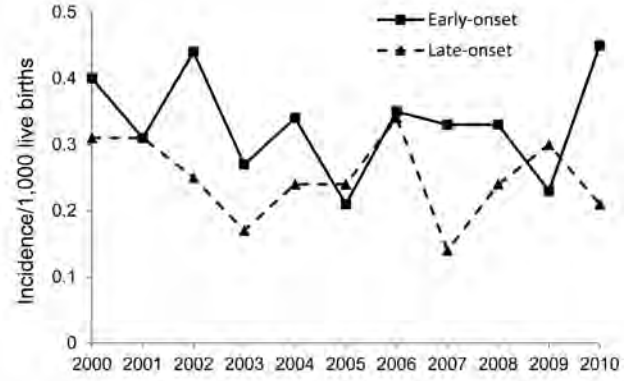

Figure 2. Incidence of early-onset and late-onset group B *Streptococcus* disease per 1,000 live births, by year, Minnesota, USA, 2000–2010.

Table 3. Patient characteristics of and clinical data for serotype IV GBS isolates from infants with invasive GBS disease, Minnesota, USA, January 2000–December 2010*

Period	Disease type†	Patient age at diagnosis	Patient outcome	Culture source	Clindamycin susceptibility‡	Isolate protein profile	Isolate PFGE profile
2001–2009	Late-onset	98 d	Survived	Joint fluid	S	C-α and BPS	37a
	Late-onset	30 d	Survived	Blood	S	C-α and BPS	37
	Late-onset	9 d	Died	Blood	S	BPS	37a
	Early-onset	1 d	Survived	Blood	S	None	40
	Late-onset	9 d	Survived	Blood	S	BPS	37a
	Early-onset	2 d	Survived	Blood	S	BPS	37a
	Early-onset	1 d	Survived	Blood	S	BPS	37a
2010	Early-onset	1 d	Survived	Blood	R	C-α	39a
	Early-onset	Newborn	Survived	Blood	R	C-α	39c
	Early-onset	Newborn	Survived	Blood	R	C-α	39a
	Early-onset	Newborn	Survived	Blood	R	C-α	39a
	Early-onset	Newborn	Survived	Blood	S	BPS	37a

*GBS, group B *Streptococcus*; PFGE, pulsed-field gel electrophoresis; C-α, C-protein α; BPS, group B protective surface protein.
†Early-onset, patient age birth–6 days; late-onset, patient age 7–180 days.
‡S, susceptible, MIC <0.25 μg/mL; R, resistant, MIC ≥1 μg/mL.

of this serotype. Figure 3, panel A, shows the PFGE DNA macrorestriction band patterns of the serotype IV prototype isolates, designated 37, 38, and 39 (*16*), and profiles we classified as 39c and 39a from 2 study isolates expressing only C-α protein. Profiles of 6 isolates with C-α protein and BPS protein or only BPS protein appeared to be identical or very similar and were designated 37 or 37a for their resemblance to the group 37 prototype (Figure 3, panel B). The profile of an isolate that did not express any of the surface proteins studied was unique and was classified as profile 40. Overall, isolates with C-α and BPS proteins or BPS protein alone were in PFGE profile group 37 or its subgroups and were susceptible to clindamycin; those with only C-α protein were in subgroups of PFGE group 39 and were resistant to clindamycin (Table 3).

To investigate further genetic relatedness among serotype IV isolates from invasive GBS disease in Minnesota, we studied 4 isolates from infants and 4 from adults by using MLST (Table 5). We found that the serotype IV prototype strain 3139, studied for comparison, was ST2 in CC1 (data not shown). Among isolates from infants, 1 was ST196, 2 were ST452, and 1 was ST468, a new sequence type (allelic profile 5,25,4,3,2,3,1). Among isolates from adults, 1 was ST196, 2 were ST291, and 1 was ST459. The 5 STs were grouped into 3 major CCs: ST196 and ST459 in CC1, ST291 in CC17, and ST452 and ST468 in CC23. ST468 was a single-locus variant of ST452, which we recently described (*16*), while ST459 was a single-locus variant of the much older ST196. Of the 8 isolates, 1 (PFGE profile 39a, ST459) was resistant to clindamycin (data not shown), as were other isolates in subgroups of PFGE profile group 39 (Table 3). The PFGE profiles of all the serotype IV isolates were classified into 5 groups or their subgroups: 37, 39, and 40 for isolates from infants; 36, 38, and 39 for isolates from adults (Tables 3, 5).

Discussion

As in the rest of the United States and other parts of the world (*8*,*12*,*13*,*27*,*28*), in Minnesota, GBS is one of the leading causes of invasive bacterial infections in infants during the first year of life. Overall, the yearly incidence rate of EO and LO disease in this state decreased modestly from 2000 to 2010; however, even with implementation of control guidelines issued by CDC, some years had an increase in incidence, as occurred for EO disease in 2010.

Our finding that GBS caused invasive disease beyond the third month of life for ≈9% of infants in our study was in keeping with previous reports (*3*). Investigators have found that premature birth was a major risk factor for LO or ULOD GBS disease (*8*,*13*,*28*,*29*). Prematurity was likely a contributing factor for LO disease in infants <90 days of age, in particular for those with delayed LO disease or ULOD. The proportion of infants with these disease types who were born preterm was ≥2× that for infants with EO

Table 4. Clindamycin susceptibility profiles of GBS isolates from 45 infants with invasive GBS disease, Minnesota, USA, 2010*

Antimicrobial susceptibility profile†			CPS serotype						
Clindamycin	Erythromycin	D-test	Ia	Ib	II	III	IV	V	Total
S	S	Not done	7	1	0	13	1	2	24
S	R	Negative	4	0	0	3	0	0	7
S→R	R	Positive	1	1	0	2	0	0	4
R	R	Not done	0	2	1	0	4	3	10
Total no.			12	4	1	18	5	5	45
No. (%) resistant			1 (8.3)	3 (75.0)	1	2 (11.1)	4 (80.0)	3 (60.0)	14 (31.1)

*GBS, group B *Streptococcus*; CPS, capsular polysaccharide serotype.
†S, susceptible, MIC <0.25 μg/mL; R, resistant, MIC ≥1 μg/mL; S→R, inducible resistance to clindamycin indicated by positive D-zone test (double-disk diffusion test).

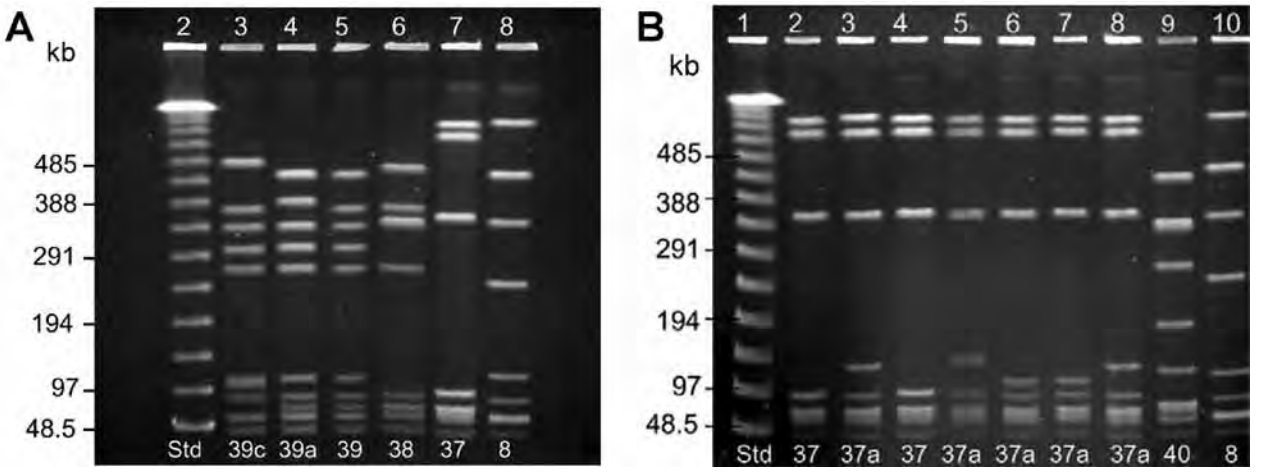

Figure 3. DNA macrorestriction profiles for serotype IV isolates from invasive group B Streptococcus (GBS) disease in infants, Minnesota. Isolates were studied by SmaI digestion and pulsed-field gel electrophoresis (PFGE) analysis and were designated as expressing C-protein α (C-α) or group B protective surface protein (BPS). Lane number is at the top and PFGE profile number at the bottom of each lane. A) Lane 2, λ molecular size standard; lanes 3 and 4, serotype IV/C-α GBS isolates from early-onset disease; lanes 5–7, prototypes of PFGE profile groups 39 (IV/C-α), 38 (IV/C-α), and 37 (IV/C-α and BPS); lane 8, internal standard 89-022 (Ib/C-α and C-β). B) Lane 1, λ molecular size standard; lane 2, PFGE profile 37 prototype (IV/C-α and BPS); lanes 3–6, isolates from late-onset disease; lanes 7–9, isolates from early-onset disease; lane 10, internal standard 89-022. Protein profile of isolates in lanes 2–4, C-α and BPS; lanes 5–8, BPS only. The isolate in lane 9 did not express any of the proteins studied.

disease; in Minnesota, as in other parts of the United States (*30*), ≈75% of infants with EO disease are born at full term.

Our results on the distribution of serotypes from all GBS invasive disease were consistent with studies showing that, worldwide, serotype III continues to predominate, followed by serotypes Ia and V (*27*,*31*). We found that the predominant serotypes in EO disease continued to be Ia, followed by III, with these serotypes in reverse order for LO disease, similar to our previous findings for Minnesota (*15*) and findings for other parts of the country (*9*). However, in marked contrast to the 1990s, when the prevalence of serotype V was 2× that for serotype II (*15*), serotype II was the third most prevalent serotype for EO disease in our study.

Characterization of GBS isolates by serotype over time enabled us to track the emergence and spread of serotype IV as a cause of invasive disease in Minnesota. These results showed that this previously less common serotype has become more common, starting in the mid-1990s, when serotype IV was found in a few adults (2 mothers in the peripartum period and 1 nonpregnant adult) (*15*); subsequently, in 2001, the first case of LO disease caused by serotype IV was found, and in 2010, this serotype became prominent in causing GBS disease. Isolation of this serotype in 2011 from 2 infants with LO disease suggests its continued presence in the community (P. Ferrieri, unpub. data).

Our findings raise the possibility that serotype IV, although reported previously from a case of EO disease in the United States (*32*) and in small numbers from other parts of the world (*6*), has the potential to emerge as a notable cause of invasive GBS disease not only among newborns but also among older infants through the first year of life, just as occurred with serotype V during the past 2 decades (*33*,*34*). The sharp increase in 2010 of disease caused by serotype IV isolates and concurrent emergence of clindamycin resistance within this serotype could foreshadow problems similar to those for serotype V; a high percentage of antimicrobial drug–resistant serotype V isolates (*14*,*26*,*35*) cause disease in infants and older adults (*33*,*36*). We found not only an increase in the percentage of nonpregnant women in the United States colonized with serotype IV in vaginal/rectal sites (*16*) but also evidence of clindamycin resistance, with 3 of 8 representative serotype IV isolates studied found to be resistant (P. Ferrieri, unpub. data). Because the vaginal tract is a reservoir for GBS causing EO disease (*13*,*37*), increased colonization of this site with clindamycin-resistant serotype IV GBS is of concern. Careful attention must be given to the type of intrapartum antimicrobial drug prophylaxis administered; GBS isolates should be assessed for inducible clindamycin resistance when penicillin, ampicillin, or cefazolin cannot be used.

Among the serotype IV isolates from invasive GBS disease, we observed association among PFGE profile, surface protein profile, and susceptibility/resistance to clindamycin. Isolates with only BPS protein or C-α and BPS proteins were in PFGE group 37 and susceptible to clindamycin; those with only C-α protein were in group 39 or its subgroups and resistant to clindamycin. Results from a study of serotype IV isolates from colonized nonpregnant women living in various areas in the United States showed a similar association between surface protein profile and

Table 5. Molecular characteristics of serotype IV GBS isolates causing invasive disease in infants and adults, Minnesota*

Isolate source†	PFGE profile	Allelic profile‡	Sequence type§	Clonal complex¶
Mother, early-onset	38d	1,1,3,1,1,12,2	196	1
Infant, early-onset	40	1,1,3,1,1,12,2	196	1
Nonpregnant adult	39a	1,1,3,1,41,12,2	459	1
Mother, early-onset	36c	2,25,1,2,1,1,1	291	17
Nonpregnant adult	36d	2,25,1,2,1,1,1	291	17
Infant, late-onset	37	5,25,4,3,2,3,3	452	23
Infant, late-onset	37a	5,25,4,3,2,3,3	452	23
Infant, early-onset	37a	5,25,4,3,2,3,1	468	23

*GBS, group B *Streptococcus*; PFGE, pulsed-field gel electrophoresis.
†Early-onset, patient age birth–6 days or mother during peripartum period; late-onset, patient age 7–180 days.
‡Multilocus sequence type of 7 housekeeping genes (*adhP, pheS, atr, glnA, sdhA, glcK, tkt*).
§From http://pubmlst.org/sagalactiae database.
¶Determined by eBurst analysis (*25*).

PFGE profile (*16*). MLST results suggested a possible association between clindamycin resistance and ST459.

In contrast to colonizing serotype IV isolates, which have limited clonal diversity (*16*), results from our molecular studies of invasive serotype IV isolates showed that the 16 isolates cultured since 1995 spanned 5 PFGE profiles, 5 STs, and 3 CCs, which suggests greater genetic diversity. Among 97 colonizing serotype IV isolates from a wider area of the United States, we found 3 PFGE profile groups (37–39) and 2 STs (452 and 459) (*16*); in contrast, among just 16 invasive isolates in this study, we found the same PFGE profiles and STs and 2 additional PFGE profile groups (36 and 40) and STs (196 and 468). In other MLST studies of GBS isolates (*4–6,14,38,39*), only small numbers of serotype IV have been included, and these have most often been placed in CC1 and a few in CC17, 2 CCs with STs seen in our study. The fact that 3 of our serotype IV isolates, particularly ST459, were in CC1 may be of importance because a large percentage of serotype V isolates, many of them antimicrobial drug resistant, have been placed in this CC (*4,14*). Two of our invasive isolates were in CC17 and 3 in CC23; these are well-known CCs that contain many isolates of various serotypes from invasive disease in infants and adults (*38,39*) or that are associated with virulent clones (*5,6,38*). ST452 and ST459 are new STs from women colonized with serotype IV (*16*).

The emergence of invasive serotype IV GBS disease in Minnesota underscores the value of monitoring prevalent serotypes in a community to detect new epidemiologic trends. In an era when antimicrobial drug prophylaxis is used during childbirth, these findings also highlight the need to be aware of antimicrobial drug–resistant isolates of all serotypes (*26,35*). Although maternal immunization with conjugate vaccines incorporating the most common GBS serotypes remains one of the most promising strategies for disease prevention (*27,40*), continued assessment of this and other approaches is essential (*8,13,30,37*).

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Transmission of Hepatitis E Virus from Rabbits to Cynomolgus Macaques

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The recent discovery of hepatitis E virus (HEV) strains in rabbits in the People's Republic of China and the United States revealed that rabbits are another noteworthy reservoir of HEV. However, whether HEV from rabbits can infect humans is unclear. To study the zoonotic potential for and pathogenesis of rabbit HEV, we infected 2 cynomolgus macaques and 2 rabbits with an HEV strain from rabbits in China. Typical hepatitis developed in both monkeys; they exhibited elevated liver enzymes, viremia, virus shedding in fecal specimens, and seroconversion. Comparison of the complete genome sequence of HEV passed in the macaques with that of the inoculum showed 99.8% nucleotide identity. Rabbit HEV RNA (positive- and negative-stranded) was detectable in various tissues from the experimentally infected rabbits, indicating that extrahepatic replication may be common. Thus, HEV is transmissible from rabbits to cynomolgus macaques, which suggests that rabbits may be a new source of human HEV infection.

Hepatitis E virus (HEV) is the causative agent of acute hepatitis E, which is endemic to many developing countries and occurs sporadically in some industrialized countries. HEV is a small nonenveloped virus with a positive-sense single-stranded RNA genome of ≈7.2 kb; it is currently classified as the sole member of the genus *Hepevirus*, family *Hepeviridae* (*1*). Thus far, at least 4 genotypes, which comprise a single serotype, of HEV have been identified in mammals: genotypes 1 and 2 are restricted to strains that infect humans, and genotypes 3 and 4 are zoonotic (*2*). More recently, a putative fifth HEV genotype was identified in wild boars in Japan (*3*). HEV from chickens, which is phylogenetically distinct from HEV from mammals, is likely to be classified as a new genus within the family *Hepeviridae* (*4*).

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The zoonotic nature of HEV was first confirmed in 1997 with the identification of HEV isolates in swine in the United States, which were most closely related to an isolate of HEV from a person in the United States, and this isolate could experimentally infect nonhuman primates (*5*,*6*). Zoonotic transmission of HEV was further substantiated with the demonstration of HEV infection in persons after they ate undercooked infected meat from wild boars and wild deer (*7*,*8*). Antibodies against HEV have been detected in numerous animal species, including dogs, cats, sheep, goats, horses, cattle, bison, and rats; and HEV strains have been genetically identified from domestic and wild pigs, chickens, deer, mongooses, and rabbits (*4*,*9*). The recent discoveries of HEV-like viruses in rats and fish have further broadened understanding of the host range and diversity of HEV (*10*–*12*).

The first strain of rabbit HEV was isolated from Rex Rabbits on 2 rabbit farms in Gansu, People's Republic of China (*13*). Additional studies indicated that rabbit HEV was prevalent among various breeds of farmed rabbits throughout much of China, and the prevalence of antibodies against HEV was 57.0% in Lanzhou and 54.6% in Beijing (*13*–*15*). Rabbit HEV has also been isolated from rabbits in Virginia, USA, which showed a high prevalence of antibodies against HEV (36%) and HEV RNA (16.5%) (*16*). Phylogenetic analyses revealed that rabbit HEV was most closely related to genotype 3 HEV, which has been confirmed to infect humans. Furthermore, a recent study indicated that rabbit HEV is antigenically related to the other known animal strains of HEV and is experimentally transmissible to swine (*17*). However, to our knowledge, no study had determined the zoonotic potential of rabbit HEV. Therefore, in this study, we endeavored to ascertain whether rabbit HEV can cross species barriers and infect nonhuman primates and to further clarify the pathogenesis and replication of rabbit HEV in its natural host.

[1]These authors contributed equally to this article.

Materials and Methods

Virus Inocula

The rabbit HEV strain (CHN-BJ-R14) used in this study was originally recovered from the feces of a farmed Rex Rabbit in the suburbs of Beijing in 2011. The fecal sample was diluted in phosphate-buffered saline (PBS) (pH 7.4) containing 1% bovine serum albumin to make a 10% (wt/vol) suspension. The clarified suspension was subsequently filtered through 0.45-μm and 0.22-μm filters. Titers of the rabbit HEV inoculum were determined by a semiquantitative nested reverse transcription PCR (RT-nPCR) (*18*), and the titer was 10^4 genome equivalents (GE) per milliliter (mL).

Animals

Two juvenile male cynomolgus monkeys (*Macaca fascicularis*), weighing 2.0–2.5 kg, designated as Cy1 and Cy2, were obtained from the Beijing Xierxing Institute of Biologic Resources (Beijing, China) for the cross-species infection study. For the rabbit infection study, four 7-week-old specific-pathogen free (SPF) New Zealand white rabbits, weighing 750–1,000 g, were obtained from the Department of Laboratory Animal Science of Peking University Health Science Center. Preinoculation serum and feces specimens were collected once a week for 3 weeks, and all animals were tested for alanine aminotransferase (ALT) to establish a baseline, and were confirmed as negative for antibodies against HEV by an ELISA and negative for HEV RNA by RT-nPCR. The animal experiments were approved by the Committee of Laboratory Animal Welfare and Ethics, Peking University Health Science Center. The regulations of the review committee of Laboratory Animal Welfare and Ethics and the protocol for the review on Laboratory Animal Welfare and Ethics, Peking University Health Science Center, were followed.

Experimental Inoculation of Nonhuman Primates

To determine whether rabbit HEV strains are transmissible to nonhuman primates, we inoculated intravenously 2 cynomolgus monkeys, housed separately, with 2 mL of the rabbit HEV inoculum. After inoculation, serial serum and fecal samples were collected 2×/week for 16 weeks.

Serum samples were tested for ALT levels and for IgM and IgG against HEV. All samples were also assayed for HEV RNA by RT-nPCR (*15*).

Experimental Infection of Rabbits

To clarify the extrahepatic replication sites of HEV, rabbits were experimentally infected with rabbit HEV as described (*19*). In brief, 4 SPF rabbits, which were housed in separate cages, were divided randomly into 2 groups (2 rabbits per group) and inoculated intravenously with either 1 mL of PBS (negative control) or 1 mL of rabbit HEV inoculum. Serum and fecal specimens were collected weekly after inoculation. Serum samples were tested for ALT activity and HEV RNA. Fecal specimens were also assayed for HEV RNA. If serum and fecal specimens became simultaneously positive for HEV RNA, a complete necropsy was performed of each rabbit. Bile and various different types of tissues and organs, including liver, kidney, small intestine, spleen, stomach, heart, brain, bladder, and lung, were collected and stored at −80°C. To prevent cross-contamination during necropsy, we used individually wrapped, sterile disposable materials and new sterile scalpel blades for each sample.

Approximately 100 mg of each tissue and organ was homogenized in 1 mL of sterile PBS (pH 7.4) to make 10% (wt/vol) suspensions and clarified by centrifugation at 4,500 *g* for 10 min at 4°C. Thereafter, 100 μL of the clarified supernatants was used for total viral RNA extraction, and positive-stranded and negative-stranded HEV RNA were detected by RT-nPCR as described below.

Determination of ALT Levels

All serum samples were tested immediately for ALT levels with a Hitachi Automatic Clinical Analyzer 7180 (Hitachi High-Technologies, Tokyo, Japan), by using chemical reagents purchased from Roche (Basel, Switzerland), according to the manufacturer's instructions. Biochemical evidence of hepatitis was recorded when the serum ALT level exceeded the baseline ALT level by >2-fold, as defined by a peak ALT value that was equal to or greater than double the prechallenge values (*19*,*20*).

ELISA to Detect Antibodies against HEV

The serum specimens collected from monkeys were tested for IgM and IgG against HEV by using an ELISA based on the virus E2 protein (amino acids 394–606 of HEV open reading frame [ORF] 2) (*20*), according to the manufacturer's instructions (Wantai, Beijing, China). The serum samples collected from rabbits were also examined for antibodies by using the same assay. Signal-to-cutoff values were calculated, and values >1 were considered positive. Preinoculation baseline serum specimens were used as negative controls for each monkey.

RT-nPCR to Detect Positive-stranded and Negative-stranded HEV RNA

RNA was extracted from 100 μL of serum, bile, tissue suspension, or 10% fecal suspension by using TRIzol reagent (Invitrogen, Burlington, ON, Canada), and purified RNA was resuspended in 11 μL of RNase-free water. To detect positive-stranded HEV RNA, 11 μL of purified

RNA was reverse transcribed at 42°C for 60 min with SuperScript II reverse transcription (Invitrogen) and the external reverse primer P4 or S4 in a reaction mixture of 20 μL. Then, nested PCRs were carried out to amplify the partial fragments of ORF1 (129–373 nt) and ORF2 (5,983–6,349 nt) of the HEV genome by using the 2 sets of specific external and internal primer pairs listed in online Technical Appendix Table 1 (wwwnc.cdc.gov/EID/article/19/4/12-0827-Techapp1.pdf). The PCR parameters for both sets of primers and both rounds of PCR were the same, with an initial incubation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 40 s, with a final incubation at 72°C for 10 min.

Tissues with detectable positive-stranded HEV RNA were then assayed for negative-sense HEV RNA by RT-nPCR with the same 2 sets of universal primers (online Technical Appendix Table 1). The extracted RNA was subjected to cDNA synthesis with the external forward primer P1 or S1. Then parental RNAs were degraded by RNaseH, and this was followed by nested PCR. The amplification conditions for negative-stranded HEV RNA detection were essentially the same as those used in the detection of positive-sense HEV RNA.

The PCR protocol used in this study could detect as few as 10 GE copies of HEV plasmid DNA. Negative and positive controls were included in each assay to exclude the possibility of contamination and failure of amplification. A recombinant plasmid containing HEV ORF1 and ORF2 fragments at a concentration of 10^2 copies per mL and serum or fecal specimens or tissues from naive rabbits were used as positive and negative controls, respectively. Samples showing a band of the expected size on a 1.5% (w/v) agarose gel were considered positive, and the positive products were directly sequenced.

Amplification of the Full-Length Genome of Rabbit HEV

To compare the complete genome sequence of the HEV passed in the macaques to that of the inoculum, the fecal sample (rHEV-Cy1) of 1 monkey at 3 weeks' postinoculation (wpi) and the inoculum (CHN-BJ-R14) were sequenced to determine the full-length genome as reported (*21*). Briefly, total RNA was extracted from 120 μL of the rabbit HEV inoculum and a 10% monkey fecal suspension in PBS by using the Total RNA Isolation System (Promega, Madison, WI, USA). cDNA was synthesized from 12 μL of purified RNA by using 1 μL (200 U) of Moloney murine leukemia virus reverse transcription (Promega) and 2 μL (10 pmol/L) of OligodT primer. With 6 sets of specific external and internal primer pairs (online Technical Appendix Table 2), a set of nested PCRs were performed by using the first-strand cDNA to amplify the entire viral genome. The nested PCR was done as described (*21*). The nucleotide sequences at the 5′ and 3′ termini of the genome were determined by using a rapid amplification of cDNA ends (RACE) kit (Invitrogen), according to the manufacturer's instructions.

Sequence Analyses

The expected PCR products amplified from the inoculum and monkey fecal sample at 3 weeks wpi were purified and ligated into a pGEM-T vector (Promega). At least 3 positive clones for each region of the viral genome were sequenced commercially in both directions by using an automated DNA sequencer (ABI model 3730 sequencer; Applied Biosystems, Foster City, CA, USA).

Nucleotide sequences were assembled and analyzed with the MEGA 4.0 and ALIGNX software (Vector NTI package version 9.0; Invitrogen). ORFs were identified by using the EMBOSS software (version 5.0.0; emboss.sourceforge.net). The full-length genomic sequences of CHN-BJ-R14 and rHEV-Cy1 reported in this study have been deposited in GenBank under accession nos. JX109834 and JX121233, respectively.

Results

Cross-Species Transmission of Rabbit HEV to Nonhuman Primates

In both of the macaques inoculated with rabbit HEV, hepatitis developed, as determined on the basis of ALT elevation, viremia, fecal shedding of viruses, and seroconversion (Figure). Dramatic elevations in serum ALT were observed 5 and 10 wpi for both monkeys, with a peak value of 135 U/L at 9 wpi for monkey Cy1 and 97 U/L at 5.5 wpi for monkey Cy2.

Before inoculation, both monkeys were seronegative for HEV and became seropositive for antibodies against HEV at 6–7 wpi. IgM against HEV was detectable from 7 to 12 wpi for Cy1 and from 6 to 8 wpi for Cy2. The rise in IgM against HEV was followed closely by a strong response of IgG against HEV for Cy1, whereas both responses occurred at about the same time for Cy2. The IgG level against HEV remained markedly elevated at the end of the 16-week experiment.

Serum and fecal samples taken before inoculation from both monkeys were negative for HEV RNA. Viremia and fecal shedding of viruses were detected in both monkeys after intravenous inoculation. Fecal excretion of rabbit HEV, indicative of replication, was first detected at 1 wpi and persisted for 5–9 weeks. HEV viremia was first detected at 5.5 wpi for Cy1 and at 2 wpi for Cy2 and lasted for 2.5–3.5 weeks. The partial sequences of the PCR products from both monkeys shared 99%–100% nucleotide identity with the original inoculum.

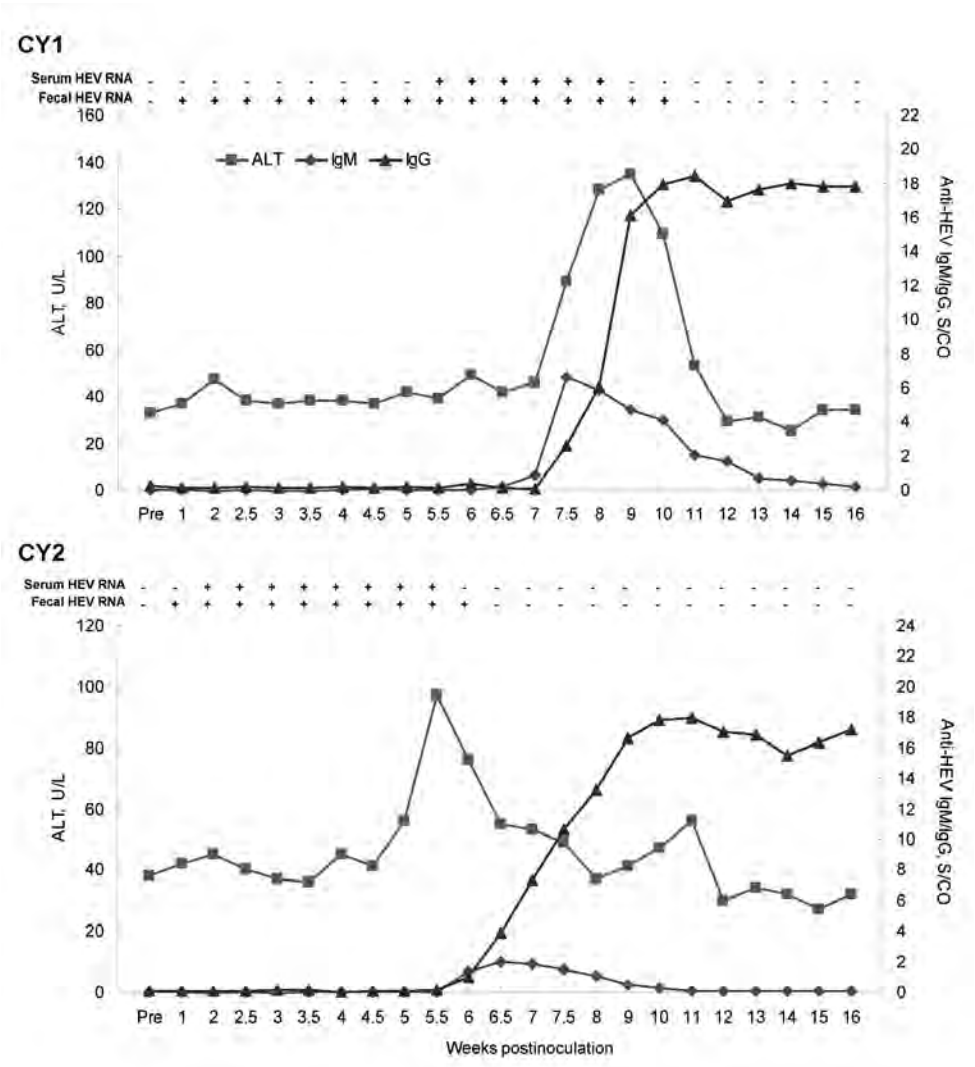

Figure. Cross-species transmission of rabbit hepatitis E virus (HEV) to 2 cynomolgus macaques (Cy1 and Cy2). Alanine aminotransferase (ALT) levels are plotted as U/L. The baseline ALT levels were 33 U/L and 38 U/L for Cy1 and Cy2, respectively. The titers of HEV IgM and IgG are plotted as ELISA signal-to-cutoff (S/CO) values. Presence and absence of HEV RNA in serum or feces are indicated by + and – signs, respectively.

Sequence Analyses of Rabbit HEV during Cross-Species Transmission

To analyze mutations in the rabbit HEV genome that appeared during a single passage between the 2 different host species, we sequenced rabbit HEV strains recovered from the inoculum (CHN-BJ-R14) and from experimentally infected cynomolgus monkeys (rHEV-Cy1) over the entire genome. The CHN-BJ-R14 and rHEV-Cy1 isolates had the same genomic length of 7,284 nt, excluding the 3′ poly (A) tail, and contained 3 ORFs—ORF1, ORF2, and ORF3—which encoded proteins of 1,722 aa (nt 26–5194), 660 aa (nt 5232–7214), and 113 aa (nt 5221–5562), respectively. The 5′ untranslated region (UTR) and 3′ UTR comprise 25 nt and 71 nt, respectively. Sequence analyses showed that CHN-BJ-R14 and rHEV-Cy1 shared 99.8% nucleotide identity with each other. Comparison of the complete genome sequence of rabbit HEV passed in the macaques (rHEV-Cy1) with that of the inoculum (CHN-BJ-R14) revealed 18 nt mutations over the entire genome, resulting in 9 nonsynonymous amino acid changes. ORF1 harbored 16 of the 18 nt mutations; 11 were in the helicase domain and in the RNA-dependent RNA polymerase domain (Table).

Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis showed that CHN-BJ-R14 and rHEV-Cy1 were most closely related to genotype 3 HEV with a maximum nucleotide identity of 81%, with the exception of 3 other rabbit HEV strains isolated in Gansu (*13*) and Beijing (*21*). However, several unique features possessed only by rabbit HEVs, but not genotype 3 or other HEV strains, were observed in the 2 rabbit HEV isolates of this study. These features, discovered in a previous study (*21*), were characterized by an insertion of 31 aa in ORF1 (929–959 aa) and a unique A residue at nt 13 (sites based on CHN-BJ-R14) in the 5′ UTR (data not shown).

Extrahepatic Replication of HEV in Experimentally Infected Rabbits

Both control rabbits remained negative for HEV RNA throughout the study. Viremia and fecal shedding of HEV were detected in rabbits inoculated with the rabbit HEV inoculum. Both rabbits were necropsied, at 5.5 wpi and 12 wpi, respectively, when ALT elevation was observed, and HEV RNA was detected simultaneously in serum and feces. Bile and 9 different types of tissues and organs were collected and tested for positive-stranded HEV RNA. Positive-stranded HEV RNA was detected in bile and in 5 of the tissues—liver, kidney, small intestine, spleen, and stomach. Detection of positive-stranded HEV RNA from various tissues and organs did not indicate that the virus was replicating in these tissues because contamination of the tissue samples by virus circulating in the blood could not be ruled out. To further identify the replicating sites of HEV, we screened for negative-stranded RNA, which is an intermediate product during HEV replication, in all tissues that were positive for the positive-stranded HEV RNA. Negative-stranded RNA was also detectable in the 5 types of tissues. The positive products were sequenced and found to be identical to the original inoculum.

Discussion

Since the first animal strain of HEV, swine HEV, was identified from a pig in the United States in 1997 (*5*), the increasing identification of HEV infection among a wide range of animals, including pigs, chickens, wild boar, and deer (*4*), has raised public health concern for zoonoses and food safety (*22*,*23*). The recent discovery of rabbit strains of HEV in China (*13*) and the United States (*16*) showed that farmed rabbits are another key reservoir of HEV. In our previous study, phylogenetic analysis of the genome of rabbit HEV suggested the potential for cross-species transmission of rabbit HEV (*21*). A recent study also demonstrated that rabbit HEV can cross species barriers and infect SPF pigs (*17*). In the study described here, we showed that under experimental conditions, rabbit HEV is transmissible to cynomolgus macaques, which can serve as surrogates for humans. This finding suggests that rabbit HEV may be a new source of human HEV infection.

In both cynomolgus monkeys infected in this study with 10^4 GEs of rabbit HEV, typical acute hepatitis E developed. The patterns of HEV infection in cynomolgus monkeys infected with rabbit HEV were similar to those of animals inoculated with HEV strains of genotypes 1–4, that is, characterized by fecal excretion of virus, followed by viremia and liver enzyme elevation and finally by seroconversion (*24*–*27*). Although the same viral doses were inoculated into both monkeys, the overall course of disease varied somewhat, findings in accord with those of previous studies (*28*). In an earlier study, cross-species infection of pigs infected with rabbit HEV showed a delayed onset and short duration of viremia and fecal virus shedding and an absence of seroconversion (*17*), which differed from findings observed in infected monkeys of this study. The differences might suggest that pigs are less susceptible than nonhuman primates to rabbit HEV. However, because the inocula in both the current study and in other studies (*17*,*19*)

Table. Comparison of the complete genome sequence of rabbit HEV passed in macaques with that of the inoculum*

		Nucleotide		Amino acid	
Nucleotide position†	Genomic region	CHN-BJ-R14‡	rHEV-Cy1§	Position†	Substitution
614	ORF1-MeT	C	T	197	Silent
957	ORF1-Y	T	C	311	Thr to Ile
1667	ORF1-PCP	T	C	548	Silent
1875	ORF1	T	C	617	Pro to Leu
2706	ORF1-X	G	A	894	Asp to Gly
3553	ORF1-Hel	A	T	1176	Silent
3571	ORF1-Hel	C	T	1182	Silent
3859	ORF1-RdRp	C	A	1278	Silent
3889	ORF1-RdRp	C	T	1288	Silent
3972	ORF1-RdRp	G	A	1316	Glu to Gly
4215	ORF1-RdRp	C	T	1397	Leu to Pro
4285	ORF1-RdRp	A	G	1420	Silent
4414	ORF1-RdRp	T	C	1463	Silent
4427	ORF1-RdRp	C	T	1468	Tyr to His
4882	ORF1-RdRp	T	C	1619	Silent
5028	ORF1-RdRp	T	C	1668	Ala to Val
5531	ORF2	C	T	100	Silent
	ORF3	C	T	104	Ala to Val
5713	ORF2	T	A	161	Ile to Asn

*HEV, hepatitis E virus; ORF, open reading frame; Thr, Threonine; Ile, Isoleucine; Pro, proline; Leu, leucine; Asp, aspartic acid; Gly, glycine; Glu, glutamic acid; Tyr, tyrosine; His, histidine; Ala, alanine; Val, valine; Asn, asparagine.
†Nucleotide or amino acid position according to the rabbit HEV CHN-BJ-R14 strain.
‡CHN-BJ-R14, HEV isolate recovered from the rabbit HEV inoculum in this study.
§rHEV-Cy1, HEV isolate recovered from the fecal sample of 1 monkey at 3 wpi in this study.
¶Putative domains in ORF1. MeT, methyltransferase domain; Y, Y domain; PCP, papain-like cysteine protease domain; X, X or macro domain; Hel, helicase domain; RdRp, RNA-dependent RNA polymerase domain.

have not yet been titrated for infectivity and because HEV infections are virus dose dependent (*18*), additional studies should be performed to determine the infectivity titer of rabbit HEV and to demonstrate whether the rate of inducing hepatitis increases with virus dose of infection.

In the current study, although comparison of the full-length sequences of rHEV-Cy1 and CHN-BJ-R14 showed 99.8% nucleotide identity, 18 nt changes, resulting in 9 nonsynonymous amino acid substitutions, were found in the genome of HEV. These results suggest that adaptation of rabbit HEV to growth in cynomolgus monkeys may be associated with a certain number of mutations. Eleven of the 16 mutations fell within ORF1, accompanied by 4 nonsynonymous substitutions, mapped to the helicase region and the RNA-dependent RNA polymerase region, which are essential for efficient replication of the genomes of HEV (*29*). Moreover, although most mutations are expected to be in the third codon position, of the 16 substitutions in ORF1, 7 occur at the first codon position and 3 at the second codon position. These facts may indicate that positive selection is operating in the infection of the cynomologus monkeys with the rabbit HEV inoculum. A recent study revealed that high-throughput sequencing of isolates from bile and feces from 2 pigs experimentally infected with human HEV of genotype 3f shared the same full-length consensus sequence as in the human sample, although a limited spectrum of mutations were observed during the interspecies transmission (*30*). The genomic sequences in this study were determined by sequencing several randomly selected positive clones, which is much less extensive than high-throughput sequencing; consequently, additional studies will be needed to verify whether the sequence changes that occurred after cross-species transmission of rabbit HEV to cynomolgus monkeys are adaptive mutations or result from the quasispecies structure of HEV.

Previous data from studies performed with pigs infected with human and swine HEV indicated that HEV can replicate in tissues and organs other than the liver (*31*). Recently, extrahepatic manifestations associated with HEV infection, including neurologic disorders (*32*) and acute pancreatitis (*33*), also suggested that HEV could replicate in extrahepatic tissues. The discovery of rabbit HEV opened a new avenue for the study of HEV replication and pathogenesis. Rabbits were used as an animal model to study the extrahepatic replication of HEV in this study. Positive-stranded HEV RNA was detected in the liver, bile, kidney, small intestine, spleen, stomach, serum, and feces from experimentally infected rabbits. Furthermore, negative-stranded HEV RNA, indicative of replication, was also discovered in the same tissues, which provided additional evidence for extrahepatic replication of HEV in its natural host. Considering the extrahepatic replication of HEV found in this study and the other reports of extrahepatic manifestations of HEV infection in humans (*34*), clinicians should consider the possibility of HEV infection in patients with nonhepatic diseases, especially patients with acute pancreatitis, neurologic syndromes, thrombocytopenia, hemolysis, and autoimmune manifestations.

In conclusion, the successful infection of cynomolgus macaques with rabbit HEV suggests that humans might be at risk for infection with rabbit HEV. Further, rabbit HEV was detectable in multiple rabbit tissues and organs, indicating extrahepatic replication may be a common feature of rabbit HEV. These findings raise additional concern for zoonotic transmission of HEV infection among persons who have occupational exposure to rabbits or persons who eat undercooked rabbit meat. Future studies should be conducted to investigate rabbit HEV infection in human populations and assess whether close contact with rabbits is a risk factor for HEV infection.

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Description and Nomenclature of *Neisseria meningitidis* Capsule Locus

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Pathogenic *Neisseria meningitidis* isolates contain a polysaccharide capsule that is the main virulence determinant for this bacterium. Thirteen capsular polysaccharides have been described, and nuclear magnetic resonance spectroscopy has enabled determination of the structure of capsular polysaccharides responsible for serogroup specificity. Molecular mechanisms involved in *N. meningitidis* capsule biosynthesis have also been identified, and genes involved in this process and in cell surface translocation are clustered at a single chromosomal locus termed *cps*. The use of multiple names for some of the genes involved in capsule synthesis, combined with the need for rapid diagnosis of serogroups commonly associated with invasive meningococcal disease, prompted a requirement for a consistent approach to the nomenclature of capsule genes. In this report, a comprehensive description of all *N. meningitidis* serogroups is provided, along with a proposed nomenclature, which was presented at the 2012 XVIIIth International Pathogenic Neisseria Conference.

Thirteen *Neisseria meningitidis* serogroups have been described on the basis of serologic differences of the capsule; of these 13 serogroups, 6 (A, B, C, W, X, Y) cause invasive meningococcal disease. The polysaccharide capsule is a key virulence determinant, and for serogroups A, C, W, and Y, it forms the basis of polysaccharide conjugate vaccines. In one of the first reports distinguishing *N. meningitidis*, disease isolates were serologically classified into types I–IV on the basis of agglutination reactions with immune rabbit serum (*1*). In 1950, the subcommittee on *Neisseria* of the Nomenclature Committee of the International Association of Microbiologists recommended that types I and III be combined into serogroup A; type II become serogroup B; a type II subgroup, termed type II-α, become serogroup C; and type IV become serogroup D. After the report of a fourth serogroup, Z′ (later shown to be serogroup E), 3 new serogroups (X– Z) were identified by using double agar diffusion (*2*,*3*). In 1981, three more serogroups (H, I, K) were proposed, and a fourth (serogroup L) was identified in 1983 (*4*,*5*).

Nuclear magnetic resonance spectroscopy enabled determination of the structure of capsular polysaccharides responsible for serogroup specificity, and structures for 12 of the 13 serogroups (all but serogroup D) from *N. meningitidis* capsular polysaccharides have been reported (*6*–*15*). Molecular mechanisms of capsular polysaccharide synthesis have been elucidated; genes involved in polysaccharide biosynthesis and cell surface translocation are clustered at a single chromosomal locus termed *cps*. Genes within this locus are divided into 6 regions: A–D, D′, and E (*16*). Genes in region A encode enzymes for biosynthesis of the capsular polysaccharide, and genes in regions B and C are implicated in the translocation of the high molecular weight polysaccharides to the cell surface.

Complete nucleotide sequences of *cps* loci encoding serogroups A–C, W, and Y have been elucidated. Serogroup-specific capsule biosynthesis genes located in region A have been published for serogroup X, and

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nucleotide sequences for serogroups E, L, and Z have been submitted to GenBank (accession nos. AJ576117, AF112478, and AJ744766, respectively) (*17*–*19*). This study provides a comprehensive description of all *N. meningitidis* serogroups and presents proposed revisions to the nomenclature.

Materials and Methods

Strain Selection

Serogroup D, H, I, and K isolates were obtained from a collection maintained at the National Institute for Biological Standards and Controls, Potters Bar, UK; the isolates were originally from a collection (1980s) from the People's Republic of China Committee for Culture Collection of Microorganisms. Serogroup E, W, X, and Y isolates were from the Bavarian *N. meningitidis* carriage collection (*20*). Three serogroup L isolates were analyzed with additional serogroup H, I, and K isolates obtained from Paula Kriz (Czech Republic), who had obtained the isolates from Fraser Ashton, who had acquired them from People's Republic of China (Table 1). The sequenced genomes from serogroup A isolate Z2491, serogroup B isolate H44/76, and serogroup C isolates FAM18 and 053442 were used (Table 1) (*21*–*24*). Isolates were grown on Mueller-Hinton agar supplemented with 5% (vol/vol) defibrinated sterile horse blood for 15 h at 37°C in a 5% (vol/vol) CO_2 atmosphere. DNA was extracted by using the Wizard Genomic DNA Purification Kit (Promega, Southampton, UK) according to the manufacturer's instructions.

PCR and Nucleotide Sequencing of the Capsule Locus

The genes for the *N. meningitidis cps* locus are located between a gene encoding a putative inner membrane transport protein (NMC0044 FAM18 genome annotation) and a gene encoding the sodium/glutamate symport carrier protein, *gltS* (NMC0069). PCR reactions were performed for isolates 29013, α707, 29031, 29043, 29046, 3608, α275, α388, α162, WUE171, WUE172, and WUE173 (Table 1) by using the Expand Long Template PCR System (Roche Applied Science, Burgess Hill, UK) according to the manufacturer's recommended protocol, with annealing at 55°C and extension at 68°C for 20 min. Initial reactions used 1 of the following primer pairs: *gltS* (NMC0069) to *tex* (NMC0059) (primers *gltS* 5′-CCGACCAAGCCGTATTGC + ATGATACTCGAAGGCGTGGTT-3′ and *tex* 5′-TGTCGAAGCCGTCCATAATCT + GCCCTGTCCAACAAGTTCGT-3′) and *tex* to NMC0044 (primers *tex* 5′-CGCCCGGTTCGTCATCC + TTGCTGCTGGTAGGCGAATCC-3′ and NMC0044 5′-CGGGCGAACACGGTAAT + TATCGTTGGTGCGCTGGTTAT-3′).

Genome Sequencing

Genomic data for serogroups D, H, I, K, and L were obtained by using the Illumina sequencing platform and de novo assembly (Illumina, San Diego, CA, USA), using the shuffle and Velvet Optimization scripts found within Velvet 1.1.03 (*25*) (Table 1). By using VelvetOptimiser version 2.1.7 (http://bioinformatics.net.au/software.velvetoptimiser.shtml), optimal k-mer lengths ranging from 41 to 65 were selected and contigs were generated. These contigs were deposited in the Bacterial Isolate Genome Sequence Database ($BIGS_{DB}$) along with the isolate provenance and name, after which contigs were scanned for genes within the *cps* locus and tagged and alleles were assigned (*26*). New alleles were manually checked for a correct start and stop codon and aligned with known alleles before assignation. Additional data (e.g., multilocus sequence type [MLST]), and PorA, PorB, and FetA designations were obtained.

Annotation and Bioinformatic Methods

Predicted proteins were clustered into homology groups by using TribeMCL algorithm with a cutoff of 1e-70 (http://doc.bioperl.org/bioperl-run/lib/Bio/Tools/Run/TribeMCL.html#General). The genes within the *cps* loci that encoded proteins with the same homology group were assigned the same name, exceptions being those genes found in Region A. BLASTp (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) searches were done by using a reference sequence database with default settings.

Annotation was performed by using the genome viewer Artemis (*27*). The *cps* nucleotide sequence locus for each serogroup was stored in $BIGS_{DB}$ and linked with the corresponding isolate record.

MEGA5 (http://megasoftware.net/) was used to calculate overall *p*-distances and G+C content and to obtain the number of polymorphisms observed within each gene. The *cps* loci were compared by using the Artemis Comparison Tool (www.sanger.ac.uk/resources/software/act/).

Results and Discussion

General Organization

All of the serogroups examined had comparable *cps* loci, and regions occurred in the following order D-A-C-E-D′-B, revealing a conserved gene synteny and a replication of the genomic rearrangements (Figure). Genes in regions B–D, D′ and E were conserved, whereas genes in region A were diverse; this finding is consistent with the distinct biochemical composition found within each serogroup (Table 2).

The lowest GC content was in regions A and C, indicative of genes in these regions resulting from horizontal recombination (Table 2; Table 3, Appendix, wwwnc.cdc.gov/EID/article/19/4/11-1799-T3.htm); regions B, D, and E displayed GC contents of 50%, 52%, and 52%,

Table 1. Description of *Neisseria meningitidis* isolates*

Isolate name	Serogroup	Origin	Disease status	Strain designation	GenBank accession no.
N. meningitidis Z2491	A	The Gambia	Invasive	A:P1.7, 13–1: F1–5: ST-4 (cc4)	AL157959
N. meningitidis H44/76	B	Norway	Invasive	B:P1.7,16: F3–3: ST-32 (cc32)	CP002420
N. meningitidis FAM18	C	United States	Invasive	C:P1.5,2: F1–30: ST-11 (cc11)	AM421808
N. meningitidis 053442	C	PRC	Invasive	C:P1.7–2,14: F3–3: ST-4821 (cc4821)	CP000381
N. meningitidis 29013	D	PRC	Unknown	C:P1.22,14–6: F3–16: ST-8723 (cc213)	ERR028660
N. meningitidis α707	E	Germany	Carrier	E:P1.5–1,2–2: F4–3: ST-254 (cc254)	HF562982
N. meningitidis 29031	H	PRC	Carrier	H:P1.21,3: F4–21: ST-4959 (-)	ERR028662
N. meningitidis H-ASH/87	H	PRC	Carrier	H:P1.21,3: F4–21: ST-4959 (-)	ERR036095
N. meningitidis H-ZANE/83	H	PRC	Carrier	H:P1.21,3: F4–21: ST-4959 (-)	ERR036096
N. meningitidis 29043	I	PRC	Carrier	I:P1.22,14–6: F5–2: ST-5594 (-)	ERR028663
N. meningitidis I-ZANE/87	I	PRC	Carrier	I:P1.22,14–6: F5–2: ST-5594 (-)	ERR036097
N. meningitidis 29046	K	PRC	Carrier	K:P1.7–2,14: F5–14: ST-8724 (-)	ERR028664
N. meningitidis K-ASH/87	K	PRC	Carrier	K:P1.7–2,14: F5–14: ST-8724 (-)	ERR036087
N. meningitidis WUE3608	L	Unknown	Carrier	L:P1.18–1,3: F1–5: ST-963	HF562986
N. meningitidis L-ASHTON/87	L	PRC	Carrier	L:P1.22,14: F1–38: ST-8902 (-)	ERR036088
N. meningitidis 21033	L	Dublin, Ireland, UK	Carrier	L:P1.7–2,13–1:F1–5: ST-3258 (-)	ERR063490
N. meningitidis α275	W	Germany	Carrier	W:P1.18–1,3: F4–1: ST-22 (cc22)	HF562987
N. meningitidis WUE171	W	Germany	Unknown	W:P1.5–9, 10: F3–6: ST-11 (cc11)	HF562992
N. meningitidis α388	X	Germany	Carrier	X:P1.5–1,2–2: F5–1: ST-765 (cc254)	HF562988
N. meningitidis α162	Y	Germany	Carrier	Y:P1.5–2,10–1: F4–1: ST-23 (cc23)	HF562989
N. meningitidis WUE172	Y	Unknown	Unknown	Y:P1.5,2: F1–1: ST-166 (cc11)	HF562992
N. meningitidis WUE173	Z	Unknown	Unknown	Z:P1.7–1,1: F1–7: ST-4443 (-)	HF562991

*PRC, People's Republic of China; UK, United Kingdom.

respectively, similar to those found in the *Neisseria* genomes (*21*,*22*,*33*). Bacterial genomic GC content varies considerably among species but remains uniform within a bacterial genome, such that genes acquired through horizontal genetic exchange will have a GC content different from the overall GC content found within the genome.

Nomenclature

Reports identifying genes and proteins involved in *N. meningitidis* capsule biosynthesis, combined with the increasing availability of bacterial genomes, have necessitated a more unified approach to the nomenclature of genes within *N. meningitidis cps* locus (Table 3, Appendix). The capsule locus from each serogroup has been uploaded to the $BIGS_{DB}$ platform, enabling sequences from each gene within the *N. meningitidis cps* locus to be indexed and multiple *cps* loci to be typed. However, during the process, it was found that the nomenclature of some genes within this locus posed a problem. For instance, genes within region B were thought to encode lipidation enzymes and, thus, have been known as *lipA* and *lipB*. However, within the annotated genomes from *N. meningitidis* FAM18, MC58, Z2491, and 05342 and from *N. lactamica* 020–06, two other distinct *lipA* and *lipB* genes have been described encoding a lipoic acid synthetase and a lipoate-protein ligase protein, respectively. Furthermore, there are, in some instances, multiple names for the same gene.

Continued surveillance of meningococcal disease combined with the use of multiple names for some genes made it apparent that a consistent approach to the nomenclature of capsule genes was needed. To meet that need, we propose a comprehensive description of all *N. meningitidis* serogroups and a nomenclature for the *cps* locus, which was presented at the 2012 XVIIIth International Pathogenic Neisseria Conference (Table 3, Appendix).

We propose that the capsule biosynthesis genes within region A should be termed cs (for capsule synthesis) followed by a letter representing the serogroup and by a capital letter defining each gene according to the Demerec system of genetic nomenclature (*34*). For example, serogroup A capsule biosynthesis genes would be termed *csaA–D* (*cs* for capsule synthesis and *a* for serogroup A) (Table 3, Appendix). Under this proposal, the sialic acid capsule biosynthesis genes would be termed *cssA–C* (*cs* for capsule synthesis and *s* for sialic acid capsule).

Serogroups B, C, W, and Y are commonly associated with invasive meningococcal disease, and rapid diagnosis of the serogroup is key in monitoring the epidemiology of the disease and in developing prevention strategies. Thus, it is necessary to have nomenclature that identifies the serogroup simply and quickly. The polysialyltransferase genes belonging to serogroups B and C share >70% sequence identity and should be termed *csb* and *csc*, respectively. The equivalent gene in serogroups W and Y is also a sialyltransferase, but it also has a glycosyltransferase function and is distinct from the serogroups B and C genes. These distinctions should be reflected in the nomenclature: we propose that the gene for serogroups W and Y be termed *csw* and *csy*, respectively. The *O*-acetyltransferases should be termed *cssE* for serogroup C and *cssF* for serogroups W and Y; the nomenclature for *ctrG*, which has been shown to have a role in surface

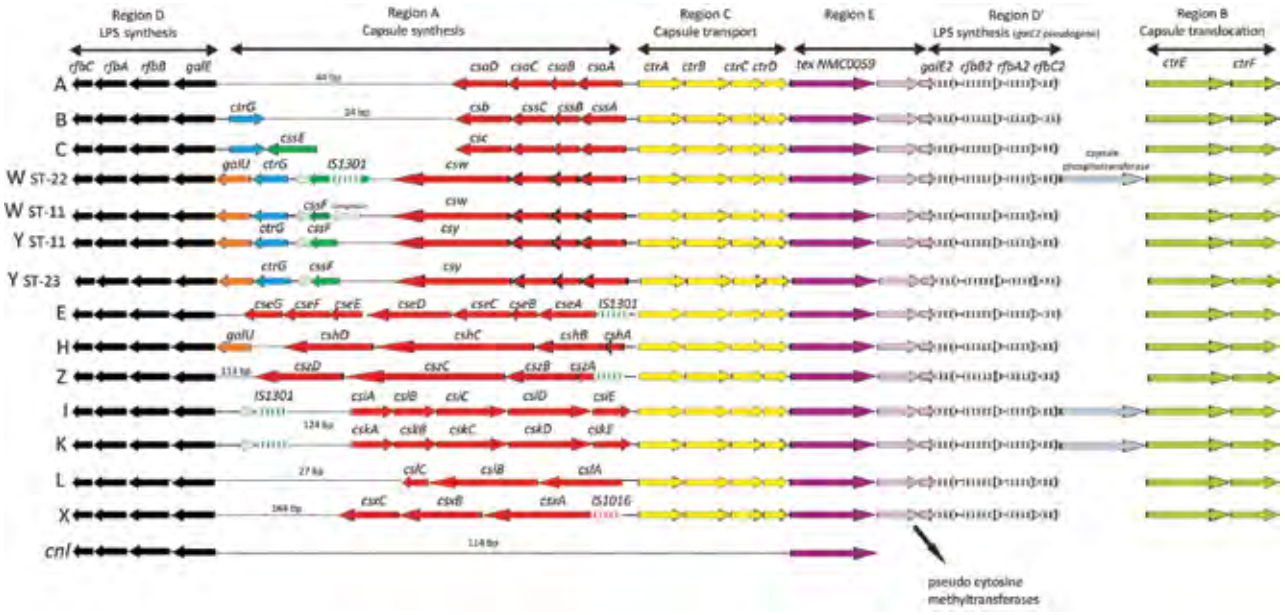

Figure. Genetic organization of the cps locus among *Neisseria meningitidis* serogroups A (*N. meningitidis* Z2491); B (*N. meningitidis* H44/76); C (*N. meningitidis* FAM18, 053442, and 29013); W α275 (clonal complex sequence type [ST] 22); W WUE171 (clonal complex ST-11); Y α162 (clonal complex ST-11); Y WUE172 (ST-23); E (*N. meningitidis* α707); H (*N. meningitidis* 29031); I (*N. meningitidis* 29043); K (*N. meningitidis* 29046); L (*N. meningitidis* WUE3608); X (*N. meningitidis* α388); and Z (*N. meningitidis* WUE173); and a cnl *N. meningitidis* isolate. Letters on left represent serogroups. Arrows depict gene orientation.

translocation of sialic capsules, should be retained (*30*). The remaining serogroups would also follow this scheme (Table 3, Appendix). Genes within region C that encode the capsule transport genes have been termed *ctrA–D* (after capsule transport), and this nomenclature should be preserved (Table 3, Appendix) (*32*). Genes within region B that are involved in capsule translocation should be designated *ctrE* and *ctrF* (*31*). Capsule genes are accessible through the PubMLST database (http://pubmlst.org/neisseria). In addition to the common gene name, these loci are allocated a value-free nomenclature consistent with the FAM18 genome annotation but using the prefix NEIS instead of NMC (Table 3, Appendix).

The W-135 and 29E serogroup designations originated at the Walter Reed Army Institute of Research as a result of a paper published by Evans et al. (*35*). We propose to rename these W and E because the numbers are historic and supply no useful information.

Region A Capsule Biosynthesis Genes

Serogroup A

The serogroup A capsule is composed of repeating units of *O*-acetylated (α1→6)-linked *N*-acetyl-D-mannosamine-1-phosphate (*13*). The capsule biosynthesis region is 4,365 bp long and contains 4 genes: *csaA–D* (also known as *mynA–D* or *sacA–D*) (*18*,*28*). The first gene, *csaA*, encodes the UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc) 2-epimerase, which converts UDP-GlcNAc into UDP-*N*-acetyl-D-mannosamine (UDP-ManNAc); *csaB* is the polymerase linking ManNAc-phosphate monomers together with *csaC*, encoding an *O*-acetyltransferase, which transfers acetyl groups to ManNAc (*18*,*28*). The fourth gene, *csaD*, is predicted to be involved in either capsule transport or in cross-linking of the capsule to the meningococcal cell surface. The serogroup A *cps* did not contain insertion sequences, and genes within region A contained some of the lowest GC content values among all serogroups (Table 3, Appendix).

Serogroups B, C, W, and Y

Capsular polysaccharides from serogroups B, C, W, and Y are composed of sialic acid derivatives; serogroups B and C express (α2→8)- and (α2→9)-linked sialic acid homopolymers, and alternating sequences of D-galactose or D-glucose and sialic acid are expressed by serogroups W and Y (*6*,*7*). Region A is 5,313 bp long in serogroup B, 6,690 bp long in serogroup C, and averages ≈7,581 bp in serogroups W and Y.

All 4 serogroups contain the conserved *cssA–C* genes for cytidine-5′-monophosphate-*N*-acetylneuraminic acid synthesis; other designations for *cssA–C* include *siaA–C*, *synA–C*, and *neuA–C* (*17*). These are followed by *csb*, *csc*, *csw*, and *csy* genes (*siaD, siaDBCWY, or synD–G*), which are the capsule polymerases and determine the functional and nucleotide specificity for the 4 serogroups.

Table 2. Genetic diversity of *Neisseria meningitidis cps* genes*

Region, locus	Size, bp	No. alleles	No. polymorphisms (%)	*p*-distance	*d*N/*d*S ratio	G+C content
MLST						
abcZ	433	11	52 (11)	0.046	0.04	51
adk	465	7	18 (4)	0.015	0.02	52
aroE	490	11	131 (7)	0.102	0.28	56
fumC	465	12	27 (6)	0.02	0.02	57
gdh	501	8	18 (4)	0.014	0.05	52
pdhC	480	13	61 (13)	0.041	0.08	56
pgm	450	10	62 (14)	0.055	0.11	54
Region B						
ctrE	2,115	13	386 (18)	0.061	0.2	51
ctrF	1,260	12	59 (5)	0.012	0.14	49
Region C						
ctrA	1,179	11	175 (15)	0.032	0.18	47
ctrB	1,164	8	264 (23)	0.051	0.10	46
ctrC	726	8	177 (24)	0.06	0.12	43
ctrD	651	7	87 (13)	0.057	0.1	46
Region D						
rfbA	867	13	75 (9)	0.034	0.26	53
rfbB	1,083	15	312 (28)	0.067	0.12	53
rfbC	558	14	124 (220	0.09	0.22	55
galE	1,020	15	382 (37)	0.119	0.12	50
Region E						
tex	2,274	13	173 (8)	0.019	0.09	59
NMC0060	1,007	8	24 (2.4)	0.003	0.91	40
NMC0061	345	5	24 (7)	0.013	0.19	36

*Genes in region A were too diverse among serogroups for direct comparison. Region D′ was omitted because *galE2* is truncated and *rfbA*, *rfbB*, and *rfbC* were duplicates. MLST, multilocus sequence type.

Serogroups C, W, and Y contain *O*-acetyltransferase genes termed *cssE* (*oatC*) and *cssF* (*oatWY*) (*29*). The 2 sequenced serogroup C and serogroup Y isolates harbored functional *cssE/cssF* genes with an *IS1301* transposase adjacent to *cssF* in serogroup Y. Serogroup W *cssF* was interrupted by the insertion sequence *IS1301*. Another gene, *ctrG*, found in all 4 serogroups, encoded a protein essential in enabling the correct expression of sialic acid polysaccharides (*30*). The orientation of this gene differed between serogroups such that it was in the same direction as the *css* genes in serogroups B and C and the opposite in serogroups W and Y (Figure).

Serogroups W and Y contained an additional gene in region A, *galU*, encoding UTP-glucose-1-phosphate uridylyltransferase, which was also found in region A of serogroup H. BLASTp searches of the derived amino acid sequence of GalU from serogroups W, Y, and H revealed that this protein shared an average of 49% sequence identity with GalU proteins from *Streptococcus pneumoniae* isolates. GalU has an essential function in capsule formation among *S. pneumoniae* isolates, catalyzing the reversible formation of UDP-Glc (uridine diphosphate glucose) and inorganic pyrophosphate from UTP (uridine 3-phosphate) and glucose 1-phosphate, and a role in virulence has been recognized for GalU in several bacterial species (*36*). Additional *galU* genes were found adjacent to the gene *argH* encoding argininosuccinate lyase in the sequenced genomes from isolates α275, 29031, H-ASH/87, and H-ZANE/83, and although the genes were conserved, they were not identical to the *galU* genes found within the *cps* locus. The genomes belonging to *N. meningitidis* Z2491, MC58, FAM18, and 053442; *N. lactamica* 020–06; and *N. gonorrhoeae* FA1090 also contained *galU* genes adjacent to *argH*. These were highly conserved (*p*-distance = 0.015) but were more distantly related to those found within the *cps* locus (*p*-distance = 0.180), indicating a different origin for *cps*-associated *galU* genes.

The serogroup D isolate was found to contain serogroup C capsule biosynthesis genes (Figure and online Technical Appendix Figure 1, panel A, wwwnc.cdc.gov/EID/article/19/4/11-1799-Techapp1.pdf). The *cps* locus from the prototype serogroup D isolates deposited by Gordon and Murray in 1917 and Branham in 1928 also contained serogroup C-specific capsule genes; however, neither isolate gave precipitins with antiserum to serogroup C, suggesting that these isolates were unencapsulated (C. Frasch. pers. comm.). The presence of internal stop codons in the *ctrA* and *ctrE* genes is consistent with this and further confirms that serogroup D does not exist.

Serogroup E

The serogroup E capsule consists of alternating D-galactosamine and 2-keto-3-deoxyoctulosonate (KDO) residues (*9*). Region A is ≈9,613 bp long, including *IS1301*, and contains 7 genes, *cseA–G* (formerly *29eA–H*); gene *cseE* encodes a putative 3-deoxy-phosphooctulonate synthase, *cseF* encodes a putative 3-deoxy-D-manno-octulosonate cytidylyltransferase, and *cseG* encodes a D-arabinose 5-phosphate isomerase protein. The deduced amino

acid sequence from *cseE* shared 83% sequence identity with a 3-deoxy-8-phosphooctulonate synthase belonging to *N. elongata* subsp. *glycolytica* and 81% identity with *N. subflava* NJ9703; *cseF* shared 66% sequence identity with a putative 3-deoxy-D-manno-octulosonate cytidylyltransferase belonging to multiple bacterial species, including *Pseudomonas putida* and *Escherichia coli*. The genes *cseD* and *cseE* were found to be fused and were predicted to form 1 protein; this prediction is in agreement with the suggestion that the genes for KDO synthesis were dispensable probably because of complementation of lipopolysaccharide–KDO synthesis elsewhere in the genome (*37*).

Serogroups H and Z

The biochemical structures of serogroups H and Z contain monosaccharide glycerol-3-phosphate repeat units and share similarities with teichoic acid polymers. Region A varied from 6,182 bp in serogroup H to 7,198 bp in serogroup Z, with the latter containing the insertion element *IS1301*. Four genes were present in both serogroups (Figure and online Technical Appendix Figure 3, panel A). BLASTp searches of the deduced amino acid sequences of the first 2 genes, termed *cshA/cszA* and *cshB/cszB*, shared 91% sequence identity with each other and also shared 76% and 63% sequence identity with the capsule biosynthesis genes *cps2B* and *cps2C* belonging to *Actinobacillus pleuropneumoniae* serovars 2, 3, 6, 7, 9, 11, and 13 (*38*). These genes are predicted to encode a glycerol-3-phosphate cytidylyltransferase, and a hypothetical protein containing a LicD domain for a role in phosphorylcholine incorporation into teichoic acid polymers has been suggested (*38*). The third genes in region A, termed *cshC* and *cszC*, each shared 65% sequence identity with a putative teichoic synthase genes (*cps2D* and *cps9D*, respectively) belonging to *A. pleuropneumoniae* serovars 2, 7, 9, and 13. Last, *cshD* and *cszD* were 75% homologous to *A. pleuropneumoniae* capsule synthesis genes (*cps7E*).

Serogroups L and X

Serogroup L capsule polysaccharides contain 2-acetamido-2-deoxy-D-glucosyl residues and phosphate groups. Region A contained 3 genes, *cslA–C* (formerly *lcbA–C*) and was 4,438 bp long. BLASTp searches of the deduced amino acid sequence from *cslA* predicted a capsule phosphotransferase sharing 73% sequence identity with LcbA proteins from *N. mucosa* C102 (GenBank accession no. ACRG00000000) and *N. subflava* NJ9703 (GenBank accession no. ACEO00000000), whereas *cslC* encoded an acetyltransferase protein. A gene similar to *cslA* was identified between regions D′ and B among serogroups I and K and a serogroup W isolate belonging to clonal complex sequence type 22 (Figure); BLASTp searches of the deduced amino acid sequence revealed that these genes shared 97% sequence identity. The longest gene, *cslB*, was 2,633 bp and putatively encoded a capsule polymerase (Figure and online Technical Appendix Figure 2).

The serogroup X capsular polysaccharide is composed of (α-1→4)-linked *N*-acetylglucosamine 1-phosphate (*15*). In agreement with findings in a previous study, we found that region A in the serogroup X isolate contained 3 genes, *csxA–C* (*xcbA–C*) and was 4,467 bp long, including the insertion sequence, *IS1016*, located upstream of *csxA* (Figure and online Technical Appendix Figure 2) (*19*). The deduced amino acid sequence from *csxA* shared 40% sequence identity with the previously mentioned LcbA protein belonging to *N. mucosa* C102, indicating that *csxA* encoded a putative capsule phosphotransferase; however, the remaining 2 serogroup X capsule biosynthesis genes did not share substantial sequence identities with any known protein.

Serogroups I and K

Different structural compositions have been described for these serogroups, with serogroup I consisting of *O*-acetylated alternating *N*-acetyl-guluronic acid and *N*-acetylmannosaminuronic acid units and serogroup K composed of *O*-acetylated disaccharide repeat units containing *N*-acetylmannosaminuronic acid (*10*,*11*). Both serogroups had almost identical capsule biosynthesis genes, *csiA–E* or *cskA–E*; region A was ≈9,026 bp long (Figure and online Technical Appendix Figure 3, panel B). MLST analysis showed that serogroup I and K isolates investigated in this study did not possess the same sequence types or PorA and FetA variable regions (Table 1). Antiserum is not commercially available to verify these serogroups; however, the immunochemical difference between serogroup I and K polysaccharides may reflect differences in the original methods used to purify them (*10*,*11*). In addition, 2 nonsynonymous substitutions were observed in the capsule biosynthesis genes *csiC/cskC* and *csiD/cskD*, for which the deduced amino acid sequence encoded putative glycosyltransferases belonging to families 1 and 2, respectively. The capsule polymerases belonging to serogroups W and Y (*csw* and *csy*) are closely related; a single amino acid substitution in the *N*-terminal glycosyltransferase domain of the capsule polymerase produces the glucose or galactose substrate specificity for the enzyme (*39*) (online Technical Appendix). It is therefore possible that the nonsynonymous changes observed in the glycosyltransferases, csiC/cskC and csiD/cskD, may produce the serogroup I and K capsules. Further investigation of these serogroups is required.

The derived amino acid sequences from *csiA*, *B*, and *E*/*cskA*, *B*, and *E* shared >70% sequence identity with capsule biosynthesis proteins belonging to *Mannheimia haemolytica* serotype A1. The capsule of *M. haemolytica* serotype

A1 is composed of a disaccharide repeat of *N*-acetylmannosaminuronic acid linked with *N*-acetylmannosamine.

Regions B and C

The genes *ctrE* and *ctrF* (formerly *lipA* and *lipB*) are required for surface expression of a properly anchored capsule polymer. The ABC (ATP binding cassette) transport system is characterized by the hydrophobic outer and inner membrane proteins CtrA and CtrB, respectively; the integral inner membrane–associated protein, CtrC; and the ATP binding protein, CtrD, and homologous genes are found among other group II capsule-expressing bacteria (*40*) (see online Technical Appendix).

Conclusions

We compared nucleotide sequence data from complete *cps* loci from all described *N. meningitidis* serogroups, revealing that the genetic organization is similar for all loci and that region A contains the capsule-specific biosynthesis genes. Distinct capsule operons corresponding to serogroups A, B, C, E, H, I, K, L, W, X, Y, and Z have been described herein, with the serogroup D capsule described as being an unencapsulated serogroup C variant. Nucleotide sequence data for each capsule locus have been deposited in $BIGS_{DB}$, with genes from each region defined and organized into schemes (*26*). However, it became apparent that a consistent approach to the nomenclature of capsule genes was required. In 2012, the approach detailed in this paper was approved at the XVIIIth International Pathogenic Neisseria Conference in Würzburg, Germany.

Horizontal genetic transfer of *cps* genes in region A was evident in serogroups H, I, K, and Z isolates, indicating acquisition of genes from external sources, including the bacterial species *A. pleuropneumoniae* and *M. haemolytica*. Combined with the low GC content observed in regions A and C, these data are consistent with acquisition of capsular genetic material from other species. Serogroup determination has been unresolved in some isolates. These isolates may contain capsule genes that are not expressed and will not be detectable by using conventional seroagglutination techniques, or they may be serogroup E, H, I, K, L, X, or Z, which are not routinely searched for and for which commercial antiserum is not available. This study provides additional tools to detect all capsule loci and may ultimately permit determination of the distribution of all serogroups among *N. meningitidis* populations and detection of *cnl* isolates.

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Detection of Spliced mRNA from Human Bocavirus 1 in Clinical Samples from Children with Respiratory Tract Infections

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Human bocavirus 1 (HBoV1) is a parvovirus associated with respiratory tract infections (RTIs) in children, but a causal relation has not yet been confirmed. To develop a qualitative reverse transcription PCR to detect spliced mRNA from HBoV1 and to determine whether HBoV1 mRNA correlated better with RTIs than did HBoV1 DNA, we used samples from HBoV1 DNA–positive children, with and without RTIs, to evaluate the test. A real-time reverse transcription PCR, targeting 2 alternatively spliced mRNAs, was developed. HBoV1 mRNA was detected in nasopharyngeal aspirates from 33 (25%) of 133 children with RTIs but in none of 28 controls (p<0.001). The analytical sensitivity and specificity of the test were good. Our data support the hypothesis that HBoV1 may cause RTIs, and we propose that HBoV1 mRNA could be used with benefit, instead of HBoV1 DNA, as a diagnostic target.

Human bocavirus 1 (HBoV1) is a small nonenveloped virus in the *Parvoviridae* family. It was discovered in human respiratory samples in 2005 (*1*). The virus does not grow in standard cell lines, and diagnosis has mainly been based on DNA detection with PCR. Detection of multiple viruses in HBoV1 DNA–positive airway samples from children with respiratory tract infections (RTIs) has been a characteristic finding in many studies (*2*–*4*). In addition, many healthy children have tested positive for HBoV1 DNA (*2*,*5*); thus whether the virus actually causes RTIs in children or is just a bystander to other infections has been debated. However, we have shown that the following 3 factors are associated with RTIs: HBoV1 viremia (HBoV1 DNAemia), a high HBoV1 DNA load in nasopharyngeal aspirates (NPAs), and monodection of HBoV1 DNA in NPAs (*5*). In addition, RTIs in HBoV1 DNA–positive children are associated with HBoV1 seroconversion (*6*). This evidence supports a causal relation between HBoV1 and RTIs in children, but DNA-based PCR tests do not seem to diagnose HBoV1 infection accurately. We propose that detection of HBoV1-specific mRNA, as a measure of actively transcribing virus, may be a better method.

The main objectives of this study were to develop a qualitative reverse transcription PCR (RT-PCR) detecting spliced mRNA from HBoV1 and to clarify whether HBoV1 mRNA detection may correlate better than DNA detection with RTIs in children. NPAs and blood samples from a group of children, with and without RTIs, who tested positive for HBoV1 DNA were used for this purpose.

Materials and Methods

Samples

HBoV1 DNA–positive NPA samples from an ongoing project on RTIs in children 0–16 years of age were used for evaluation of the test (*5*). In particular, 161 NPA samples collected at admittance from 161 children at the Department of Pediatrics, St. Olav's Hospital, Trondheim University Hospital (Trondheim, Norway), during June 2007–June 2010 were included. A blood sample was also available for 63 of the children. All samples had been stored at −70°C.

Children with RTIs

Of the 161 HBoV1 DNA–positive NPA samples, 133 were from children with RTIs. Median age was 17 months (range 3 months–5 years) and 60% were boys. They were classified as having either lower (86 children) or upper (47 children) RTI (LRTI; URTI). LRTI was diagnosed in the presence of dyspnea, signs of lower airway obstruction (wheezing, retractions), and/or a chest roentgenogram

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with positive results (infiltrates, atelectasis, air trapping). URTI was diagnosed when rhinitis, pharyngitis, and/or otitis media was found without signs of LRTI. In addition, 3 children with RTIs, admitted during winter 2011–12, were followed up on 3 occasions, each over 2 months.

Children without RTIs (Controls)

Twenty-eight HBoV1 DNA–positive NPA samples were collected from a group of children who were admitted for elective surgery and who had exhibited no signs or symptoms of RTI during the previous 2 weeks. The children were included prospectively during the same period in 2007–2010. Median age was 31 months (range 15 months–6 years), and 70% were boys.

Tests for Other Respiratory Agents

All NPA samples from patients and controls were also tested with PCRs for adenovirus, coronavirus (OC43, 229E, and NL63), enterovirus, parechovirus, human metapneumovirus (HMPV), influenza A and B viruses, parainfluenza virus types 1–4, respiratory syncytial virus (RSV), rhinovirus, *Bordetella pertussis*, *Chlamydophila pneumoniae*, and *Mycoplasma pneumoniae*. The PCRs were in-house, real-time assays with TaqMan probes (Roche Diagnostics, Basel, Switzerland) (*5*). The analyses were conducted as part of the daily laboratory routine and performed within 24 hours after sample collection. The target for the HBoV1 DNA PCR was the nuclear phosphoprotein-1 gene. This PCR has been described (*4*). A semiquantitative approach was chosen, and a cutoff value of 10^6 copies/mL was used to distinguish between high and low HBoV1 DNA load in NPAs.

Spliced HBoV1 mRNA-PCR

We developed a real-time RT-PCR on the basis of TaqMan technology (Roche). The following primers were designed: forward 5′-CGGCGAGTGAACATCTCTGGA-3′ (positions 203–223) and reverse 5′-TGCTTGTCTTTCATATTCCCT-3′ (positions 2438–2418). The estimated PCR product spanned a spliced segment from positions 241 to 2236 of the complete genome for HBoV1 (GenBank accession no. NC007455), which gives a theoretical PCR product of 242 bp and an alternative product, including a short segment from positions 2044 to 2164, yielding a product of 363 bp (Figure 1). These estimations were based on in vitro studies performed by Chen et al. (*7*). The probe targeting the untranslated region upstream of the nuclear phosphoprotein-1 gene had the following sequence: 5′-FAM-TGTCCACCCAAGAAACGTCGTCTAA-TAMRA-3′ (positions 2295–2319). The PCR for every sample was also run without reverse transcription to test for potential unspecific reactions with viral DNA. The theoretical PCR product from HBoV1 DNA would be 2,236 bp in length, which is too long for amplification by real-time PCR under normal conditions.

Total DNA and RNA were extracted by using NucliSens easyMag extractor (bioMérieux, Marcy l'Etoile, France), and reverse transcription was carried out with Universal RiboClone random primers (Promega, Fitchburg, WI, USA) and M-MLV Reverse Transcriptase (Life Technologies Corp., Carlsbad, CA, USA) at 37°C for 60 min. followed by 94°C for 10 min. The PCR was performed for 45 cycles at 95°C for 5 s., 55°C for 10 s, and 72°C for 20 s.

Amplification efficiency was calculated by using the formula $E=10^{(-1/S)}-1$, where S is the slope of the standard curve. A human DNA PCR (specific for the γ-glutamyltransferase light chain 1 gene on chromosome 20) was used as amplification control (*8*). Nucleic acid extract from a clinical sample positive for RSV was used as cDNA control. To make sure that mRNA had not been degraded during storage, we used an RT-PCR to detect human β actin mRNA (*9*).

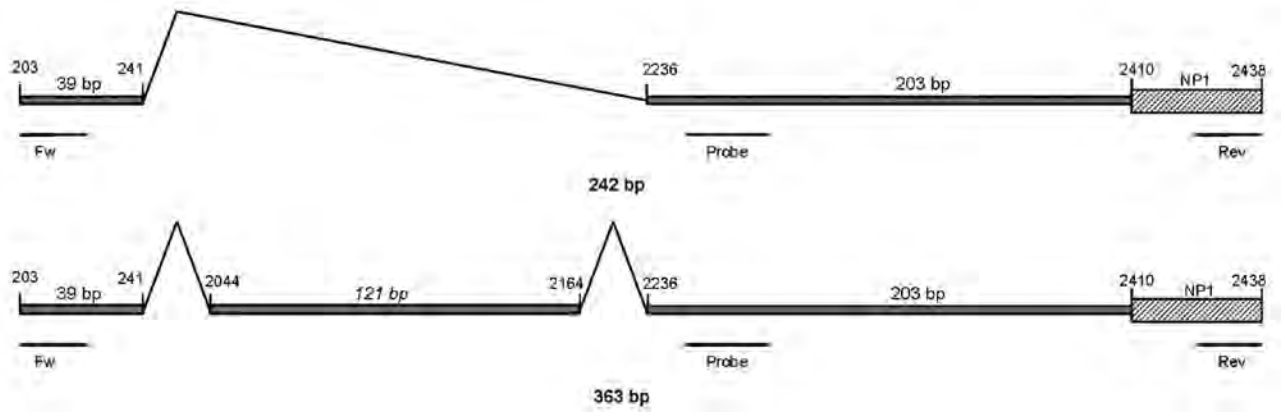

Figure 1. Schematic representation of the 2 human bocavirus 1 (HBoV1) mRNA PCR products, illustrating alternative splicing. Positions of primers and probe are shown. The total length of the upper product is 242 bp, and the length of the lower is 363 bp (reference sequence: GenBank accession no. NC007455).

RNA stability was studied by using clinical NPA samples and nucleic acid extracts from the easyMag extractor (bioMérieux). Four clinical NPA samples collected within the previous 2 hours were stored for 0, 1, 3, and 5 days at 4°C before nucleic acid extraction and testing with the HBoV1 mRNA PCR. One NPA sample was stored at room temperature and tested likewise. Two other clinical NPA samples were frozen and thawed 0, 1, 2, and 4 times before extraction and testing with the HBoV1 mRNA PCR (3 times was skipped to save NPA material). Furthermore, HBoV1 mRNA PCR results from 3 HBoV1-positive NPA samples stored at −70°C for 3 years were compared with nucleic acid extracts from the same samples stored under equal conditions for the same period. This was done to determine whether the stability of RNA in clinical NPA samples added to virus transport medium was comparable to the stability of RNA in nucleic acid extracts from the easyMag at this temperature. Relative changes in RNA load were measured by comparing logarithmically transformed cycle threshold values (Ct values) obtained from the same experiment.

Quantitative standards for the real-time HBoV1 mRNA PCR were made by cloning a plasmid (pCR4-TOPO; Life Technologies Corp.) containing the PCR product. The amount of nucleic acid was measured, and serial dilutions covering a range of 7 logs were made to measure the analytical sensitivity of the HBoV1 mRNA PCR.

Analytical specificity was evaluated by using cDNA from NPA samples positive for all respiratory agents included in the study. cDNA from NPA samples containing viruses that can be reactivated in the respiratory tract were also included (i.e., herpes simplex virus, cytomegalovirus, Epstein-Barr virus, and human herpes virus 6). Finally, cDNA from NPA samples positive for the more closely related parvovirus B19 and cDNA from fecal samples positive for human bocaviruses 2 and 3 (HBoV2 and HBoV3) were tested. The primers and probe described by Kantola et al. were used for detection of HBoV2 and HBoV3 (*10*). Two samples positive for each agent were used, and all samples had undergone extraction within 2–20 hours after sample collection. Sequence analysis on the PCR products was performed by using the BigDye Terminator Cycle sequencing method and the ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

Statistical analysis was by χ^2 test for categorical variables and Student *t* test for continuous variables. Multiple logistic regression analysis was used to evaluate the association between detection of HBoV1 mRNA and LRTI, controlling for differences in age, sex, and the presence of other viruses among case-patients and controls. We report the odds ratio (OR) with 95% CIs and the corresponding p value as a measure of the strength of the association. All analyses were performed by using IBM SPSS Statistics version 19.0 (SPSS Inc., Chicago, IL, USA).

Results

Spliced HBoV1 mRNA PCR

HBoV1 mRNA was detected in 33 of the 161 HBoV1 DNA–positive NPA samples (Table 1). Gel electrophoresis showed that primarily 2 PCR products were amplified with sizes of ≈250 bp and ≈400 bp (Figure 2). Sequence analysis showed that they were spliced products with either 1 or 2 introns cut out as expected (schematically illustrated in Figure 1). The exact product sizes were 242 bp and 363 bp. Direct PCR analysis for HBoV1 mRNA on the nucleic acid extracts, without initial reverse transcription, was negative for 30 of 33 NPA samples. For the remaining 3 samples, however, weak signals were detected. These 3 samples had very high HBoV1 DNA loads (range 4×10^8 copies/mL to $>10^{10}$ copies/mL), and were also strongly positive by the HBoV1 mRNA PCR after cDNA synthesis. The products of the 3 PCRs that were done without cDNA synthesis were sequenced. Product sizes were 145, 261, and 457 bp, and sequence analysis showed gaps at different positions, all of them lacking splice site characteristics (data not shown).

Amplification efficiency of the HBoV1 mRNA PCR was calculated on the basis of dilutions of both the nucleic acid extract and cDNA. It was measured to 100% in both cases, indicating a high efficiency of both the PCR and cDNA synthesis (data not shown). The assays' reportable range was from 500 copies/mL (10 copies/reaction) to 10^{10} copies/mL.

Results of the β-actin PCR performed after DNase treatment were positive for all samples studied. Results of the HBoV1 mRNA PCR were negative for all other respiratory agents, herpesviruses, and parvoviruses tested.

Table 1. HBoV1 mRNA PCR results in NPAs from children with and without RTIs, Norway, 2007–2010*

Sample source	Total no.	No. (%) HBoV1 mRNA$^+$	No. (%) HBoV1 mRNA$^-$	p value
Children with RTIs	133	33 (25)	100 (75)	p<0.001
Controls (without RTIs)	28	0	28 (100)	
Children with LRTIs	86	27 (31)	59 (69)	p = 0.02
Children with URTIs	47	6 (13)	41 (87)	

*HBoV1, human bocavirus 1; NPAs, nasopharyngeal aspirates; RTIs, respiratory tract infections; LRTIs, lower RTIs; URTIs, upper RTIs.

mRNA Stability

The HBoV1 mRNA load in NPA remained stable for 5 days at 4°C. At room temperature, it was unaltered after 24 h but was reduced by 1 log after 3 days and by 1.5 log after 5 days. Freezing and thawing of the NPA samples once or twice did not affect yield, but after 4×, it was reduced by ≈0.5 log. For 3 NPA samples that had been stored at −70°C for 3 years, the results were equal for both the nucleic acid extract and the original sample. HBoV1 mRNA PCR results for nucleic acid extracts were stable for weeks when samples were stored at 4°C (samples stored for up to 8 weeks were tested; data not shown).

Performance of Spliced HBoV1 mRNA PCR on Samples

First, we compared the rates of positive test results for HBoV1 mRNA among children with positive results for HBoV1 DNA, with and without RTIs. Only one fourth of the patients and none of the controls had test results positive for HBoV1 mRNA (Table 1). More children with LRTI (27/86 [31%]) than with URTI (6/47 [13%]) had positive test results for HBoV1 mRNA (Table 1). After we adjusted for age, sex, and presence of other viruses, this difference persisted (OR 3.5, 95% CI 1.3–9.8, p = 0.02).

We previously found that 3 factors (HBoV1 DNAemia, high HBoV1 DNA in NPAs, and monodection of HBoV1 DNA in NPAs) were each associated with RTIs in children (*5*). In the present study, these factors were strongly associated with a positive test result for HBoV1 mRNA (Table 2). The close relationship between HBoV1 DNA load and HBoV1 mRNA detection in NPAs is also illustrated in Figure 3. Of the 100 RTI patients who were negative for HBoV1 mRNA, 75 were positive for ≥1 other respiratory viruses. Twenty-eight (37%) of these children were infected with the highly pathogenic RSV. Distribution of the viruses most commonly co-detected with HBoV1 is shown in Table 3.

Follow up of 3 Children with RTIs during Winter 2011–12

During winter 2011–12, sequentially collected samples from 3 HBoV1 DNA–positive children made it possible to gain some information about changes in HBoV1 mRNA and HBoV1 DNA over time. One of these children (boy 1) was a 2-year-old boy with cerebral palsy who had been admitted with bronchiolitis. On admission, he had HBoV1 DNAemia and analysis of NPAs showed that he had 1) a high HBoV1 DNA load, 2) monodection of HBoV1 DNA, and 3) positive HBoV1 mRNA PCR results. He recovered slowly, and after 10 days a new NPA sample was taken. The HBoV1 DNA load was still high, but the results for the HBoV1 mRNA PCR were negative. Two months later, his NPAs still were positive for HBoV1 DNA and negative for HBoV1 mRNA. The other patients were two 1.5-year-old boys (boys 2 and 3), who had been included in an HMPV follow-up study. Their NPA samples became positive for HBoV1 DNA 1 week after HMPV infection was diagnosed. The initial samples were negative for HBoV1 DNA. The clinical condition was unaltered for both when HBoV1 DNA appeared. HBoV1 DNA loads reached moderate levels for boy 2 and high levels for boy 3, but for both boys, the PCRs were negative for HBoV1 mRNA in 2 consecutive NPA samples taken 5 days apart. Unfortunately, no blood samples were taken from these 2 boys.

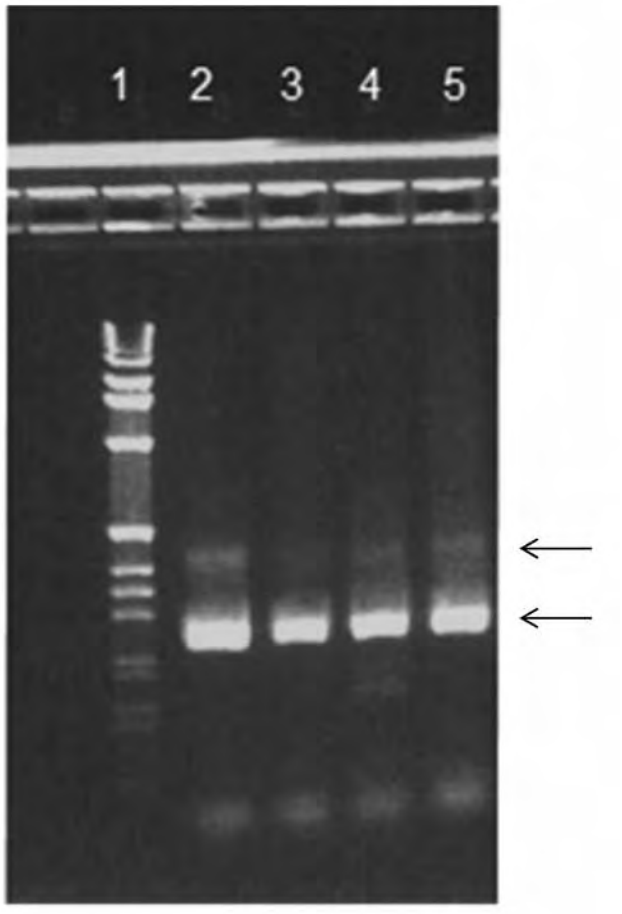

Figure 2. Agarose gel stained with ethidium bromide. Reverse transcription PCR products from 4 patients are shown in lanes 2–5 and 1-kb DNA Ladder (Life Technologies Corp., Carlsbad, CA, USA) in lane 1. Arrows indicate 2 bands corresponding to ≈250 and ≈400 bp.

Discussion

We report here the development of a robust PCR for detection of spliced mRNA from HBoV1. We found that HBoV1 mRNA correlated significantly better with RTIs in children than did HBoV1 DNA, indicating that this PCR may diagnose HBoV1 infection more accurately than PCR for HBoV1 DNA.

Table 2. HBoV1 mRNA PCR results in NPAs in relation to HBoV1 DNAemia, a high HBoV1 DNA load, and monodection of HBoV1 DNA, Norway, 2007–2010*

Factor	Total no.	No. (%) HBoV1 mRNA$^+$	No. (%) HBoV1 mRNA$^-$	p value
HBoV1 DNAemia, n = 63	17	13 (77)	4 (23)	p<0.001
No HBoV1 DNAemia	46	5 (11)	41 (89)	
HBoV1 DNA load, n = 161				
$\geq 10^6$ copies/mL	59	33 (56)	26 (44)	p<0.001
$<10^6$ copies/mL	102	0	102 (100)	
$\geq 10^8$ copies/mL	18	17 (94)	1 (6)	p<0.001
$<10^8$ copies/mL	143	16 (11)	127 (89)	
Monodection of HBoV1 DNA, n = 161	43	14 (33)	29 (67)	p = 0.022
Multiple virus detections	118	19 (16)	99 (84)	

*HBoV1, human bocavirus 1; NPAs, nasopharyngeal aspirates; HBoV1 DNAemia, HBoV1 viremia.

Splicing is a process specific for mRNA synthesis, and with use of a primer pair spanning an intron, spliced viral mRNA should be specifically detected within the frame of the familiar and robust RT-PCR. This diagnostic technique has been used to diagnose parvovirus in dogs and may also be an option for diagnosing HBoV1 infections in humans (*11*). Furthermore, detection of mRNA is routinely used for diagnosing human papillomavirus infections and has been studied for diagnosing human herpesvirus 6 and HIV infections (*12–15*). However, the mRNA tests for these viruses have been based on either specific mRNA extraction, nucleic acid sequence–based amplification technology, or pretreatment with DNase. The advantage with our approach is that no pretreatment, other than cDNA synthesis, is needed. The procedure is performed as a regular RT-PCR with standard equipment and will be easy to use in most routine laboratories. The analytical performance of the test was good with high analytical specificity and sensitivity.

The probe target was chosen to detect the 2 products (Figure 1) and thereby to maximize analytical sensitivity. A probe spanning the spliced segment between positions 241 and 2236 would have been an alternative approach, ensuring specific detection of mRNA spliced at this exact location (Figure 1). However, because our results indicated high specificity with the chosen probe, we did not develop this approach further.

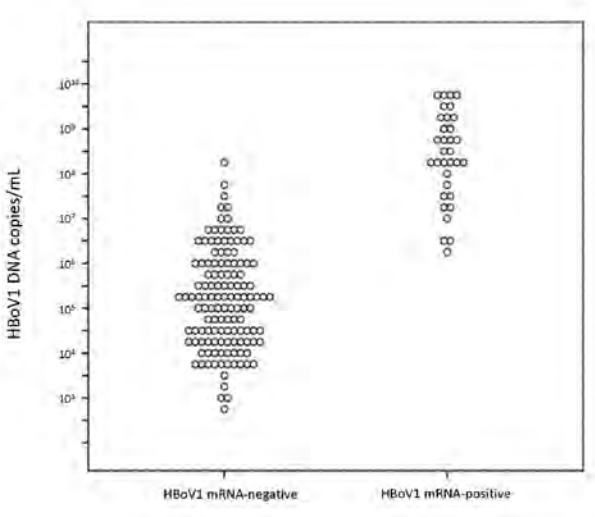

Figure 3. Distribution of human bocavirus 1 (HBoV1) DNA loads in nasopharyngeal aspirates either positive (n = 33) or negative (n = 128) for HBoV1 mRNA. Each dot indicates 1 sample.

Previously, RNA molecules were believed to have short half-lives because RNases may be present everywhere and easily degrade RNA. Recent studies, however, have suggested that mRNA may be stable when molecules are kept in the original biologic material (*16*,*17*). We found that the mRNA content in NPA samples was stable during a 5-day period at 4°C. This stability suggests that NPA samples kept in a refrigerator and processed within 1–2 days, which is standard in our laboratory, are safe to use for HBoV1 mRNA testing.

In 3 samples that had strong signals in the HBoV1 mRNA test, a weak signal was detected also without prior cDNA synthesis, evoking the question of whether HBoV1 DNA could give false-positive reactions in the HBoV1 mRNA test. The PCR was designed so that the theoretical DNA product would be 2,236 bp—too large for amplification to occur in a regular, real-time PCR. For this reason, the PCR products were expected to result from recombination events. Gel and sequence analysis showed that all 3 products had different sizes, ranging from 145 to 457 bp. Moreover, no common sequence profiles were found near the gap junctions, which seemed to be located at random. Homologous recombination, a concentration-dependent process, may explain this phenomenon because it occurred only in NPA samples with extremely high levels of HBoV1 DNA. We speculate that the PCR products might have been subpopulations of nonviable HBoV1 mutants that appeared when virus replication was at its highest. However, the specificity of the test was not affected because it happened only in patients with very high viral DNA loads and strong HBoV1 mRNA signals.

In addition to being a diagnostic test, this method may be used to gain information on HBoV1 transcription in vivo. Our data confirmed previous in vitro results on the splicing pattern at the 5′ end of the HBoV1 genome (Figure 1) (*7*,*18*).

Table 3. Most commonly co-detected viruses in NPAs from children with HBoV1 DNA, distributed by presence of RTI and HBoV1 mRNA, Norway, 2007–2010*

	HBoV1 DNA+ children with RTIs		HBoV1 DNA+ controls	
Virus	No. (%) HBoV mRNA+, n = 33	No. (%) HBoV1 mRNA–, n = 100†	HBoV mRNA+, n = 0	HBoV1 mRNA–, n = 28†
Respiratory syncytial virus	4 (12)	28 (28)	–	0
Rhinovirus	7 (21)	25 (25)	–	10 (36)
Enterovirus	5 (15)	24 (24)	–	15 (54)
Adenovirus	1 (3)	20 (20)	–	9 (32)

*NPAs, nasopharyngeal aspirates; HBoV1, human bocavirus 1; RTIs, respiratory tract infections; +, positive; –, negative.
†Triple and quadruple infections were common, and percentages within the columns may therefore add up to >100%.

For evaluation of the HBoV1 mRNA test, we were able to use available clinical samples from children with or without RTIs who had been tested for 18 respiratory agents and had HBoV1 DNA in NPAs (*5*). None of the children without RTIs had detectable HBoV1 mRNA. Because mRNA is a marker of active viral transcription, this finding indicates that the 28 asymptomatic children carried inactive HBoV1 or HBoV1 with low activity. The strong association found between active HBoV1 transcription and RTI in children supports the hypothesis that HBoV1 may cause RTIs. The hypothesis is further supported by the associations found between HBoV1 mRNA and the 3 factors: HBoV1 DNAemia, high HBoV1 DNA load in NPAs, and monodection of HBoV1 DNA—all factors strongly related to RTIs in children (*5*,*6*,*19*). In addition, the fact that HBoV1 mRNA was more frequently detected in children with LRTIs than with URTIs indicates that LRTI is a prominent manifestation of HBoV1 infection.

Only one fourth of the HBoV1 DNA–positive children with RTIs had detectable HBoV1 mRNA. Similar findings were recently reported by Proenca-Modena et al. (*20*). The absence of HBoV1 mRNA in most of the children with RTI may indicate that these children did not have a clinical HBoV1-infection, despite positive test results for HBoV1 DNA. Indeed, other respiratory viruses were frequently detected among the children who had a negative HBoV1 mRNA test result; RSV accounted for one third of infections.

The previously mentioned strong relation between HBoV1 DNA load in NPAs and HBoV1 mRNA is illustrated in Figure 3. It shows 2 distinct populations with little overlap, and good discrimination between HBoV1 mRNA–positive and –negative samples can be achieved with cutoff values from 10^6 to 10^7 HBoV1 DNA copies/mL. We suggest that, for clinical purposes, HBoV1 mRNA is more accurate than HBoV1 DNA in diagnosing active HBoV1 infection, but a high HBoV1 DNA load (>10^7 copies/mL) may also be useful in diagnosis.

Previously, HBoV1 has been found to persist in NPAs for many months (*21*–*23*). The molecular basis for this persistence is largely unknown, but 2 recent studies have given evidence in support of persistent circular HBoV episomes (*24*–*26*). The NPA samples in our study which were negative for HBoV1 mRNA and positive for HBoV1 DNA could be from patients with past HBoV1 infections who were still shedding viral DNA. Boy 1, who was followed up during winter 2011–12, may illustrate this. Results of PCR on NPA samples from this boy were positive for HBoV mRNA only for a short period (<10 days), coinciding with the acute symptomatic infection, whereas HBoV1 DNA persisted for months. An alternative hypothesis might be that the samples negative for HBoV1 mRNA and positive for HBoV1 DNA were from children with a latent HBoV1 infection. The findings in boys 2 and 3, who were followed up during the same winter, may support this hypothesis. HBoV1 DNA in NPAs appeared during an ongoing HMPV infection in both children, but results of PCR for HBoV1 mRNA remained negative in 2 consecutive samples. The lack of detectable HBoV1 mRNA may indicate that HBoV1 did not play a role in these infections. Release of latent HBoV1 DNA from cells disrupted by inflammation caused by HMPV may be a better explanation. More longitudinal studies, including serologic analyses, are needed to further study these relationships.

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Predicting Hotspots for **Influenza Virus Reassortment**

Trevon L. Fuller, Marius Gilbert, Vincent Martin, Julien Cappelle, Parviez Hosseini, Kevin Y. Njabo, Soad Abdel Aziz, Xiangming Xiao, Peter Daszak, and Thomas B. Smith

The 1957 and 1968 influenza pandemics, each of which killed ≈1 million persons, arose through reassortment events. Influenza virus in humans and domestic animals could reassort and cause another pandemic. To identify geographic areas where agricultural production systems are conducive to reassortment, we fitted multivariate regression models to surveillance data on influenza A virus subtype H5N1 among poultry in China and Egypt and subtype H3N2 among humans. We then applied the models across Asia and Egypt to predict where subtype H3N2 from humans and subtype H5N1 from birds overlap; this overlap serves as a proxy for co-infection and in vivo reassortment. For Asia, we refined the prioritization by identifying areas that also have high swine density. Potential geographic foci of reassortment include the northern plains of India, coastal and central provinces of China, the western Korean Peninsula and southwestern Japan in Asia, and the Nile Delta in Egypt.

Simultaneous infection with multiple influenza virus strains can affect virus fitness components, such as virus growth performance, and thus affect virus pathogenicity, transmission, or recombination (*1*). In a host infected with 2 closely related influenza viruses, the stains can reassort, exchanging gene segments to produce new strains, some of which might have increased virulence. Virulence might also trade off with transmission such that more pathogenic viruses spread more slowly (*2*). However, in some instances, a reassortant virus can have high transmissibility and high pathogenicity. For example, reassortment between influenza viruses of humans and birds resulted in the 1957 and 1968 pandemic viruses, each of which is estimated to have killed ≈1 million persons (*3*,*4*). The exchange of genes between pairs of influenza virus subtypes increased virulence in animal models, including reassortment between subtypes H9N2 and H1N1, between H5N1 and H1N1, and between H3N2 and H5N1 (*5*,*6*). We focus on reassortment between subtypes H3N2 and H5N1 because extensive data are available, but given sufficient data, our approach could be extended to other subtypes.

For seasonal influenza virus A subtype H3N2, person-to-person transmissibility and prevalence among humans are high (*7*). Furthermore, subtype H5N1, which is primarily found in birds, can be highly pathogenic; the fatality rate among humans is 60% (*8*). In mice, ≈8% of reassortant viruses formed from human subtype H3N2 and avian subtype H5N1 resulted in increased virulence and a mortality rate of 100% (*5*). This finding among mice raises the possibility that among humans reassortment events between subtypes H3N2 and H5N1 could generate a novel influenza virus that could spread rapidly, resulting in many deaths. To prioritize areas where future reassortment is most likely to occur, we analyzed surveillance data for subtype H5N1 among poultry in the People's Republic of China and Egypt and subtype H3N2 among humans. We chose China and Egypt because both countries have had recent outbreaks of subtype H5N1 infection among poultry, human deaths from subtype H5N1 infection, and extensive spatial data on cases of infection with subtype H5N1. This information would help decision makers implement policies to reduce spillover in these areas (*9*). Areas with high risk for co-occurrence of these 2 influenza virus subtypes along with high densities of susceptible hosts, such as swine, quail, or turkeys, could benefit from enhanced monitoring and farm and market biosecurity.

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Materials and Methods

Influenza Data

Egypt

All data for Egypt were aggregated to the scale of the markaz, an administrative district that includes several villages. The dataset for subtype H5N1 infections

during 2009–2012 consisted of 453 cases among poultry in backyard flocks, farms, and live-bird markets in 35 markazes. Screening assays are described elsewhere (*10*). See online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/19/4/12-0903-Techapp1.pdf, for a workflow analysis. Most (72%) positive samples came from chickens in backyard flocks. The available geographic data on subtype H3N2 in Egypt are limited (online Technical Appendix Table 3), so we used human population density as a surrogate for cases of infection with subtype H3N2 because virtually all humans (except those who have been vaccinated or infected recently) will be susceptible to infection with subtype H3N2.

China

All data for China were aggregated to the spatial scale of the prefecture, an administrative unit within a province that typically contains several towns and villages. We examined 2 independent datasets for cases of subtype H5N1 infection on the basis of outbreaks on poultry farms and active surveillance of live-bird markets (*11*). The data on cases of subtype H3N2 infection were retrieved by querying GenBank and the EpiFlu database available from the Global Initiative on Sharing All Influenza Data (GISAID) website (http://platform.gisaid.org) for all occurrences of subtype H3N2 in China that included fine-scale geographic data on the prefecture in which the sample was collected (online Technical Appendix Table 1). Data on subtype H3N2 cases were available for 35 prefectures and comprised 632 human cases collected over 14 years. However these data are limited because in a typical influenza year in China, hundreds of millions of cases might occur. Therefore, we also compared the GenBank and GISAID data on subtype H3N2 cases with human population density, assuming that the density is a proxy for the true number of subtype H3N2 cases.

Ecologic Variables

Subtype H3N2

For China, we predicted the probability of occurrence of subtype H3N2 cases by using environmental factors hypothesized in previous studies to be major drivers of human influenza: human population density, percentage urban area, precipitation, and temperature (online Technical Appendix Table 2). For instance, we incorporated population into the model because we hypothesized that human influenza cases would be more likely to occur in high-density urban areas with a large number of susceptible human hosts (*12*). For Egypt, we used human population density as a proxy for subtype H3N2 infections.

Subtype H5N1

To predict occurrence of subtype H5N1 cases, we used the following as environmental covariates: chicken and duck density, human population density, percentage of each prefecture occupied by bodies of water, and percentage of cultivated cropland per prefecture or markaz (online Technical Appendix Table 2). These variables were included because results of previous studies have associated them with risk for subtype H5N1 (*11*,*13*,*14*). For example, human population was included as a predictor of subtype H5N1 because it serves as an indirect measure of intensity of poultry trade (*15*). For Egypt, we used overall poultry density because density of chickens and ducks separately was not available.

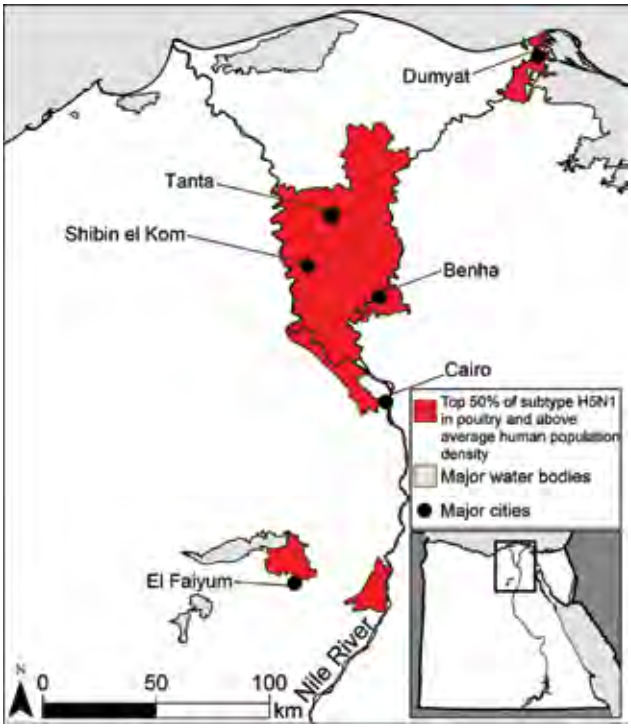

Figure 1. Potential influenza reassortment areas in Egypt. Districts in red are predicted to have an above average number of cases of influenza subtype H5N1 virus in poultry and an above average human population density, which is a proxy for subtype H3N2 virus infections.

Swine Density

To identify potential areas of influenza reassortment, we refined the co-occurrence maps by also incorporating swine density. We used density data from the Food and Agriculture Organization of the United Nations, which constructed these data by extrapolating from agricultural censuses and livestock surveys by using regression models (*16*). The rationale was to focus on areas where subtypes H3N2 and H5N1 might exchange genes in livestock because swine support co-infections with multiple lineages of the influenza virus, which occasionally generate novel strains (*17*,*18*).

Statistical Models

Egypt

We constructed a Poisson regression model in which the dependent variable represents the count of a rare event. The dependent variable was the number of cases of subtype H5N1 in poultry per district. The independent variables were poultry density, human population density, percentage cropland, and percentage water per district. The dataset included sites where poultry were negative for subtype H5N1. We identified districts predicted to have an above average number of cases of subtype H5N1 among poultry and above average human population density. Such districts could be the site of double infections with subtypes H3N2 and H5N1 in humans and of in vivo reassortment.

China

We used multivariate logistic regression to relate occurrence of subtypes H3N2 and H5N1 to the aforementioned ecologic variables (online Technical Appendix Table 2). The logistic regression models were built by using population density, percentage urban area, temperature, and precipitation as predictors of subtype H3N2 presence and chicken and duck density, human population density, percentage cropland per prefecture, and percentage water as predictors of subtype H5N1 presence. The datasets comprise occurrences of subtypes H5N1 or H3N2 but lack negative occurrences. Therefore, we selected negative sites at random and then fitted a logistic regression model to the positive and random negative occurrences. To reduce the bias of random negative occurrences, we selected negative sites at random 10,000× and calculated the average of the parameters of the logistic regression model over these randomizations.

Results

Egypt

Areas with a high number of cases of subtype H5N1 in poultry and high human population density, which we used as a surrogate for subtype H3N2 infections in humans, were located in the Nile Valley and Delta in Lower Egypt (Figure 1). These areas could be sites of human co-infection with subtypes H3N2 and H5N1, leading to the evolution of novel influenza strains. Major cities located within 10 km of potential reassortment hotspots are Benha, Cairo, Dumyat, El Faiyum, and Shibin el Kom, which could be prioritized for increased surveillance to detect reassortment events and prevent spread. Poultry density per district was a highly statistically significant predictor of subtype H5N1 in poultry (Table), probably because high bird densities facilitate transmission of the virus among flocks in a village. The percentage of cropland per district was highly correlated with poultry density ($\rho = 0.72$), so we included only the latter in the regression model. The percentage of water per district also approached significance, which could be because family compounds in rural areas where backyard flocks are raised are typically located near canals and irrigated fields.

China

Cases of subtype H3N2 in humans in China were mostly concentrated along the east coast (online Technical Appendix Figure 2, panel A). The association between subtype H3N2 and human population density was significant (Table). Human population density, climate, and the percentage of urban areas per prefecture collectively explained ≈60% of the risk for subtype H3N2 occurrence ($R^2 = 0.596$, area under the curve [AUC] = 0.902). Subtype H3N2 is expected to occur primarily in central, eastern, and southern China (online Technical Appendix Figure 2, panel B). In the surveillance and the outbreak datasets, statistically significant drivers of subtype H5N1 occurrences were human population, duck density, and percentage of water. The models for subtype H5N1 had moderate predictive power (R^2 surveillance = 0.604, AUC surveillance = 0.918, R^2 outbreak = 0.424, AUC outbreak = 0.848).

After creating maps of the probability of occurrence of subtypes H3N2 and H5N1, we multiplied the maps by one

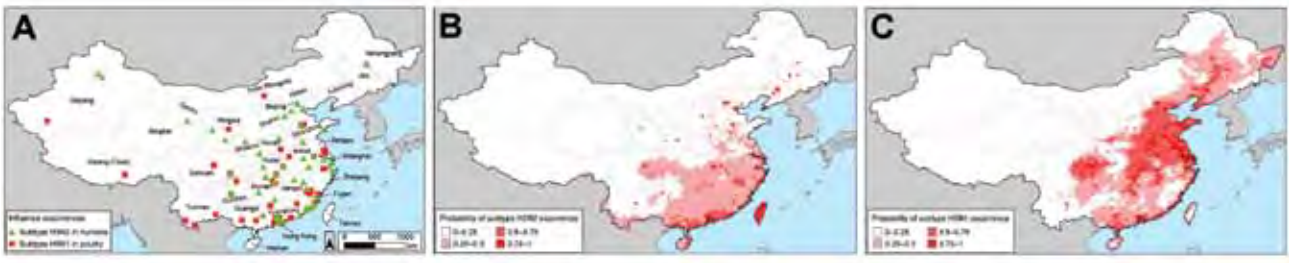

Figure 2. Influenza empirical data and occurrence maps for influenza virus subtypes H3N2 and H5N1. A) Observed cases of subtypes H3N2 and H5N1 in People's Republic of China, according to outbreaks reported to the Chinese Ministry of Agriculture. B) Spatial model of the probability of subtype H3N2 at the prefecture scale predicted by using logistic regression. C) Risk for subtype H5N1 according to the outbreak dataset. See online Technical Appendix Figure 2, wwwnc.cdc.gov/EID/article/19/4/12-0903-Techapp1.pdf, for the corresponding map for the surveillance dataset.

Table. Effect of environmental variables on occurrence of influenza virus subtypes*

Location, subtype (data source)	Coefficient	SE	p value
China			
Subtype H3N2			
Intercept	−5.184	0.946	**8.7×10^{-8}**
Human population	9.47×10^{-4}	3.23×10^{-4}	**4.47×10^{-2}**
Percentage urban	0.113	0.117	0.284
Precipitation	4.59×10^{-3}	8.87×10^{-3}	0.573
Temperature	1.24×10^{-2}	8.27×10^{-3}	0.195
Subtype H5N1 (surveillance dataset)			
Intercept	−7.93	1.66	**3.01×10^{-5}**
Chicken density	-6.94×10^{-2}	0.438	0.572
Duck density	1.53	0.433	**3.45×10^{-3}**
Human population	1.41×10^{-3}	2.85×10^{-4}	**1.77×10^{-4}**
Percentage agriculture	2.18×10^{-4}	1.52×10^{-4}	0.218
Percentage water	7.32×10^{-2}	1.7×10^{-2}	**2.36×10^{-3}**
Subtype H5N1 (outbreak dataset)			
Intercept	−9.24	1.14	**3.15×10^{-12}**
Chicken density	1.24	0.277	**2.87×10^{-4}**
Duck density	0.542	0.24	0.117
Human population	1.37×10^{-3}	2.67×10^{-4}	**1.41×10^{-3}**
Percentage agriculture	2.13×10^{-4}	8.84×10^{-5}	5.4×10^{-2}
Percentage water	0.101	1.91×10^{-2}	**4.53×10^{-3}**
Egypt			
Subtype H5N1			
Intercept	1.83	0.516	**4×10^{-4}**
Poultry density	7.86×10^{-4}	2.31×10^{-4}	**7×10^{-4}**
Human population	3.36×10^{-2}	6.49×10^{-2}	0.605
Percentage water	0.752	0.41	6.64×10^{-2}

*__Boldface__ indicates p<0.05.

another to predict the probability of co-occurrence of the 2 subtypes (Figure 3, panel B; online Technical Appendix Figure 3, panel B). We classified an area as a potential reassortment hotspot if the probability of both subtypes occurring at the site was >50% and the density of swine was above average. Analysis of other swine density thresholds yielded similar results. The consensus of the spatial models (Figure 3, panel C; online Technical Appendix Figure 3, panel C) is that in China, there are 2 main geographic foci of risk for reassortment of subtypes H3N2 and H5N1: 1) the coastal provinces bordering the South China Sea and East China Sea (Guangdong, Jiangsu, Shanghai, and Zhejiang Provinces) and 2) central China (Hunan and Sichuan Provinces). The added value of modeling areas where subtypes H3N2 and H5N1 co-occur versus modeling based exclusively on subtype H5N1 is that the former approach pinpoints a smaller geographic region that can be prioritized for increased surveillance or farm biosafety. Mapping areas based on the probability of subtype H5N1 occurrence alone would prioritize additional provinces to the southwest (Henan, Hebei, and Hubei) and to the north (Beijing, Hebei, Liaoning, and Tianjin) of the 6 provinces that we identified as potential areas for reassortment between subtypes H3N2 and H5N1 (Figure 2, panel C; online Technical Appendix Figure 2, panel C). Our prioritization of a smaller geographic area is valuable if the resources for surveillance are insufficient to enable sampling of all of the provinces that are at risk for subtype H5N1.

East Asia

We applied the influenza virus subtypes H3N2 and H5N1 logistic regression models that were fitted to the data from China to neighboring countries for which chicken and duck density data were available (*19*). As in the analysis for China, we multiplied the subtype H3N2 and H5N1 models to predict areas of co-occurrence between the subtypes and overlaid swine density. To the extent that these areas have above average swine density and a >50% chance for co-occurrence of subtypes H3N2 and H5N1, potential reassortment hotspots are the northern plains of India (Uttar Pradesh), the western Korean Peninsula (Daejeon, Gyeonggi, Jeollabuk Provinces of South Korea and Pyonganbuk and Pyongannam Provinces of North Korea), and southwestern Japan (Saga Prefecture on Kyushu Island) (Figure 4, panel B; Technical Appendix Figure 4, panel B). Major cities with >500,000 persons near these hotspots include Kanpur, India; Chengdu, Sichuan, central China; Hangzhou and Shanghai, eastern China; and Seoul, South Korea. Risk is higher in these cities because they have high densities of swine, which could be a mixing vessel for reassortment of subtypes H5N1 and H3N2, and a high potential for infection with subtype H5N1 and H3N2 according to our regression models; for example, the models indicate that the ecologic suitability of Shanghai is 0.97 for subtype H3N2 and 0.996 for subtype H5N1. Incorporating population density as a proxy for infection with subtype H3N2 results in predictions that are compatible with the models based on swine density but also identifies 2

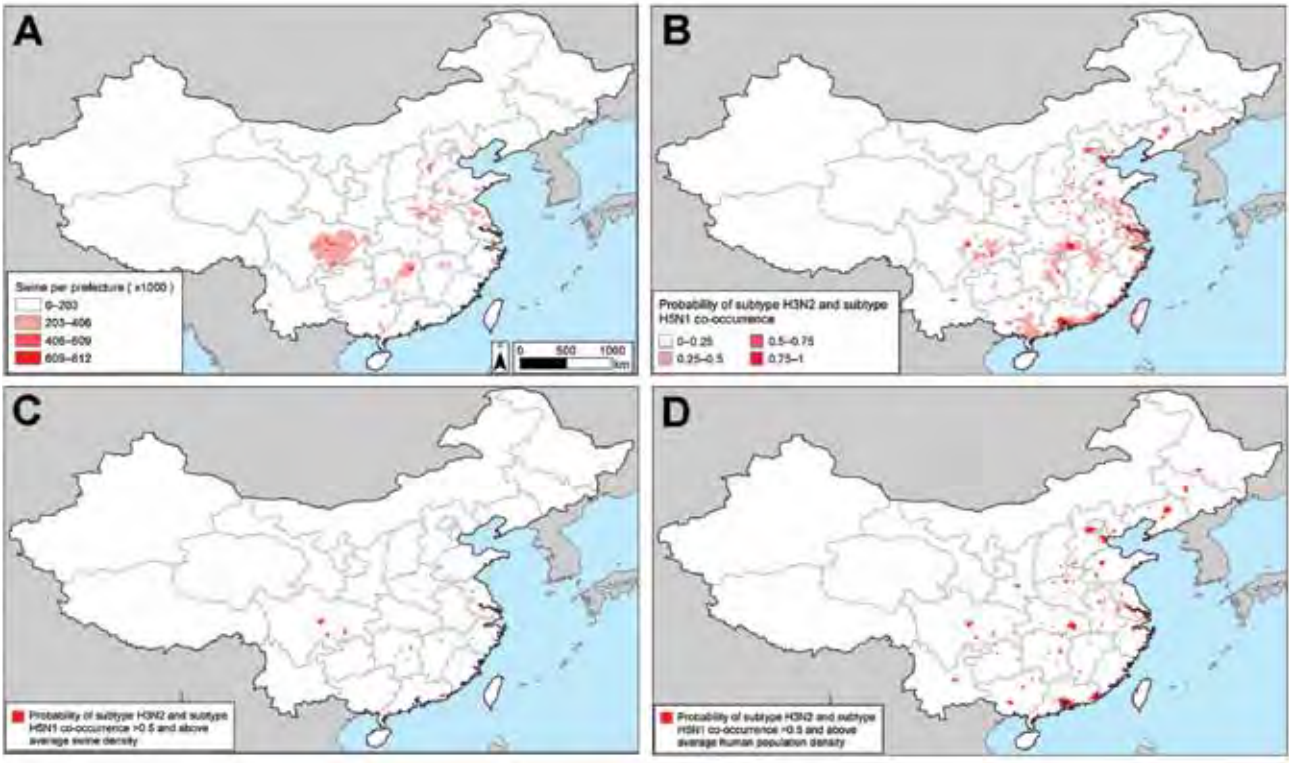

Figure 3. Potential influenza reassortment areas in People's Republic of China determined by using the influenza virus subtype H5N1 outbreak dataset. A) Density of swine. B) Spatial model of the risk for subtype H3N2 and H5N1 co-occurrence according to the outbreak dataset. C) Areas with a probability of subtype H5N1 and H3N2 co-occurrence >50% and above average swine density. D) Areas with a probability of subtype H5N1 and H3N2 co-occurrence >50% and above average human population density. See online Technical Appendix Figure 3, wwwnc.cdc.gov/EID/article/19/4/12-0903-Techapp1.pdf, for corresponding maps based on the subtype H5N1 surveillance dataset.

other megacities of >10 million persons that could be at high risk for virus reassortment: Dhaka, Bangladesh and Delhi, India (Figure 4, panel C; online Technical Appendix Figure 4, panel C).

Discussion

The spatial models presented here predict that a reassortant influenza (H3N2/H5N1) virus is most likely to originate in the coastal and central provinces of China or the Nile Delta region of Egypt. The probability that subtypes H3N2 and H5N1 will co-occur in these regions is high (Figure 1; Figure 3, panel C; online Technical Appendix Figure 4, panel C), which could lead to dual infection in mammalian hosts, such as swine or humans in China or humans in Egypt. Co-infection could subsequently result in in vivo reassortment. Although the influenza A(H1N1) pdm09 virus is hypothesized to have originated from Mexico (*20*), southern China remains a major hotspot for the generation of novel influenza viruses (*21*). Our spatial models are compatible with this longstanding observation insofar as we predict that the southern coastal province of Guangdong is a potential hotspot for the evolution of novel influenza viruses by reassortment.

A caveat is that even if virus subtypes H3N2 and H5N1 were to reassort in swine, the spread of the reassortant virus among humans might require further virus adaptation events; for example, mutations might be required for the virus to replicate efficiently in humans or to be transmitted among humans (*22*). Recent work has shown that as few as 5 aa substitutions are required for aerosol spread of subtype H5N1 among mammals (*23*). With these qualifications in mind, this analysis provides actionable recommendations about which areas to target for intensified farm and market surveillance. Such surveillance could enable early detection of a reassortant influenza (H3N2/H5N1) virus, should it arise in swine, and facilitate containment of the virus before it crosses the species barrier to humans.

Our finding that in China the probability of subtype H3N2 infection increases with human population density is compatible with previous studies that detected a positive association between population, influenza cases, and mortality rates (*12*,*24*). Reasons for this association could be that the number of susceptible human hosts increases with population (*11*) or that surveillance efforts are greater in populous areas (*25*). Our results with regard to subtype H5N1 in birds are also largely consistent with those of previous

Figure 4. Reassortment areas elsewhere in Asia based on the People's Republic of China model constructed from the influenza virus subtype H5N1 outbreak dataset. A) Probability of subtype H3N2 and H5N1 co-occurrence (according to the subtype H5N1 outbreak dataset). B) Areas with a probability of subtype H5N1 and H3N2 co-occurrence >50% and above average swine density. C) Areas with a probability of subtype H5N1 and H3N2 co-occurrence >50% and above average human population density. See online Technical Appendix Figure 4, wwwnc.cdc.gov/EID/article/19/4/12-0903-Techapp1.pdf, for corresponding models based on the surveillance dataset.

studies that mapped subtype H5N1 hotspots in China and Egypt. In China, several provinces identified as having high ecologic suitability for subtype H5N1 (including Shandong, Jiangsu, and Sichuan) were also identified as subtype H5N1 hotspots in a previous study that used a different statistical model and different predictor variables (*11*). In China, previous analyses have concluded that risk for subtype H5N1 increases with the density of domestic ducks (*26*). In Egypt, earlier studies identified high-intensity crop production as a statistically significant predictor of subtype H5N1 in poultry (*27*). Similarly, we found that subtype H5N1 infections in poultry were associated with poultry density, which was highly correlated with crop production. In a previous study, models constructed from satellite images of vegetation predicted that the highest environmental suitability for subtype H5N1 is along the Nile River and in the Nile Delta (*28*). Our models were constructed from different predictor variables, such as poultry density, but yielded similar results: the highest number of subtype H5N1 cases in poultry were predicted to occur in districts in the Nile Delta.

Efforts to contain the A(H1N1)pdm09 virus would have been more effective if the virus had been detected in animal populations before it was transmitted to humans (*29*). Continuous zoonotic influenza surveillance is needed in China and Egypt and requires a network of laboratories to screen surveillance samples and requires financial incentives to encourage poultry producers and sellers to report outbreaks. One strategy for early detection of a reassortant virus could involve increasing farm and market surveillance in the identified areas (i.e., live-bird markets in 6 provinces in China [Guangdong, Hunan, Jiangsu, Shanghai, Sichuan, and Zhejiang] that have a >50% chance of subtype H3N2 and H5N1 co-occurrence and above average swine density). Increased monitoring could identify hotspots where subtype H5N1 is circulating, leading to more efficient targeted vaccination of poultry, and could pinpoint prefectures at high risk for a reassortant virus. In China, sanitary practices, such as cage disinfection and manure disposal, would substantially reduce risk for subtype H5N1 in live-bird markets (*30*).

In Egypt, our results support increased surveillance of backyard flocks near Benha, Cairo, Dumyat, El Faiyum, Shibin el Kom, and Tanta, where suitability for subtypes H5N1 and H3N2 is predicted to be high. Control measures could include compensation plans and vaccination of poultry with a recently developed subtype H5N1 vaccine that is more effective than previous vaccines against strains circulating in Egypt (*10*). Reporting of poultry disease outbreaks in Lower Egypt is poor (*31*), probably because farmers fear loss of income if authorities cull their flocks. Indeed, birds suspected to be infected with subtype H5N1 are often sold quickly at a discount, resulting in virus transmission to buyers' flocks and families (*32*). If equitable compensation schemes were implemented, reporting of subtype H5N1 might increase and outbreaks could be contained more quickly, reducing opportunities for subtypes H5N1 and H3N2 to co-infect humans or domestic animals and, thus, for reassortment.

In general, policies such as culling must have a scientific basis because these measures have major effects on the economy and animal welfare. For example, when part of a swine herd is culled to contain an outbreak, it might become necessary to euthanize the entire herd, including animals with no influenza exposure, because buyers will not accept them (*33*). Furthermore, influenza outbreaks among livestock can trigger major global declines in meat prices, and the nature and timing of veterinary health authorities' responses to an outbreak can affect the extent to which demand recovers after the crisis. In particular, when control measures such as culling are scientifically well justified and explained to the public soon after the start of an outbreak, consumer confidence is restored more quickly (*34*).

Although our maps suggest a risk for reassortment in Lower Egypt and eastern and central China, in vivo reassortment of subtypes H3N2 and H5N1 has not been detected in humans in these areas. On the other hand, numerous infections with influenza (H3N2)v, a reassortant virus that contains genes from a subtype H3N2 virus circulating in swine and from the A(H1N1)pdm09 virus, have been detected in humans in North America (*35*,*36*). This finding raises the question of why subtype H3N2v

has spread but subtype H3N2/H5N1 reassortants have not. Spread of subtype H3N2v could result from the fact that the reassortant virus contains the M gene from the A(H1N1) pdm09 virus, which increases aerosol transmission (*35*,*37*). Our models might explain why, in contrast with subtype H3N2v reassortants, no subtype H3N2/H5N1 reassortants have been detected in humans. For example, we predict that subtypes H3N2 and H5N1 occur in Hunan, China, a province that has high swine density and was the geographic origin of subtype H5N1 viruses in clade 2.1 (*38*). Influenza (H3N2/H5N1) reassortants in which the nonstructural gene comes from a clade 2.1 virus replicate poorly in mice (*5*). Thus, subtype H3N2/H5N1 reassortants might not have emerged as often as subtype H3N2v reassortants because the provinces where subtypes H3N2 and H5N1 overlap contain a clade of subtype H5N1, whose genes reduce the fitness of reassortant viruses. If this hypothesis is correct, if subtypes H5N1 and H3N2 infect a pig in central China and exchange genes, the hybrid virus might not replicate efficiently or transmit to other hosts. Furthermore, a reassortant virus with surface proteins similar to those of subtype H3N2 viruses that have circulated in humans recently might have poor transmissibility because of preexisting immunity (*18*).

Applying our modeling framework to other zoonotic influenza subtypes, such as H3N2v, could yield insight about geographic hotspots of reassortment and the pattern of spatial spread of reassortants. To accomplish this, 2 data limitations must be overcome. First, to be incorporated into spatial models, influenza sequences submitted to GenBank or GISAID should be accompanied by geographic data at relatively high spatial resolution, for example, names of cities or counties where sampling was conducted. However, such sequences are often accompanied by only the state or country of the sample, which reduces the usefulness of the data for fine-scale spatial modeling (*39*). For example, we searched online databases and confirmed that the geographic data available for Indonesia are insufficient to construct a spatial model to predict sites with a high risk for reassortment. Second, more extensive surveillance of livestock is needed to provide sufficient sample sizes to parameterize geographic models. Currently, the number of influenza subtype H1, H3, and H5 viruses from swine in major databases is an order of magnitude lower than that available for humans (online Technical Appendix Table 3). Additional surveillance of swine could lead to better predictions about hotspots of influenza in livestock and sites of potential swine-to-human transmission. Livestock surveillance campaigns should sample large geographic areas and include regions where production is high (*35*).

The potential for reassortment between human and avian influenza viruses underscores the value of a One Health approach that recognizes that emerging diseases arise at the convergence of the human and animal domains (*29*,*40*). Although our analysis focused on the influenza virus, our modeling framework can be generalized to characterize other potential emerging infectious diseases at the human–animal interface.

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Effect of 10-Valent Pneumococcal Vaccine on Pneumonia among Children, Brazil

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Pneumonia is most problematic for children in developing countries. In 2010, Brazil introduced a 10-valent pneumococcal conjugate vaccine (PCV10) to its National Immunization Program. To assess the vaccine's effectiveness for preventing pneumonia, we analyzed rates of hospitalization among children 2–24 months of age who had pneumonia from all causes from January 2005 through August 2011. We used data from the National Hospitalization Information System to conduct an interrupted time-series analysis for 5 cities in Brazil that had good data quality and high PCV10 vaccination coverage. Of the 197,975 hospitalizations analyzed, 30% were for pneumonia. Significant declines in hospitalizations for pneumonia were noted in Belo Horizonte (28.7%), Curitiba (23.3%), and Recife (27.4%) but not in São Paulo and Porto Alegre. However, in the latter 2 cities, vaccination coverage was less than that in the former 3. Overall, 1 year after introduction of PCV10, hospitalizations of children for pneumonia were reduced.

Streptococcus pneumoniae infections are the leading cause of bacterial pneumonia, meningitis, and sepsis among children (*1*,*2*); in developing countries, these infections account for almost a half million deaths among children <5 years of age (*3*). In Brazil, the largest country in South America, the role of *S. pneumoniae* in pneumonia in children is considerable (*4*,*5*).

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Brazil is composed of 5 administrative regions with different climatic and socioeconomic characteristics. In 2010, the estimated population of infants (children <12 months of age) was ≈2,800,000, and the infant mortality rate was 17 deaths per 1,000 live births (*6*,*7*). In Brazil, the main reason for hospitalization of infants is pneumonia (*6*).

Vaccination with pneumococcal conjugate vaccine (PCV) is a public health intervention to prevent pneumococcal disease. PCV has been in use since 2000, when a 7-valent vaccine (PCV7) was licensed in the United States for routine use in children. In 2010, PCV7 was replaced by a 13-valent vaccine. Recently, a 10-valent pneumococcal conjugate vaccine (PCV10) was licensed in Brazil; this vaccine includes the same serotypes that are in PCV7 (4, 6B, 9V, 14, 18C, 19F, 23F), plus 3 more (1, 5, and 7F) (*8*).

In 2010, Brazil introduced PCV10 into its routine National Immunization Program. Previously, no PCV had been incorporated into the routine immunizations. The vaccination was introduced in all cities from March through September 2010; 3 doses (at 2, 4, and 6 months of age) plus 1 booster (at 12–15 months of age) were recommended. Two routine catch-up schedules were also in place: 1) two doses for children 7–11 months of age plus a booster at 12–15 months of age, and 2) one dose for children 12–24 months of age. PCV10 is not given to children >24 months of age (*9*).

In Brazil, vaccination of children with PCV10 is free through the National Unified Health System (*10*). By October 2011, the mean vaccination coverage rapidly reached 80% for a full primary series for children <12 months of age in >5,000 municipalities (Brazilian Ministry of Health, unpub. data).

Studies that assessed the effect of PCV7 found a statistically significant reduction in the overall incidence of

invasive pneumococcal disease and hospitalizations for pneumonia among children <2 years of age shortly after the first year of vaccination (*11–14*). Our aim was to assess the effectiveness of PCV10 for reducing hospitalizations for all-cause pneumonia. We analyzed trends in rates of hospitalization for pneumonia among children soon after the introduction of PCV10 in Brazil. Ethical approval was granted by the Ethics Committee, Federal University of Goiás, Goiania, Brazil.

Methods

Data Sources

We conducted an interrupted time-series analysis by using individual-level secondary data from the Hospitalization Information System of the National Unified Health System from January 2005 through August 2011. The Hospitalization Information System records ≈75% of all hospitalizations in Brazil and 60%–80% of the hospitalizations for the cities in the analyses (*15*). During the study period, there were no major changes in the amount of hospital care provided by the National Unified Health System.

Variables in the Hospitalization Information System are demographics, date of admission/discharge, residential address, hospital code, and International Classification of Diseases 10th Revision (ICD-10) codes for primary and secondary diagnoses. Because the Hospitalization Information System database is mainly used for reimbursement purposes, the likelihood that hospitalizations would be underreported and that data would be missing are small (*16*).

The structure of the Hospitalization Information System made it possible for 1 episode of hospitalization for a given patient to be recorded multiple times. Additional records might be generated when patients remain hospitalized longer than anticipated. To avoid including duplicate records, we used a deterministic record linkage algorithm to find records for the same patient (*17*). We then considered that consecutive records of the same patient with a 14-day interval between discharge and reentry belonged to the same episode of disease (*18*).

We studied 5 state capital cities in Brazil: Belo Horizonte, Curitiba, Recife, São Paulo, and Porto Alegre (Figure 1). The cities were initially selected from a list of 10 cities participating in an ongoing case–control study evaluating the effect of PCV10 vaccination on pneumococcal disease. The selection of cities for the case–control study was based on data quality and willingness of the local surveillance teams to participate in the study. Of the initial 10 cities, 5 were excluded a priori from the time-series analysis; 3 cities had not reached vaccination coverage of at least 75% for the first dose of vaccine (primary series) 3 months after vaccine introduction, and 2 cities were excluded because of poor data quality in the initial descriptive analyses. The 5 chosen cities account for 50% of the population of the state capitals of the country and are located in 3 of the 5 administrative regions of the country.

The annual numbers of live births were obtained from the Live Birth Information System and used to calculate the annual population of children 2–24 months of age. The monthly population was calculated by interpolating an exponential growth model to the annual data.

Figure 1. Capital cities of Brazilian states, and their populations, in which effectiveness of 10-valent pneumococcal vaccine was studied. Population data obtained from Brazilian Census 2010.

Definitions

We identified Hospitalization Information System records of children 2–24 months of age who were hospitalized from January 2005 through July 2011 with specific ICD-10 codes: pneumonia (J12-J18), bronchiolitis (J21), respiratory causes (J00–J99), nonrespiratory causes, and all causes (*19*). We considered nosocomial pneumonia more likely to be reported as a secondary discharge diagnosis; therefore, only the primary diagnosis of the first record of each episode of disease was used in all data analyses.

In a descriptive analysis, which included only the prevaccination period, we obtained average annual numbers and rates of pneumonia hospitalizations for each city and the proportion of pneumonia out of all respiratory causes and out of all causes of hospitalizations. Specific pneumococcal pneumonia–coded cases in the Hospitalization Information System represented only 0.06% of the pneumonia cases reported in the system because confirming bacteriologic pneumonia in children is difficult; thus, we considered use of pneumococcal pneumonia–coded cases not appropriate in a time-series analysis.

Vaccination Coverage

In Brazil, the National Immunization Program, established in 1973, led to high rates of coverage (*20*). PCV10

vaccination was introduced in March 2010 in all selected cities except Porto Alegre, where it started in June 2010. PCV10 coverage data for each city were obtained from the National Immunization Program vaccine coverage database of the National Unified Health System, in which number of vaccine doses and administrative vaccination coverage are made available for all municipalities in the country. Numerator data are obtained from the number of doses administered in the vaccination rooms, by vaccine type, patient age, and municipality. PCV10 coverage for a full PCV10 primary series (3 doses) was estimated as the number of third doses of PCV10 administered (numerator) to children <12 months of age divided by the number of births in a population over time in each municipality (denominator) multiplied by 100 (see online Technical Appendix, wwwnc.cdc.gov/EID/article/19/4/12-1198-Techapp1.pdf, for sources of data on vaccination coverage).

We calculated the moving average of vaccine coverage for every 3-month period. The value attributed to a given month was the average vaccine coverage in that month and the coverage for the months before and after the given month. This calculation was done to smooth out short-term vaccination coverage fluctuations (Figure 2).

Data Analyses

In the interrupted time-series analysis, 3 immunization periods were defined: prevaccination, transition, and postvaccination. The prevaccination period was January 2005–February 2010 and had 62 time points (monthly data) in the final model (except for Porto Alegre, which had 65 time points because vaccination introduction was delayed for 3 months). The transition period was the time of vaccine introduction through 4 months after. The postvaccination period was the time after the transition period; it comprised 14 time points in the analysis (3 fewer for Porto Alegre). The transition period was excluded from analysis, although it is shown in the figures. The time-series analysis was based on a generalized linear model for rates of hospitalizations for pneumonia and for nonrespiratory causes by using the negative binomial distribution with a logarithmic link function and an offset equal to the log of the population divided by 100,000 (*21*). Residual analyses showed no substantial deviations from model assumptions. The main outcome was rates of hospitalization for all-cause pneumonia. The explanatory variables in the model were calendar month (to control for seasonality), linear trend over time (to control for preexisting trends), and a variable equal to 1 after vaccination and 0 otherwise. After estimation of the models, 2 outputs were presented: 1) the percentage change in hospitalization rates, which compare the prevaccination and postvaccination periods and their corresponding p values and 95% CIs, and 2) a graph showing the predicted hospitalization rates for pneumonia and their 95% CIs for the postvaccination period based on models fitted with data for the prevaccination period. With the latter output, it is possible to visually

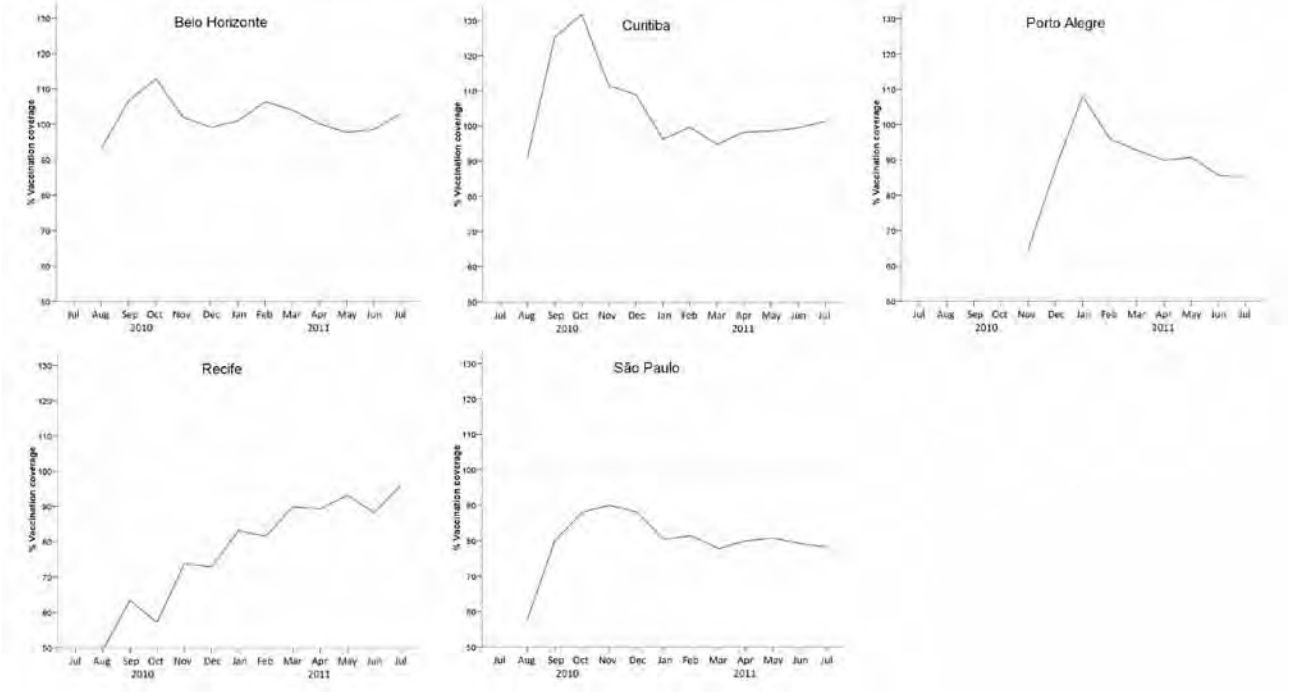

Figure 2. Monthly coverage for third dose of 10-valent pneumococcal vaccine achieved 11–14 months after vaccination among children <12 months of age in 5 cities in Brazil. Dotted horizontal lines represent 100% vaccination coverage.

evaluate the observed and the predicted monthly hospitalization rates for the postvaccination period; that is, the rates that would have resulted had the changes during the transition period not taken place.

Sensitivity analyses for each city compared models with and without removal of the months of July and August 2009. This comparison was an attempt to reduce potential bias resulting from the influenza epidemic. To compare the estimated percentage change for hospitalizations for pneumonia and nonrespiratory causes, we performed separate time-series analyses. In theory, the vaccination-induced percentage change would be higher for hospitalizations for pneumonia than for nonrespiratory causes, although a minority of possible pneumococcal disease codes was included in nonrespiratory causes (such as meningitis). The percentage change for hospitalizations for nonrespiratory causes was expected to reflect influences other than the PCV10 vaccination effect that might have concomitantly affected the data series. We expected the effect of reporting/processing delays to still be present during the postvaccination period, therefore inducing an artificially lower number of hospitalizations in more recent months (although we had discarded the most recent ones from analysis). To compensate for this effect, we calculated the differences between the percentage changes in rates of hospitalizations for pneumonia and nonrespiratory causes for each city. The equality of the percentage changes for hospitalization for pneumonia and nonrespiratory causes was tested by using the Wald test (*22*).

The observed trends for bronchiolitis, respiratory, and all-cause hospitalizations are shown for comparison. The linkage/classification procedures were conducted by using STATA version 12.0 (www.stata.com/), and the statistical analysis was done by using R (www.r-project.org/).

Results

In the 5 cities, 197,975 hospitalizations of children 2 months to 2 years of age were identified during the study period; 109,155 (55.1%) were for respiratory causes, including 59,636 (30.1%) for pneumonia. During the prevaccination period, the rates of pneumonia hospitalizations varied substantially by city (Table 1).

Figure 2 shows the moving average for PCV10 coverage (percentage of children <12 months of age who received all 3 doses of PCV10). Vaccination coverage varied by city. Belo Horizonte and Curitiba rapidly reached 100% coverage and maintained stable rates of ≈100% from September 2010 on. Recife showed a tendency toward sustained and continuously rising coverage over the study period, eventually reaching ≈100%. Porto Alegre reached 100% coverage on January 2011, followed by a gradual decrease to 85% in July 2011. São Paulo coverage increased to 90% in November 2010, after which it continuously declined, reaching 75% in July 2011.

Trends in patterns of hospitalization rates for pneumonia, respiratory causes, and all causes are shown for each city (Figure 3). Seasonal variations are evident for all cities. The contribution of pneumonia to the total number of hospitalized patients varied widely by city but not by years. Rates of hospitalization for all causes, particularly from mid-2007 on, decreased notably for Belo Horizonte and Recife; however, the observed reductions in rates of hospitalization for pneumonia for these cities and for Curitiba seem to be restricted to the postvaccination period (from mid-2010 on).

Rates of hospitalization for bronchiolitis were lower than those for pneumonia in all cities except Porto Alegre (Figure 4). The seasonal variations of bronchiolitis and pneumonia were mostly parallel and were found for all cities. Hospitalization rates progressively increased in the more recent years for Porto Alegre, São Paulo, and possibly Curitiba.

Table 2 and Figure 5 show results derived from the same time-series models. During the postvaccination period, rates of hospitalization for pneumonia decreased significantly ($p<0.001$) in Belo Horizonte (–40.3%), Curitiba (-37.6%), and Recife (–49.3%). Rate reductions were borderline significant for São Paulo (-13.4%; $p = 0.074$) and Porto Alegre (-23.5%; $p = 0.052$) (Table 2). Rates of hospitalization for nonrespiratory causes also decreased in all cities, albeit at a lower rate. The following differences between the percentage changes in hospitalization rates for pneumonia and nonrespiratory causes represent our best estimate of the vaccination effect: Belo Horizonte (-28.7%), Curitiba (-23.3%), Recife (-27.4%), São Paulo (-1.8%), and Porto Alegre (-2.3%). During the postvaccination period, reductions in rates of hospitalization for pneumonia did not differ significantly from rates of hospitalization for nonrespiratory causes in São Paulo ($p = 0.827$) and Porto Alegre ($p = 0.845$).

Table 1. Rates of hospitalization for pneumonia among children 2 months–2 years of age, Brazil, prevaccination period (2005–2009)*

City	No. cases, annual mean (± SD)	Rates, annual mean (± SD)	% Hospitalizations for pneumonia/hospitalizations for all respiratory causes	% Hospitalizations for pneumonia/hospitalizations for all causes
Belo Horizonte	939 (133)	1.643 (217)	53.4	34.8
Curitiba	359 (49)	790 (114)	72.2	27.9
Recife	538 (41)	1.304 (107)	47	23.4
São Paulo	3999 (312)	1.247 (103)	61.4	36.1
Porto Alegre	292 (38)	863 (112)	25.8	15

*Pneumonia identified by International Classification of Diseases, 10th Revision, codes: J12–J18.

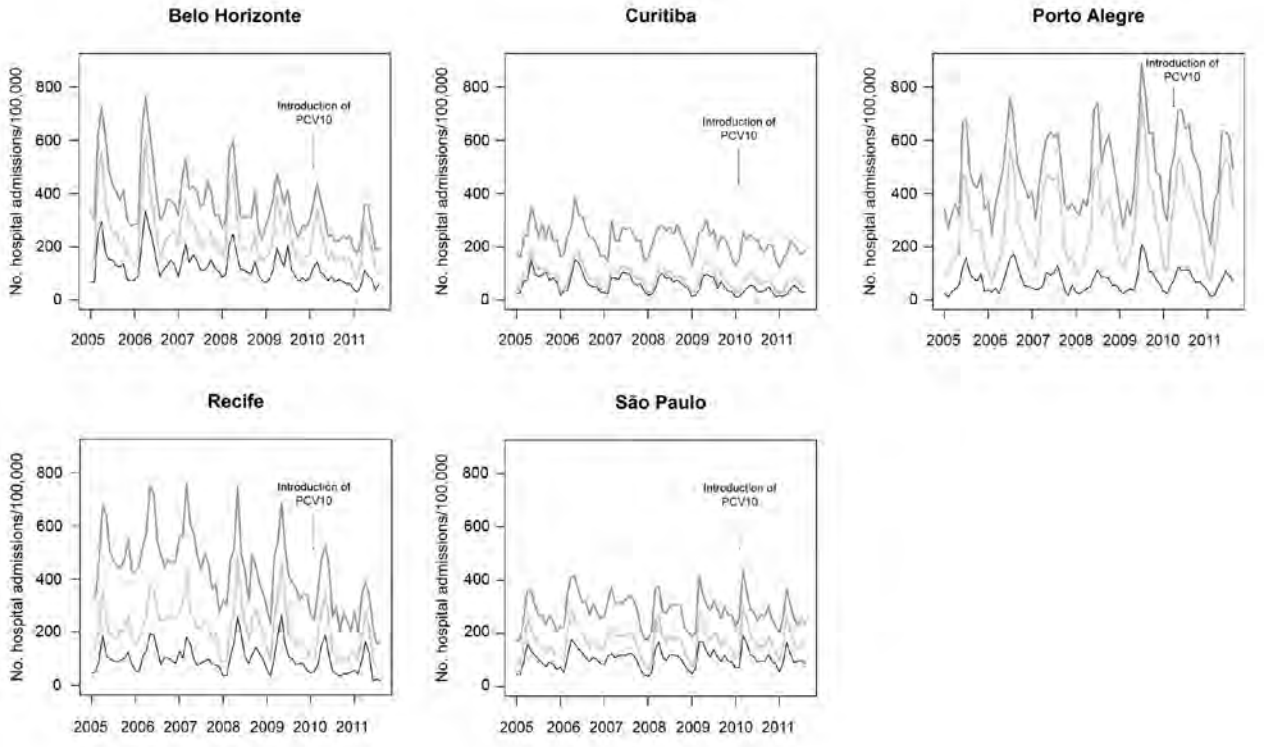

Figure 3. Trends in rates of hospitalization for pneumonia (black) and for all respiratory causes (light gray) and all causes (dark gray) among children 2 months–2 years of age in 5 cities, Brazil, January 2005–August 2011. PCV10, 10-valent pneumococcal vaccine.

Figure 5 compares the observed monthly rates of hospitalization for pneumonia with the forecasted values that were modeled with use of data exclusively from the prevaccination period. For Belo Horizonte, Curitiba, and Recife, the observed numbers are close to or below the lower limit of the 95% CI, particularly for the most recent months.

Discussion

This study indicates that the introduction of PCV10 through the routine immunization program in Brazil has effectively lowered rates of hospitalization for pneumonia among children. Rates of hospitalization for all causes declined in 3 of the 5 cities studied (Belo Horizonte, Curitiba, and Recife). In the other 2 cities (São Paulo and Porto Alegre), these rates did not decline significantly, possibly because vaccination coverage for these 2 cities in 2011 was lower (≈80%) than it was in the other 3 cities (>90%). Another possible reason is that Porto Alegre started its vaccination program 3 months after the other cities, so its postvaccination period was shorter, leaving less time for the vaccination to become effective.

Comparison of our results with those of other studies is not straightforward because, to our knowledge, no comparable studies have been published (e.g., effects of PCV10 on rates of hospitalization for all causes). PCV10 has recently been introduced in some countries in North America and Europe. Preliminary evaluations indicate a reduction of invasive pneumococcal disease. In the province of Quebec, Canada, PCV10 was introduced to the routine immunization schedule 5 years after PCV7 was introduced. Data obtained by a sentinel laboratory surveillance network showed lower incidence of invasive pneumococcal disease among children vaccinated with PCV10 than with PCV7 (35.3 vs. 64.1 cases/100,000 person-years) (*23*). In Finland, results of a recent field trial found a marked decrease in the incidence of invasive pneumococcal disease among children who were vaccinated according to a 3+1 or a 2+1 immunization schedule; vaccine effectiveness reached 100% (95% CI 83%–100%) and 92% (95% CI 58%–100%), respectively, after 2 years (*24*). The Clinical Otitis Media and Pneumonia Study conducted at urban sites in Argentina, Colombia, and Panama showed that the efficacy of PCV10 for reducing community-acquired pneumonia and alveolar consolidation among children was 7.3% and 23.4%, respectively (*25*).

For PCV7, studies have already documented its effect on rates of hospitalization for pneumonia among children (*11*,*12*,*26*–*28*). In the United States, the rates of hospitalization for pneumonia were reduced 39%–52%. However, aside from the use of different vaccines, our study is not directly comparable. Vaccination coverage was generally lower in the United States, increasing from

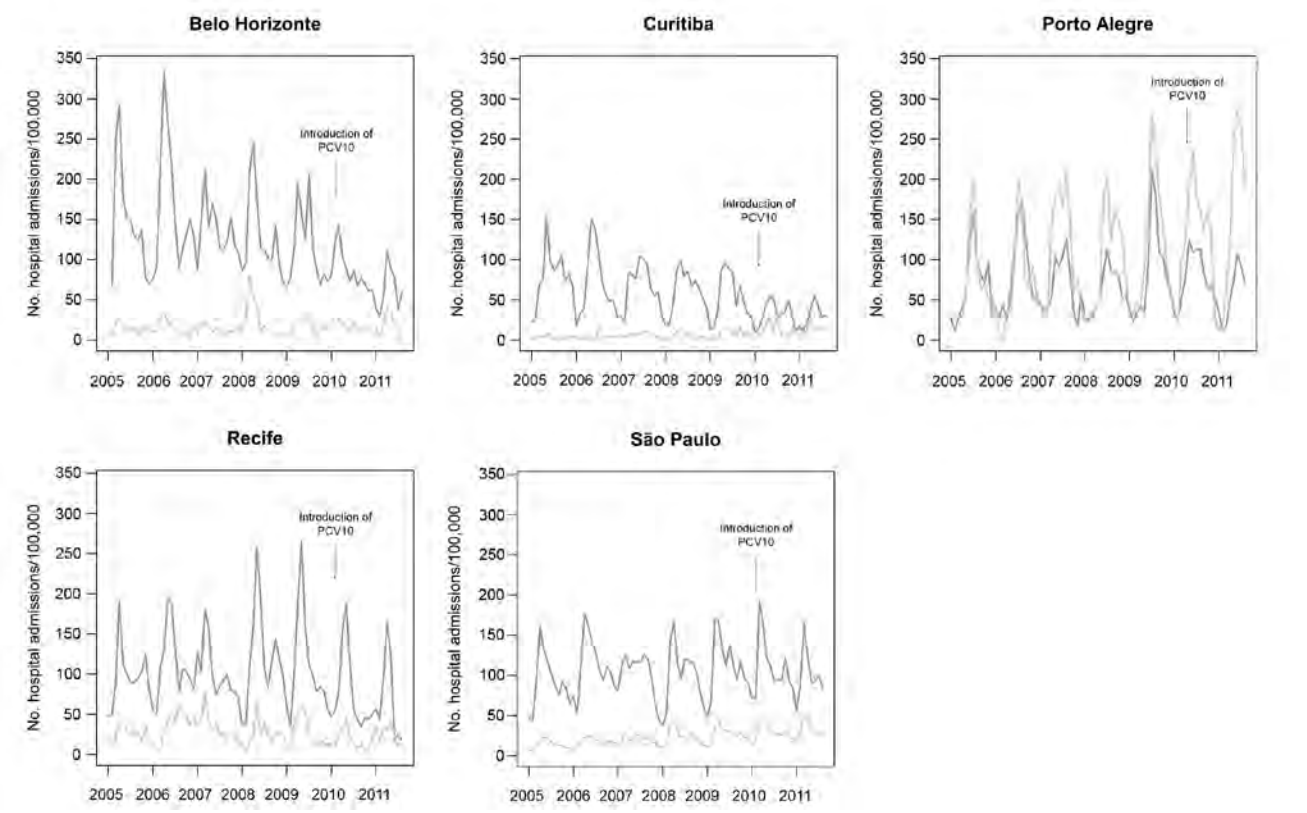

Figure 4. Trends in rates of hospit-alization for pneumonia (dark gray) and bronchiolitis (light gray) among children 2 months–2 years of age in 5 cities, Brazil, January 2005–August 2011. PCV10, 10-valent pneumococcal vaccine.

68% to 83% during the postvaccination period, which was much (4 years) longer. This longer time might have allowed time for herd immunity to protect the nonvaccinated population (*26*,*28*). Also, the illnesses compared in each study were not the same; the United States study evaluated dehydration and diarrhea, whereas our study evaluated all nonrespiratory conditions since the rotavirus vaccine was introduced in 2006 to Brazil.

The introduction of rotavirus vaccination might actually be one of the best explanations for the decreasing trends of all-cause hospitalizations in the target age group during the study period. Another explanation is the rapid increase in coverage of the Family Health Programme. This program reached 85% of Brazilian municipalities in 2010 and greatly reduced deaths and hospitalizations of infants for primary-care sensitive diseases like diarrhea and for lower respiratory tract diseases (*29*–*31*).

Across all 5 cities, we found differences in rates of hospitalization for pneumonia before introduction of PCV10. Marked regional differences had already been documented (*32*). Possible reasons, other than differences in health care provision, are variations in epidemiology, demographics,

Table 2. Annual percent change (trend) and percentage change in rates of hospitalization among children 2 months–2 years of age, Brazil, postvaccination period (January 2005–August 2011)

City	Hospitalizations for pneumonia		Hospitalizations for nonrespiratory causes		Difference in change	p value
	% Change (95% CI)	p value	% Change (95% CI)	p value		
Belo Horizonte	–40.30 (–50.88 to –27.44)	<0.001	–11.61 (–23.48 to 2.10)	0.093	–28.69	0.002
Curitiba	–37.59 (–49.63 to –22.68)	<0.001	–14.27 (–23.94 to –3.38)	0.012	–23.32	0.011
Recife	–49.32 (–61.63 to –33.05)	<0.001	–21.93 (–32.18 to –10.13)	0.001	–27.39	0.007
São Paulo	–13.38 (–26.02 to 1.42)	0.074	–11.60 (–19.31 to –3.15)	0.008	–1.78	0.827
Porto Alegre	–23.51 (–41.60 to 0.18)	0.052	–21.18 (–31.08 to –9.86)	0.001	–2.33	0.845

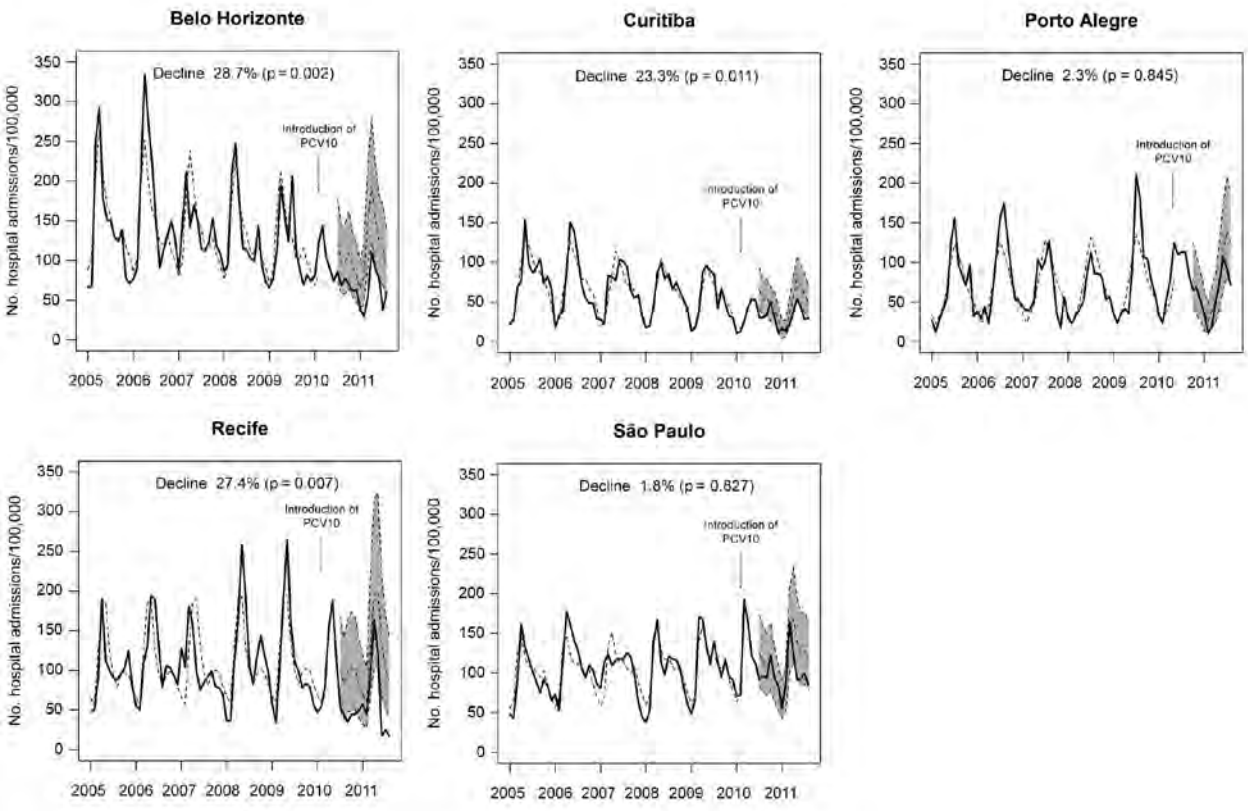

Figure 5. Observed (solid lines) and predicted (dashed lines) rates of hospitalization for pneumonia and 95% CIs (shaded area) among children 2 months–2 years of age in 5 cities, Brazil, January 2005–August 2011. The 95% CIs are shown only for the 4 months after start of vaccination. Decline represents the reduction in hospitalizations for pneumonia. PCV10, 10-valent pneumococcal vaccine.

socioeconomic status, and climate. Health care provisions might play a progressively lesser role in explaining the differences in rates of hospitalization for pneumonia because the results of National Household Sample Surveys show a trend toward equity in access to and use of health care facilities (*31*,*33*).

Several potential limitations of our study should be highlighted. Our data represent only the population served by National Unified Health System in Brazil. The findings observed for the 5 capital cities cannot be considered representative of the entire country. We attempted to include mostly community-acquired cases of pneumonia by restricting our analysis to the primary diagnosis for hospitalization. By doing so, we hypothetically increased the proportion of hospitalizations for pneumococcal pneumonia out of all hospitalizations for pneumonia. Because only a few cases have pneumonia listed as a secondary diagnosis, we missed only a few community-acquired cases of pneumonia by not including it.

Information about the extent of coding errors in Brazil is scarce. None of the information is specific to pneumonia. However, the clinical diagnosis of pneumonia has been shown to have high sensitivity and low specificity for ascertaining pneumococcal pneumonia; thus, any bias resulting from misclassification of ICD-10 would be toward reduction of the observed effect of vaccination (*34*).

Our results could also have been influenced by changes in disease diagnosis and management over time. We observed an increase in hospitalizations for bronchiolitis in the cities of Curitiba, Porto Alegre, and São Paulo. Although this increase might represent a real increase in disease incidence and/or severity, a more likely reason could simply be improvements in the diagnosis of bronchiolitis. Thus, hospitalizations for bronchiolitis, which would otherwise be coded as nonspecific pneumonia or other lower respiratory infections, could be increasingly coded correctly in these locations. Potentially, this reduction in misclassification over time would tend to increase the observed effect of vaccination in these cities, but no vaccination effect was observed in Porto Alegre and São Paulo. We have not identified other major changes in diagnosis and reporting habits, including those motivated by knowledge of PCV introduction or the fact that its effect was being assessed.

The study was conducted at the time of the 2009 influenza pandemic, and an influenza A (H1N1) vaccination campaign was conducted in Brazil as a time-limited intervention. This campaign took place from March through June 2010 and achieved high vaccination coverage among children <2 years of age (Brazilian Ministry of Health, unpub. data). This age group was only slightly affected by the pandemic, as evidenced by the lack of a temporary increase in the rates of hospitalization for pneumonia and rates of hospitalization for influenza (data not shown because numbers were so small). Therefore, we consider it unlikely that the pandemic or its vaccination campaign have biased our results.

Any study that uses a time-series method to determine the early effects of a vaccine can be challenged by fluctuations in vaccination coverage and by the natural lag period between vaccination and protection. Moreover, the limitations of using vaccine coverage estimates derived from secondary data collected for administrative reasons are obvious because of the fact that coverage goes beyond 100% for the initial months after start of a vaccination program (Figure 2). The entry of a new vaccine into the immunization program in Brazil usually attracts infants <6 months of age and infants from areas surrounding the municipality. For both situations, the number of doses administered per month are higher than the number of live births per month.

A major challenge to our analysis was dealing with unavoidable delays for reporting the estimated early effect of PCV10 vaccination. Although we found that the number of hospitalizations for all causes decreased during the most recent months of our series, to run the time-series models we still needed as many data points as possible after the vaccine was introduced. The chosen strategy was to subtract the declines for the nonrespiratory hospitalization rates from the pneumonia rates. By doing so, we accounted for as much of the effect of reporting delays as possible.

In conclusion, our data demonstrate that 1 year after its introduction to Brazil, PCV10 reduced hospitalizations for pneumonia among children in 3 of the 5 cities studied. To ascertain the sustainability of this reduction, prospective analyses covering a longer time after introduction of the vaccination program are needed.

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Occult Hepatitis B Virus Infection in Chacma Baboons, South Africa

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During previous studies of susceptibility to hepatitis B virus (HBV) infection, HBV DNA was detected in 2/6 wild-caught baboons. In the present study, HBV DNA was amplified from 15/69 wild-caught baboons. All animals were negative for HBV surface antigen and antibody against HBV core antigen. Liver tissue from 1 baboon was immunohistochemically negative for HBV surface antigen but positive for HBV core antigen. The complete HBV genome of an isolate from this liver clustered with subgenotype A2. Reverse transcription PCR of liver RNA amplified virus precore and surface protein genes, indicating replication of virus in baboon liver tissue. Four experimentally naive baboons were injected with serum from HBV DNA–positive baboons. These 4 baboons showed transient seroconversion, and HBV DNA was amplified from serum at various times after infection. The presence of HBV DNA at relatively low levels and in the absence of serologic markers in the baboon, a nonhuman primate, indicates an occult infection.

Hepatitis B virus (HBV) is a 3.2-kb partially double-stranded virus belonging to the family *Hepadnaviridae*. The outcome of infection with this virus is determined mainly by the immune response of the host and can be acute, chronic, or occult. HBV is divided into 9 genotypes (A–I); an additional genotype, J, has also been proposed (*1*–*3*). Several genotypes are further divided into subgenotypes. In sub-Saharan Africa, subgenotypes A1 and D3 and genotype E circulate (*4*).

Hepadnaviruses can infect avian and mammalian hosts but have a limited host range, infecting only their natural hosts and a few closely related species. Naturally occurring infections have been found in several Old and New World nonhuman primates, such as chimpanzees (*5*), gorillas (*6*), gibbons (*7*), orangutans (*8*) and woolly monkeys (*9*). HBV, whose natural host is humans, also infects chimpanzees (*10*); Barbary macaques (*11*); and tree shrews (*12*), in addition to humans.

Baboons (*Papio* species) have been proposed as a possible animal model of HBV infection. Phylogenetically, baboons are close to humans, showing ≈96% homology at the DNA level, and they have an immune system similar to that of humans (*13*). Early studies involving injection of baboons with HBV-positive serum failed to detect any clinical or biochemical signs of infection in these primates, and initial serologic surveys failed to detect HBV surface antigen (HBsAg) in serum, leading to the conclusion that baboons were not susceptible to HBV infection (*14*). This supposed lack of susceptibility of baboons to infection with HBV, and the fact that unlike chimpanzees, baboons are not an endangered species, intimated that baboons were good candidates for sources of liver for xenotransplants. The use of xenotransplants from pigs and nonhuman primates to humans was considered to overcome the donor shortage and to bridge patients with terminal hepatic failure until a human donor organ became available (*15*).

To confirm that baboons were not susceptible to HBV infection, Kedda et al. injected 6 wild-caught Chacma baboons (*Papio ursinus orientalis*) with pooled HBV-positive serum and analyzed the baboons for 52 weeks by using sensitive molecular techniques to detect evidence of transmission (*16*). HBV DNA was detected by nested PCR in serum and liver of 4 of the baboons ≤52 weeks after injection. Liver function and histologic results were within reference ranges, and HBsAg was not detected in serum (*16*). However, during that study, HBV DNA was detected by using nested PCR in serum of 2 of the 6 baboons at baseline, before injection with HBV. The presence of HBV DNA was confirmed by retesting samples in independent laboratories. This finding raised the possibility that baboons were naturally infected with a hepadnavirus. The aims of the present study were to determine the prevalence of HBV in wild baboons, molecularly characterize the virus isolated from these baboons, determine whether the virus replicates in the baboon liver, and demonstrate viral transmission to experimentally naive baboons.

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Methods

Serum samples obtained from 49 adult and 20 juvenile Chacma baboons caught in the Western Cape, Eastern Cape, and Limpopo Provinces of South Africa were stored at −70°C. Liver tissue (fresh frozen and formalin-fixed) was obtained from 1 of the adult wild-caught baboons (hereinafter referred to as baboon 9732), which was euthanized for medical and ethical reasons. Permission for this study was obtained from the Animal Ethics Committee of the University of the Witwatersrand. All procedures were approved by this Committee. Baboons were provided care according to the guidelines of the South African Medical Research Council.

Serologic and Immunohistochemical Analysis

Baboon serum samples were tested for HBsAg and for antibodies against HBV core antigen (anti-HBc) by using commercially available assays (Abbott Laboratories, Abbott Park, IL, USA). Alanine and aspartate aminotransferase levels were measured by using a 747 Automatic Analyzer (Hitachi, Tokyo, Japan).

Formalin-fixed liver tissue from baboon 9732 was used for histologic and immunohistologic preparations. Liver tissue was embedded in paraffin and sectioned. The sections were stained with hematoxylin and eosin for histologic examination or with immunoperoxidase and polyclonal antibodies to HBsAg and HBV core antigen (HBcAg) to detect HBsAg and HBcAg in hepatocytes.

Southern Hybridization Slot Blot

Baboon serum samples were blotted onto a nylon membrane by using a slot-blot manifold according to the protocol described by Zaaiger et al., which has a detection limit of 2.5 × 10^7 HBV genomes/mL (*17*). HBV DNA was detected by Southern blot hybridization with a ^{32}P-labeled HBV DNA probe (HBV DNA in pBV325 vector, *adr* subtype).

DNA Extraction and Amplification and Phylogenetic Analysis

DNA was extracted from 200 μL of baboon serum by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA was also extracted from baboon liver tissue by using a phenol-chloroform extraction method (*18*). Extracted DNA was quantified by using spectrophotometric analysis with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA).

Subgenomic nested PCR amplifications of the precore/core (nt 1732–2045 and nt 1765–1968), core (nt 1687–2498 and nt 2267–2436), polymerase (nt 2540–2896 and nt 2566–2858), and surface (nt 255–759 and nt 459–710) regions were used to confirm the presence of HBV DNA in the serum extracts by using 40 cycles (*16*). The sensitivity of these amplifications is 40–400 HBV genomes/mL (*19*).

The complete viral genome was amplified by using 8 overlapping subgenomic fragments (Figure 1). Thermocycling

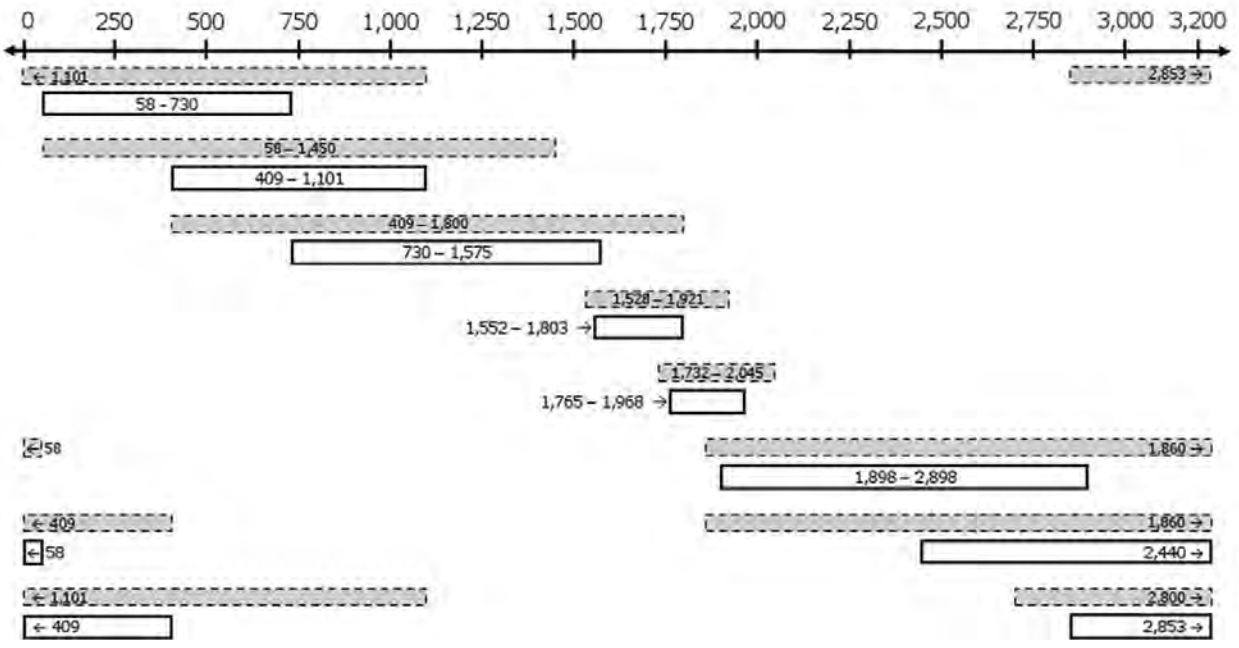

Figure 1. Amplification of the hepatitis B virus (HBV) genome by using overlapping subgenomic fragments. Shown are 8 overlapping subgenomic fragments amplified by nested PCR, These fragments were used to generate the complete HBV sequence isolated from liver tissue of Chacma baboon 9732, South Africa. Dashed gray boxes indicate first-round PCRs, and white boxes indicate second-round PCRs. Values along the 2-headed arrow at the top are in basepairs from the *Eco*RI site of the circular genome of HBV. Small arrows within boxes indicate the direction of amplification.

conditions and sequences for primers 58, 409, 730, 1101, 1450, 1860, 2440, and 2853 were obtained from Hu et al. (*20*). Additional primers used were 1575 (5′-CCGGCAGATGAGAAGGCACAGACGG-3′), 1528 (5′-ACCTCTCTTTACGCGGTCTC-3′), 1552 (5′-TCTGTGCCTTCTCATCTGCC-3′), 1800 (5′-AGACCAATTTATGCCTACAGCCTCCTA-3′), 1803 (5′-CGCAGACCAATTTATGCCTAC–3′), 1898 (5′-GGCATGGACATTGACCCGTA-3′), 1921 (5′-TTTATACGGGTCAATGTC-3′), 2800 (5′-CAGGTAGCGCCTCATTTTGTGGGTCACCATATTCT-3′), and 2898 (5′-GAGGATTGGGAACAGAAAGATT-3′). All nucleotide numbering refers to the position from the *Eco*RI site as position 1.

Amplicons were sequenced directly by using the BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems., Foster City, CA, USA) and sequenced on an ABI3130*xl* Genetic Analyzer with 16 capillaries (Applied Biosystems) and the same primers that were used for amplification. The sequence has been deposited in GenBank under accession no. JX507080. The HBV genomic sequence obtained from the baboon was compared with corresponding sequences of HBV from GenBank as described (*4*).

RNA Extraction, Reverse Transcription, and Amplification

RNA was extracted from varying amounts of baboon liver tissue by using the guanidinium-acid-phenol method (*21*) and digested with RNase-free DNase I (Fermentas, Waltham, MA, USA) to remove any contaminating DNA. The RNA concentration was determined by spectrophotometry with the NanoDrop ND-1000 Spectrophotometer. cDNA was generated by using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo$(dT)_{18}$ primers (Invitrogen) in accordance with the manufacturer's instructions. Non–reverse transcribed negative controls were prepared in an identical manner except that diethylopyrocarbonate–treated water (Invitrogen) was added to each reaction instead of reverse transcriptase. The success of the reverse transcription reaction was confirmed by PCR amplification of a portion of the glyceraldehyde-3-phosphate dehydrogenase gene (*22*). Subgenomic nested PCR amplifications of the precore/core (nt 1732–2045 and nt 1765–1968) and surface (nt 255–759 and nt 459–710) regions were also performed.

Detection of Covalently Closed Circular DNA

DNA was extracted from baboon liver tissue by using the QIAamp DNA Mini Kit (QIAGEN). DNA extracts were treated with Plasmid-Safe ATP-Dependent DNase (Epicenter Biotechnologies, Madison, WI, USA) to selectively hydrolyze linear double-stranded chromosomal DNA while leaving HBV covalently closed circular DNA (cccDNA) intact. The cccDNA was detected by real-time PCR (*23*) with the Power SYBR Green PCR Master Mix (Applied Biosystems).

Transmission of HBV to Experimentally Naive Baboons

Transmission of HBV to experimentally naive baboons was performed at the National Institutes of Health (Bethesda, MD, USA). Animals were housed and maintained at Bioqual, Inc. (Rockville, MD, USA). Housing and care of animals complied with all relevant guidelines and requirements, and the animals were housed in facilities that are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. All protocols were reviewed and approved by the Institutional Animal Care and Use Committees of the National Institute of Allergy and Infectious Diseases of the National Institutes of Health and Bioqual, Inc. Experimentally naïve, domestically raised baboons were obtained from a domestic breeder (Mannheimer Foundation, Homestead, FL, USA). Before inclusion of animals in the study, serum was free of all markers of HBV replication when tested serologically and by nested PCR as described above. A liver biopsy to detect HBV DNA was not performed.

Four experimentally naive baboons were each inoculated with 500 μL of serum obtained from HBV DNA–positive wild-caught baboons from South Africa. Each baboon was injected with serum from a single wild-caught baboon. After injection, serum was obtained from each of the 4 newly injected baboons at weekly intervals. Serum was used to measure levels of alanine aminotransferase, isocitrate dehydrogenase, γ-glutamyltranspeptidase, HBsAg, HBV e antigen, antibodies against HBV e antigen, antibodies against HBsAg, and anti-HBc.

DNA extracted from serum samples was used for nested PCR amplification of a region of the surface gene (nt 459–710). Twenty-five weeks after injection, the study was terminated and the baboons were euthanized. At necropsy, liver tissue and serum was obtained from each baboon for further testing to confirm that virus extracted from experimentally naive baboons was the same as that found in the original baboons.

Results

Prevalence of HBV in Wild-caught Baboons in South Africa

The prevalence of HBV in the baboon (*P. ursinus orientalis*) population in South Africa was determined by extracting DNA from serum of 69 wild-caught baboons and amplifying 4 regions of the viral genome (the precore/core, core, polymerase, and surface regions) by nested PCR.

HBV DNA could not be amplified with a single-round PCR, indicating that HBV DNA was present at low levels in baboon serum.

Using the criterion of ≥3 of the 4 regions being PCR positive, we found that 11 (22.4%) of 49 adult and 4 (20.0%) of 20 juvenile wild-caught baboons were positive for HBV. The overall prevalence of hepadnaviral DNA in baboons was 21.7% (15/69). Furthermore, the presence and specificity of the HBV DNA was confirmed when detected directly in the serum of 5 of the 69 baboons by using Southern blot analysis. Only 5 of the 15 PCR-positive serum samples were positive by Southern hybridization because of the relatively lower sensitivity of this method.

Serologic, Liver Function, and Immunohistologic Tests

Serum samples from 4 of the 15 HBV DNA–positive baboons, for which additional serum was available, were tested and found to be negative for HBsAg and anti-HBc. Alanine and aspartate aminotransferase levels were within reference ranges, and after high-speed centrifugation and treatment with antibody against HBsAg, no viral particles were observed in the serum by electron microscopy. Histologic examination of liver tissue from 1 of these baboons (9732) showed mild focal lobular hepatitis but no evidence of interface hepatitis, bridging necrosis, dysplasia of hepatocytes, cirrhosis, or hepatocellular carcinoma (Figure 2, panel A). Immunohistochemical staining of liver tissue showed HBcAg in nuclei of some hepatocytes with a patchy distribution (Figure 2, panel B). HBsAg and 42-nm enveloped (Dane) particles were not detected in the cytoplasm.

Amplification, Sequencing, and Phylogenetic Analysis of HBV Genome from Baboon Liver

The low viral loads indicated that the complete HBV genome could only be amplified subgenomically and because of the small volumes of serum available, liver tissue obtained from baboon 9732 was used to further characterize HBV in baboons. The complete HBV genome was amplified by nested PCR of 8 overlapping subgenomic fragments (Figure 1).

Phylogenetic analyses of the complete genome showed that HBV from the baboon was closely related to HBV subgenotype A2 (Figure 3). This finding was confirmed by comparison of mean ± SD nucleotide divergence calculations compared to subgenotype A2 (1.00 ± 0.55) and to subgenotype A1 (4.52 ± 0.42). Similar results were obtained for each of the 4 open reading frames. The baboon HBV had mutations in the basic core promoter and precore regions not found in subgenotype A2. These mutations included the G1809T/C1812T double mutation in the Kozak sequence preceding the precore protein start codon and G1888A in the precore region. G1809T/C1812T mutations are characteristic of subgenotype A1, and G1888A is unique to subgenotype A1 (*4*,*24*). Translation of the 4 open reading frames showed them to be well conserved relative to the consensus sequence of subgenotype A2 with the following exceptions: T380C resulting in an rtV84A in conserved region A of the polymerase and a C76R in HBsAg; A2019G resulting in an E40G in the core protein; and C1470T resulting in a P33G in the X protein and T1765C resulting in a P145S in the X protein.

Expression of HBV RNA in Baboon Liver Tissue

RNA extracted from liver tissue of baboon 9732 was reverse transcribed and amplified in precore/core (nt 1765–1968) and surface (nt 459–710) open reading frames (Figure 4). Sequences of these amplicons were identical to sequences of the DNA isolated from liver of baboon 9732.

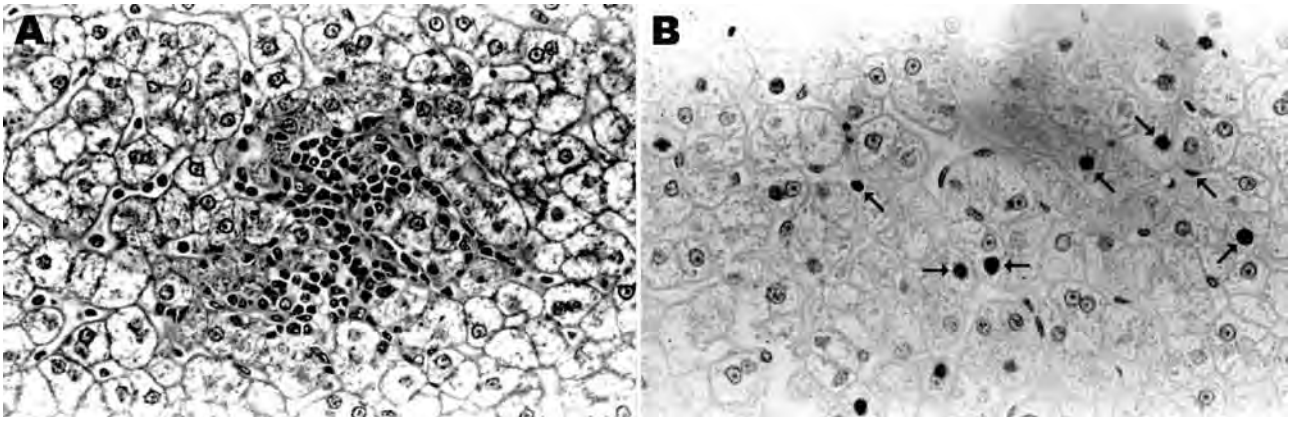

Figure 2. Liver tissue from Chacma baboon 9732, South Africa, showing lobular hepatitis. Liver tissue was obtained at necropsy, fixed in formalin, embedded in paraffin, and sectioned. A) Hematoxylin and eosin staining, showing a focus of mild lobular hepatitis but no evidence of interface hepatitis or bridging necrosis. Portal tracts are normal. B) Immunoperoxidase staining with polyclonal antibody against hepatitis B core antigen. Core antigen was detected in the occasional hepatocyte nucleus. Arrows indicate selected positive nuclei. Original magnifications ×400.

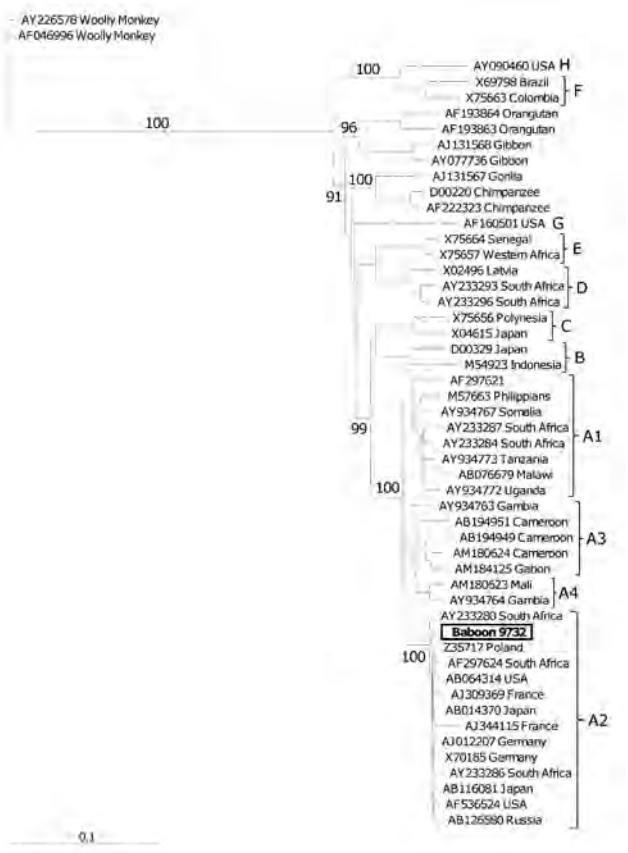

Figure 3. Dendogram of the complete hepatitis B virus (HBV) genome isolated from Chacma baboon 9732, South Africa. Samples representative of all 8 HBV genotypes and primate hepadnaviruses are included. Samples are numbered according to their GenBank accession numbers, followed by their country of origin. The sample from baboon 9732 (**boldface**) clusters strongly with the subgenotype A2 isolates (bootstrap value 100). Letters along the right indicate genotypes. Values along branches are bootstrap values. Scale bar indicates nucleotide substitutions per site.

Identification of cccDNA in Baboon Liver Tissue

cccDNA was detected in liver tissue of baboon 9732 by using real-time PCR. DNA from HBV DNA–positive tumorous and nontumorous human liver and plasmid DNA containing a greater than full-length HBV genome were used as positive controls, and DNA from rat liver tissue was used as a negative control. HBV cccDNA–positive controls had melting temperatures of 82.7°C–84.0°C, and negative controls had melting temperatures <80.0°C. Samples from the baboon liver tissue showed similar melting temperatures as positive controls (82.7°C–83.1°C) indicating that HBV cccDNA was present.

Transmission of HBV from Wild-caught Baboons to Experimentally Naive Baboons

Each of 4 experimentally naive, domestically raised baboons was injected with serum from 1 of 4 wild-caught HBV DNA–positive baboons. Serum samples from the experimentally naive baboons were HBV DNA negative before injection, and baseline levels of aspartate aminotransferase, alanine aminotransferase, and IgG were also determined. The baboons were transiently positive for several of these markers and were intermittently positive for HBV DNA in serum. Results for a representative sample (baboon 2) infected with serum from baboon 9732 are shown in Figure 5. DNA extracted from liver tissue obtained at necropsy from baboon 2 was used to amplify and sequence a portion of the surface gene (nt 409–1101). The sequence of virus extracted from liver tissue of baboon 2 six months after injection was identical to the HBV sequence of this region found in original baboon 9732.

Discussion

Detection of HBV DNA in serum samples of 2 Chacma baboons before injection with human HBV (*16*) suggested that baboons might be chronically infected with HBV. Our objective was to determine the prevalence of HBV in wild-caught Chacma baboons and to characterize the virus isolated from these animals. The overall prevalence (21.7%) of HBV in the baboons is similar to the HBV prevalence in other nonhuman primates in areas to which HBV is highly endemic, including sub-Saharan Africa (*25*).

The baboons were serologically negative, and the 1 baboon examined histologically had mild focal lobular hepa-

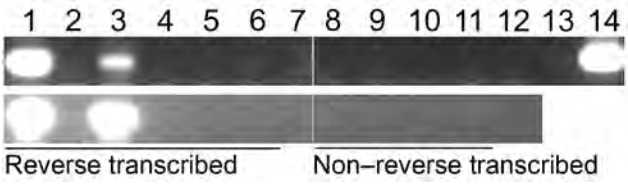

Figure 4. Nested PCR amplification of a subgenomic region of cDNA for baboon hepatitis B virus, South Africa. Reverse transcribed, DNase I–treated cDNA products were amplified by PCR, and amplicons were resolved by electrophoresis on a 1% agarose gel containing ethidium bromide. Non–reverse transcribed samples (in which diethyl pyrocarbonate [DEPC]–treated water was added instead of enzyme during reverse transcription) were included as negative controls. Top panel: nested PCR 1: 255F–759R; PCR 2: 459F–710R. Bottom panel, 550-bp region of glyceraldehyde-3-phosphate dehydrogenase gene amplified to assess quality of mRNA. Lanes 1 and 8, 100 mg of baboon liver tissue used for RNA extraction; lanes 2 and 9, RNA extraction negative control; lanes 3 and 10, 200 mg of baboon liver tissue used for RNA extraction; lanes 4 and 11, RNA extraction negative control; lane 5, DNase I treatment negative control in which DEPC-treated water was added instead of RNA; lane 6, reverse transcription negative control in which DEPC-treated water was added instead of cDNA; lanes 7 and 12, double-round nested PCR negative control containing best-quality water instead of cDNA; lane 13, single-round PCR negative control containing best-quality water instead of cDNA; lane 14, PCR-positive control containing DNA extracted from liver tissue of baboon 9732 as template.

titis, and HBcAg was detected in liver tissue by immunohistochemical staining. Detection of low levels of HBV DNA in baboon liver tissue and in serum in the absence of HBsAg and at relatively low levels of HBV DNA classifies this infection as occult (*26*). Lack of any serologic markers of HBV infection further distinguishes this infection as a seronegative occult HBV infection (*26*). This finding is analogous to a secondary occult infection in the woodchuck (*27*). Furthermore, HBV DNA was detected in juvenile and adult baboons, suggesting lifelong persistence of the virus, which was successfully transmitted to experimentally naive baboons. Detection of HBV DNA alone does not necessarily correspond to an HBV infection (*28*). Thus, detection of cccDNA and viral RNA in liver tissue of baboon 9732 showed that HBV was replicating. The low levels of viral nucleic acids detected in baboon liver are a further characteristic of occult infections (*27*). This study demonstrates a naturally occurring occult HBV infection in a nonhuman primate.

Phylogenetic analysis of the baboon HBV genome showed that it belonged to genotype A, clustering with subgenotype A2. This finding is an unexpected result because subgenotype A1 predominates in South Africa (*24*). However, in the basal core promoter/precore region, the baboon HBV had distinct characteristics of subgenotype A1. Four additional mutations in the polymerase, surface, X, and core regions of the baboon HBV strain differentiated the baboon isolate from most subgenotype A2 isolates. These mutations are not known to cause any major functional or conformational changes.

Paradoxical identification of subgenotype A2 in the baboon when subgenotype A1 predominates in Africa might indicate that subgenotype A2 is an older strain that previously circulated in Africa, which has been replaced by other strains, including subgenotype A1 and genotypes D and E (*29*). An analogous trend might have occurred in the Mediterranean region where genotype D now predominates over genotype A (*30*). Similarly, a change in the prevalent HBV genotype in central and western Africa has been postulated to have occurred over the past 200 years, and genotype E, originally restricted to the west coast of Africa, now spreads over a large crescent stretching from The Gambia, through Nigeria and the Democratic Republic of the Congo into Namibia and Mozambique (*31*). There is a paucity of sequencing data for subgenotype A2 from Africa. Only 4 complete subgenotype A2 genomes from South Africa have been deposited in GenBank. Shorter subgenomic sequences of subgenotype A2 from South Africa (*32*), Tunisia (*33*), and Kenya (*34*) have also been deposited. More extensive molecular epidemiologic studies in Africa might uncover a higher circulation of subgenotype A2 in more remote regions.

Another explanation for the paradoxical finding of subgenotype A2 in the baboon could be that in Africa, as

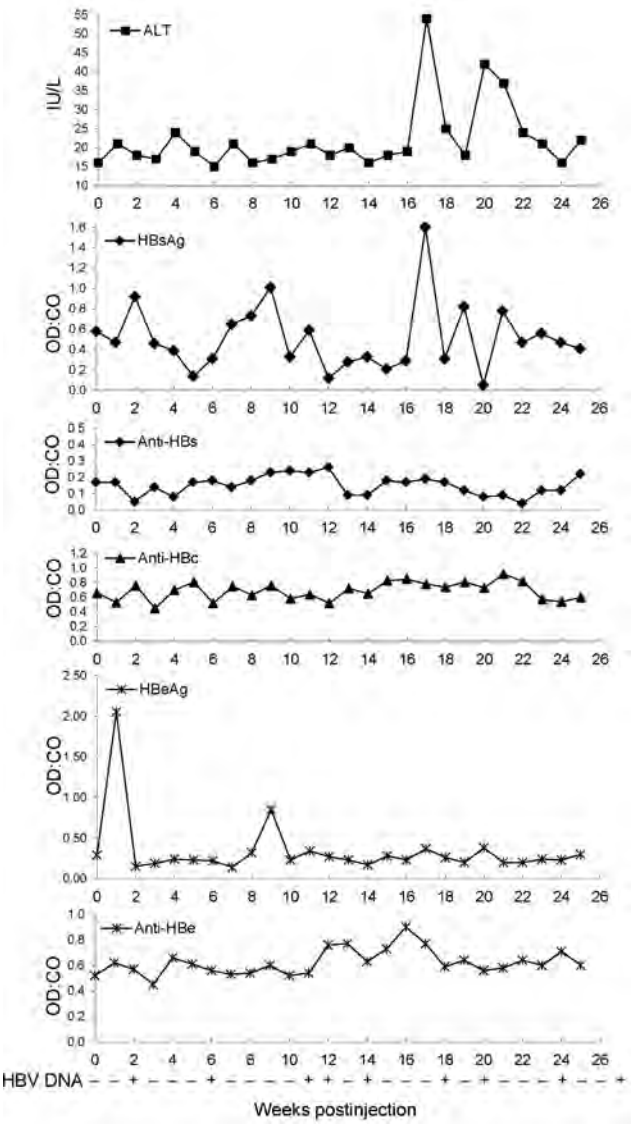

Figure 5. Levels of alanine aminotransferase (ALT) and hepatitis B virus (HBV) serologic markers and detection of HBV DNA in baboon 2. Serum obtained from baboon 2 (which was injected with serum from baboon 9732), and ALT and HBV serologic marker levels were measured at weekly intervals after injection. Serum for week 0 levels was obtained just before injection. OD:CO indicates optical density:cutoff value ratios. An OD:CO >1 indicates a positive result. HBV DNA was detected by nested PCR amplification (255–761 in the first round and 459–710 in the second round) of a 252-bp region of the virus surface gene by using DNA extracted from serum obtained from the infected baboon at weekly intervals. HBsAg, HBV surface antigen; Anti-HBs, antibody against HBV surface antigen; Anti-HBc, antibody against HBV core antigen; HBeAg, HBV e antigen; Anti-HBe, antibody against HBV e antigen. + indicates that the sample amplified successfully at this time point, and – indicates no amplification of virus DNA. The week 26 time point indicates the result of amplification by using DNA extracted from serum at necropsy, and the final time point indicates successful amplification of the virus product from DNA extracted from liver tissue obtained from baboon 2 at necropsy.

shown in India, subgenotype A2 isolates are confined to peripheral blood leukocytes (PBLs). In India, subgenotype A1 and genotype D circulate, but actively replicating HBV subgenotype A2 isolates have been detected in PBLs (*35*). These subgenotype A2 isolates were confined to PBLs and were not detected in serum. Differential immune pressures are believed to compartmentalize HBV to different parts of the body in which different strains evolve independently (*35*). Further study will be needed to determine whether subgenotype A2 is compartmentalized to PBLs in Africa.

In sub-Saharan Africa, most human HBV carriers are negative for HBV e antigen, and in these persons, spread of HBV is mainly horizontal (*36*). In baboons, their natural habits indicate that horizontal baboon-to-baboon transmission of HBV is highly probable. Baboons are social animals, and bonds are strengthened by daily grooming with several related and unrelated partners, including offspring (*37*). Young baboons, like their human counterparts, spend much of the day playing together. The games can become quite physical, often leading to mock fights. Physical fights among male and female adult baboons are rare. Alpha males tend to be aggressive, and displays of dominance and chases occur daily.

The well-documented interactions between humans and baboons make cross-species transmission of this virus extremely plausible (*37*). Baboons are the most widely distributed nonhuman primates in Africa and are found in virtually all parts of sub-Saharan Africa. They often appear in ancient Egyptian mythology and art, depicted as captives brought from southern Africa or as pets on leashes (*37*). In southern Africa, baboons have been kept as pets and trained to work as oxcart drivers, railway laborers, and as goat herders on farms (*37*). However, more common are reports of conflict between humans and baboons. In the rural areas, baboons raid orchards, destroy irrigation pipes, and kill sheep and goats. Because baboons can become aggressive when challenged, they are often killed by farmers for being pests. Slaughter of baboons for bush meat could be another source of exposure to the virus (*38*).

From the results of the present study, it is impossible to determine whether baboons were infected with HBV by humans, as has been hypothesized (*39*), or whether humans were infected by baboons. However, as noted by Michael Lai, a virus expert, "When we expose ourselves to exotic animals, there is always a risk of being exposed to something unknown…When we perturb the existing peace between human beings and nature, we are opening a Pandora's box, which may contain surprises" (*40*).

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Risk Factors for Influenza among Health Care Workers during 2009 Pandemic, Toronto, Ontario, Canada

Stefan P. Kuster, Brenda L. Coleman, Janet Raboud, Shelly McNeil, Gaston De Serres, Jonathan Gubbay, Todd Hatchette, Kevin C. Katz, Mark Loeb, Donald Low, Tony Mazzulli, Andrew Simor, and Allison J. McGeer, on behalf of the Working Adult Influenza Cohort Study Group[1]

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Release date: March 18, 2013; Expiration date: March 18, 2014

Learning Objectives

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

- Define an influenza pandemic in this study
- Report signs and symptoms of influenza
- Determine whether the risk of influenza was higher in health care workers (HCWs) than in non-HCWs
- Report risk factors for influenza among HCWs

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This prospective cohort study, performed during the 2009 influenza A(H1N1) pandemic, was aimed to determine whether adults working in acute care hospitals were at higher risk than other working adults for influenza and to assess risk factors for influenza among health care workers (HCWs). We assessed the risk for influenza among 563 HCWs and 169 non-HCWs using PCR to test nasal swab samples collected during acute respiratory illness; results for 13 (2.2%) HCWs and 7 (4.1%) non-HCWs were positive

[1]Additional members of the Working Adult Influenza Cohort Study Group are listed at the end of this article.

for influenza. Influenza infection was associated with contact with family members who had acute respiratory illnesses (adjusted odds ratio [AOR]: 6.9, 95% CI 2.2–21.8); performing aerosol-generating medical procedures (AOR 2.0, 95% CI 1.1–3.5); and low self-reported adherence to hand hygiene recommendations (AOR 0.9, 95% CI 0.7–1.0). Contact with persons with acute respiratory illness, rather than workplace, was associated with influenza infection. Adherence to infection control recommendations may prevent influenza among HCWs.

The numerous outbreaks of influenza described in acute care hospitals indicate that influenza transmission in this setting is of major concern (*1*–*3*). Nonetheless, it remains unclear whether health care workers (HCWs) are at higher risk for infection than are adults working in nonclinical settings (non-HCWs). Vaccination recommendations for HCWs are intended primarily to protect patients from hospital-acquired influenza and influenza-associated death (*4*,*5*). Although working in hospitals has been proposed as a risk factor for influenza (*6*), findings that support that working in health care settings poses an occupational risk (*7*), or that performing particular activities or working in specific health care disciplines are associated with an increased risk for influenza infection, are sparse.

Better understanding of risk factors for infection among HCWs would support decision-making regarding priorities for seasonal influenza vaccination, antiviral treatment or prophylaxis programs, implementation of other measures to reduce influenza transmission in hospitals, and planning for pandemics. Therefore, we aimed to assess risk factors for influenza among HCWs and to determine whether, during the first 2 waves of influenza A(H1N1)pdm09, HCWs working in acute care hospitals were at higher risk than non-HCWs for symptomatic influenza.

Materials and Methods

Participants and Setting

The Influenza Cohort Study, initiated by the Working Adult Influenza Cohort Study Group, a research team based in Toronto, Ontario, Canada, was started in May 2009. The purpose of the study was to examine incidence, clinical features, and epidemiology of infection caused by A(H1N1) pdm09 among HCWs and other working adults in Canada. For this analysis, participants were enrolled during May 29–September 27, 2009. Participants were eligible if they were 18–75 years of age and either worked ≥8 hours per week in 1 of 5 acute care hospitals (HCW) or in an office-based setting in Toronto (non-HCW). Non-HCWs were intended to provide a sample of working adults at low occupational risk for influenza, so as to bias the study toward the ability to identify an occupational risk in health care. Details of the recruitment of these control participants are included in the online Technical Appendix (wwwnc.cdc.gov/EID/article/19/4/11-1812-Techapp.pdf). The study was approved by the Research Ethics Boards of all participating hospitals and universities and by the human resources departments of participating employers.

Upon enrollment, participants received a collection kit, an illustrated guide, and instruction from a nurse for mid-turbinate nasal swab sample self-collection. They also completed a Web-based questionnaire detailing influenza vaccination history, underlying medical conditions, demographic data, potential work- or school-related risk factors for respiratory virus infection, and potential community risk factors. Blood samples were taken from consenting participants at enrollment and again in April or May of 2010.

Participants were asked to complete weekly Web-based diaries from enrollment until March 31, 2010, detailing respiratory symptoms and acute respiratory illness (ARI) or febrile illnesses and documenting time-dependent risk factors (e.g., contact with persons with ARI symptoms). Per the study protocol, if any signs or symptoms suggestive of an ARI developed, participants provided a self-collected mid-turbinate nasal swab sample as soon as possible after onset to be tested for influenza by using PCR. ARI was defined as 1) fever without another obvious source; or 2) new symptoms, including ≥2 of the following: runny or stuffy nose, sneezing, sore or scratchy throat, hoarseness, or cough; or 3) one local (runny/stuffy nose, sneezing, sore/scratchy throat, hoarseness, or cough) and 1 systemic symptom (fever, malaise, myalgia, headache, or fatigue).

Participants whose specimens tested positive for influenza were offered treatment in accordance with public health recommendations (*8*). All participants with undetermined A(H1N1)pdm09 vaccine status as of March 31, 2010, were contacted again to confirm whether they had received it and, if so, when. For logistical reasons, participants with unconfirmed 2009–2010 seasonal influenza vaccine status could not be contacted again; instead, these participants were assumed not to have received it. In Canada, vaccine for A(H1N1)pdm09 became available for HCWs and patients at high risk for complications of influenza during calendar week 43 (starting October 25, 2009) and was available for healthy adults during calendar week 47 (starting November 22).

Definitions

For this study, HCW were defined as persons working in an acute care hospital. A non-HCW was defined as a person working in an office-type environment not associated with the provision of health care. The first and second waves of the influenza pandemic in Ontario were

Table 1. Characteristics of study participants in the Influenza Cohort Study performed during 2009 pandemic, Toronto, Ontario, Canada*

Characteristic	Participant values by cohort, n = 732		
	No. HCWs in acute care facilities, n = 563	No. non-HCWs in office settings, n = 169	p value
Mean age, y (± SD)	42.2 (11.3)	45.4 (10.8)	0.001
Female sex	478/563 (84.9)	133/169 (78.7)	0.06
Recipient of vaccine			
A(H1N1)pdm09 vaccine†	469/554 (84.7)	66/165 (40.0)	<0.001
Seasonal influenza vaccine 2009–10	226/563 (40.1)	41/169 (24.3)	<0.001
Seasonal influenza vaccine 2008–09	407/552 (73.7)	76/164 (46.3)	<0.001
Underlying health conditions			
Asthma	55/555 (9.9)	14/167 (8.4)	0.56
Diabetes mellitus	22/555 (4.0)	5/165 (3.0)	0.58
Allergies to airborne irritants	235/509 (46.2)	60/155 (38.7)	0.10
Current smoker or smoker in household	73/551 (13.3)	27/163 (16.6)	0.28
Potential exposure conditions			
Hand-to-face habits	275/557 (49.4)	76/168 (45.2)	0.35
Wearing of prescription eyeglasses	386/561 (68.8)	119/168 (70.8)	0.62
Reusable water bottle use ≥1/week	232/556 (41.7)	81/169 (47.9)	0.15
Public transit: ≥8 trips per week	196/558 (35.1)	45/168 (26.8)	0.04
Group gathering attendance	524/563 (93.1)	150/167 (89.8)	0.17
Face-to-face contacts/d, median (IQR)	10 (5, 20)	10 (5, 20)	0.94
>1 person/bedroom in household	192/551 (34.9)	51/166 (30.7)	0.33
Children in workplace	62/558 (11.1)	9/167 (5.4)	0.03
Child <5 y in household	77/563 (13.7)	10/169 (5.9)	0.006
Child <18 y in household	212/563 (37.7)	59/169 (34.9)	0.52
Child in household attends day care	77/555 (13.9)	16/164 (9.8)	0.17

*Data are no./total (%) unless otherwise specified. HCW, health care worker; A(H1N1)pdm09, pandemic influenza A(H1N1) 2009 virus; IQR, interquartile range.
†Participants who had acquired A(H1N1)pdm09 ≤7 d after vaccination were considered unprotected.

defined as the periods for which the weekly proportion of respiratory specimens that were positive for A(H1N1) pdm09 was >5%, as reported by the Ontario Agency for Health Protection and Promotion. Similarly, seasonal influenza waves were defined as periods for which >5% of weekly specimens tested positive for seasonal influenza. By this definition, the first pandemic wave occurred during calendar weeks 21–31 of 2009 (May 17–August 8); the second wave occurred during calendar weeks 39–48 (September 27–December 5). Peak weeks were defined as weeks during which positivity rates were >15% and comprised calendar weeks 21–27 (May 17–July 11) during wave 1 and calendar weeks 41–46 (October 11–November 21) during wave 2. As expected, few cases of seasonal influenza were identified during the study period.

Aerosol-generating medical procedures were defined as any of the following: administration of nebulized therapy or humidified oxygen at >40%, use of bag-valve mask, manual ventilation, noninvasive ventilation, open airway suctioning, bronchoscopy or other upper airway endoscopy, tracheostomy, endotracheal intubation, cardiopulmonary resuscitation, oscillatory ventilation, or any procedure that involved manipulation of open ventilator tubing in a mechanically ventilated patient or sputum induction or other deliberate induction of coughing.

Adherence to hand hygiene and facial protection recommendations was defined as the self-reported proportion of situations during which hand hygiene and facial protection were performed according to infection control recommendations (*9*). Symptomatic influenza infection was defined as influenza-positive PCR results for a participant-collected mid-turbinate nasal swab sample.

Antibody Assays and Interpretation

Serum specimens were extracted from blood samples and 1 mL aliquots frozen at −70°C. Aliquots were tested by hemagglutination-inhibition (HAI) assay to determine antibody titers against the A(H1N1)pdm09 strain (A/California/07/2009-like) and the 2008–09 seasonal A(H1N1) strain (A/Brisbane/59/07) to identify potential cross-reactivity by using a protocol adapted from World Health Organization methods (*10*). Two HAI assays were performed per aliquot by using 0.5% turkey erythrocytes and 4 hemagglutination units per 25 μL of virus. For discordant pairs, the higher of the 2 geometric mean titers was used. Serum specimens were tested at the Queen Elizabeth II Health Sciences Centre, Halifax, Nova Scotia, Canada. Seroprotection was defined as having HAI antibody titers of ≥40. Seroconversion was specifically defined as a prevaccination HAI titer of <10 and a postvaccination titer of ≥40 or a 4-fold change in titers for participants with a prevaccination titer of ≥10 (*11*,*12*).

Data Management and Statistical Analyses

Data were entered online by the participants, then cleaned and manually inspected for errors and outlying

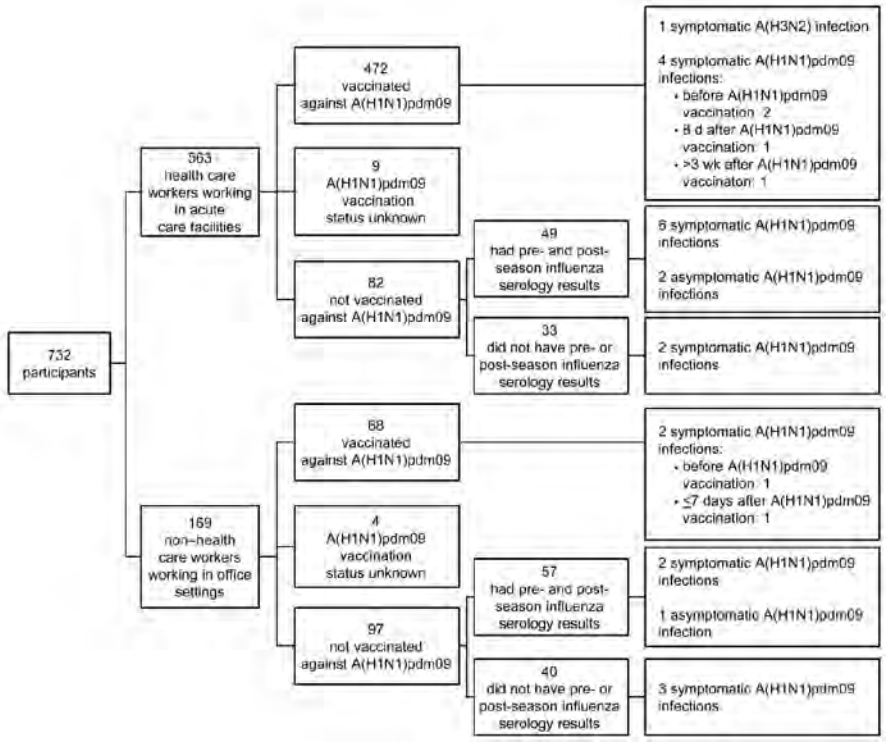

Figure. Flowchart of 732 persons enrolled in the Influenza Cohort Study, Toronto, Ontario, Canada.

values. Differences in group proportions were assessed by the χ^2 or Fisher exact test, as appropriate, and differences in means (for normally distributed data, on the basis of the Shapiro-Wilk test for normality) and medians (for non-normally distributed data) were calculated by using Student *t* test and Wilcoxon rank-sum test, respectively.

The analysis for the primary objective (i.e., to determine whether the risk for laboratory-confirmed symptomatic influenza was higher in HCWs than in non-HCWs) included all participants who were enrolled by the start of the second wave of the 2009 H1N1 influenza pandemic (calendar week 39, starting September 27, 2009). Multivariable generalized estimating equation logistic regression analysis was used to determine adjusted odds ratios with 2-sided 95% CIs for constant and time-dependent risk factors for symptomatic influenza infection on the basis of information from baseline questionnaires and weekly diaries. Model construction was performed on the basis of the method proposed by Harrell (*13*) including A(H1N1) pdm09 vaccination status and changing risk for influenza infection over time (community influenza activity). Our a priori approaches to adjust for changing risk for influenza infection over time were to 1) adjust for weekly percentage of specimens positive for influenza reported to the Ontario Agency for Health Protection and Promotion (continuous variable) and 2) adjust for peak weeks (defined as weeks during which >15% of specimens were positive for influenza; [dichotomous variable]). Vaccine failure among participants was defined as acquiring A(H1N1)pdm09 infection after receipt of A(H1N1)pdm09 vaccine >7 days before symptom onset. Participants who acquired A(H1N1) pdm09 within 7 days after vaccination were considered not fully protected. To evaluate the validity of this assumption, we performed sensitivity analyses by calculating lags of 0 days and 14 days, respectively. The same criteria were used in the analysis of the secondary objective (i.e., to determine risk factors for laboratory-confirmed symptomatic influenza among HCWs). The models with the lowest quasi-likelihood under the independence model criterion were preferred.

Data were analyzed in SAS, version 9.1 for PC (SAS Institute, Cary, NC, USA). We considered p values <0.05 as statistically significant.

Sample Size

This study was initiated at the onset of the 2009 influenza pandemic; because the expected incidence of infection was unknown, a formal sample size was not established. Details of the sample size estimate for the planned seasonal study can be found in the online Technical Appendix.

Results

Study Population, Symptomatic Influenza Case-patients and Community Influenza Activity

The first participant was enrolled in the study on May 28, 2009 (calendar week 21). By October 11 (calendar week 41), at the start of the second wave of the pandemic, 732

Table 2. Results of influenza virus testing of nasal swab specimens from 732 study participants in the Influenza Cohort Study performed during 2009 pandemic, Toronto, Ontario, Canada*

Characteristic	Influenza test status		
	No. negative or not ill, n = 712†	No. positive, n = 20‡	p value
Mean age, y (± SD)	42.9 (11.3)	43.9 (9.3)	0.69
Female sex	595/712 (83.6)	16/20 (80.0)	0.76
Worker in acute care hospital	550/712 (77.3)	13/20 (65.0)	0.28
Recipient of vaccine			
A(H1N1)pdm09 vaccine§	533/699 (76.3)	2/20 (10.0)	<0.001
Seasonal influenza vaccine 2009–10	258/712 (36.2)	9/20 (45.0)	0.42
Seasonal influenza vaccine 2008–09	470/696 (67.5)	13/20 (65.0)	0.81
Underlying health conditions			
Asthma	67/702 (9.5)	2/20 (10.0)	1.00
Diabetes mellitus	26/700 (3.7)	1/20 (5.0)	0.54
Allergy to airborne irritants	284/646 (44.0)	11/18 (61.1)	0.15
Current smoker	63/704 (9.0)	3/20 (15.0)	0.42
Potential exposure conditions			
Hand-to-face habits¶	337/705 (47.8)	14/20 (70.0)	0.05
Wearing of prescription eyeglasses	493/709 (69.5)	12/20 (60.0)	0.36
Reusable water bottle use >1×/ wk	307/705 (43.6)	6/20 (30.0)	0.23
Public transit ≥8 trips per week	236/706 (33.4)	5/20 (25.0)	0.43
Group gathering attendance ≥1×	655/710 (92.3)	19/20 (95.0)	1.00
Face-to-face contacts/d, median (IQR)	10 (5, 20)	15 (8, 20)	0.53
Household crowding index >1#	233/697 (33.4)	10/20 (50.0)	0.12
Children in workplace	68/705 (9.7)	3/20 (15.0)	0.43
Child <5 y in household	83/712 (11.7)	4/20 (20.0)	0.28
Child <18 y in household	258/712 (36.2)	13/20 (65.0)	0.009
Child in household attends day care	87/699 (12.5)	6/20 (30.0)	0.03

*Data are no./total (%) unless otherwise specified. A(H1N1)pdm09, pandemic influenza A(H1N1) 2009 virus; IQR, interquartile range.
†Participants who did not report any illness or whose nasal swab samples tested negative for influenza.
‡All participants who tested positive were symptomatic.
§Participants who had acquired A(H1N1)pdm09 ≤7 d after vaccination were considered unprotected
¶Defined as biting one's nails or cuticles or habitually putting one's fingers in his or her mouth or nose.
#Household crowding index is defined as number of persons per household divided by the number of bedrooms.

participants were enrolled in the Influenza Cohort Study: 563 (76.9%) were HCWs who worked in 1 of 5 community and teaching acute care hospitals in the Toronto area and 169 (23.1%) were non-HCWs who worked in an office environment not associated with the provision of health care (Table 1; Figure). Of the 2 cohorts, HCWs were younger and were more likely to have been vaccinated against seasonal and pandemic influenza, to work with children, to have children <5 years of age in their households, and to use public transportation ≥8 times per week. Of 422 HCWs who were vaccinated against A(H1N1)pdm09, 403 (95.5%) received vaccine within 2 weeks after its availability; of 61 non-HCWs, 28 (45.9%) were vaccinated during the same time period (p<0.001).

A total of 334 (45.6%) study participants submitted 436 nasal swab samples. More than half (52.1%) of these samples were collected on the day of symptom onset (day 1), 19.4% on day 2, 9.9% on day 3, and 12.1% on or after day 4. Among the 20 (4.6%) specimens yielding influenza, 12 (60.0%) were collected on day 1, four (20.0%) on day 2, three (15.0%) on day 3, and one (5.0%) on day 4 of illness. Thirteen (2.2%) of 563 HCWs and 7 (4.1%) of 169 non-HCWs submitted samples that tested positive for influenza. A(H1N1)pdm09 was detected in 19 (95%) of the 20 positive participants: 1 case during each of calendar weeks 24, 25, 31, 39, 40, and 47; two cases during each of calendar weeks 42, 44, and 45; and 7 cases during calendar week 43. Thus, 16 of 19 cases occurred during weeks of peak A(H1N1)pdm09 activity. Seasonal influenza A(H3N2) virus was isolated in a sample from 1 participant during calendar week 43.

Risk Factors for Symptomatic Influenza Infection

The probability of symptomatic influenza infection did not differ between HCWs and non-HCWs (p = 0.28) (Table 2). Study participants who had a child <18 years of age living in the household (36.2% of influenza negative/ untested participants vs. 65.0% of influenza positive participants; p = 0.009), a child who attended day care living in the same household (12.5% vs. 30.0%; p = 0.03), and who were not vaccinated against A(H1N1)pdm09 >7 days before onset of infection (76.3% vs. 10.0%; p<0.001) were more likely to have respiratory illness with positive test results for influenza.

After adjusting for A(H1N1)pdm09 vaccination history and community influenza activity, we found no difference in the risk for influenza infection between persons working in an acute care hospital (HCWs) and other healthy adults (non-HCWs) (Table 3). Rather, contact with a family member with an ARI in the previous week was the main risk factor for symptomatic influenza infection, irrespective of the method of adjusting for

changing risk over time. In general, quasi-likelihood under the independence model criterion statistics were lower in models adjusting for weekly percentage of specimens yielding influenza than in those adjusting for weeks of peak influenza activity (results not shown). A sensitivity analysis calculating lags of 0 and 14 days (vs. 7 days) from the time of receipt of A(H1N1)pdm09 vaccine did not alter these results.

Analyses restricted to HCWs and including potential occupational risk factors in health care are shown in Table 4. During the study period, 49.6% of HCWs worked in emergency departments, medical inpatient wards, intensive care units, or pediatric wards; 12.9% were present during >1 and 9.4% performed >1 aerosol-generating medical procedure per week. Approximately one quarter (26.5%) of HCWs reported providing direct care for >1 patient per week who had ARI. The analysis of risk factors for infection indicates that, similar to the combined study population, HCWs with symptomatic influenza infection confirmed by positive nasal swab sample were more likely to have children <18 years of age in their households (69.2% of HCWs who tested positive vs. 36.9% who tested negative or were untested; p = 0.02) and less likely to have been vaccinated against A(H1N1)pdm09 >7 days before onset of infection (15.4% vs. 86.3%; p<0.001) (Table 4). Compared with other HCWs, those with symptomatic influenza infection were more likely to be present during aerosol-generating medical procedures >1× per week (38.5% vs. 12.7%; p = 0.02) and reported lower adherence to hand hygiene recommendations (77.5% vs. 95%; p = 0.02). After adjustment for changing risks for influenza infection over time, risk factors for influenza infection among HCWs were: contact with a family member with ARI in the previous week, performing or assisting with aerosol-generating medical procedures, and lower adherence to hand hygiene recommendations (Table 5).

HAI Antibody Assays

Among the combined study population, 450 (61.5%) of 732 participants provided pre- and post-influenza season blood samples. Among those, 3.6% had protective HAI titers against A(H1N1)pdm09 at baseline. There was no association with workplace and baseline HAI titers. Of the 142 (31.6%) participants who tested positive after enrollment, 137 (96.5%) had received the A(H1N1)pdm09 vaccine, 2 (1.4%) submitted a nasal swab that tested positive by PCR, and 3 (2.2%) did not submit a swab for testing or report an ARI (consistent with asymptomatic infection).

Analysis of data collected during the period after vaccine became available for unvaccinated participants without known previous A(H1N1)pdm09 infection showed that 8 (16.3%) of 49 HCW and 3 (5.3%) of 57 non-HCWs seroconverted or had a positive mid-turbinate nasal swab sample. Although persons working in an acute care hospital were 3.1× as likely as other working adults to be infected with influenza, the results were not significant in this small unvaccinated group (95% CI 0.9–11.1). Influenza among unvaccinated participants was not associated with age, sex, or any of the other characteristics listed in Table 1.

Discussion

In this prospective cohort study conducted in Canada during the 2009 influenza A(H1N1) pandemic, we found no association between working in an acute care hospital and risk for influenza infection. Our findings are similar

Table 3. Risk factors for symptomatic influenza infection among health care workers in acute care hospitals and non–health care workers in office settings during 2009 pandemic, Toronto, Ontario, Canada*

Risk factor	OR (95% CI), adjusted†	OR (95% CI), multivariable‡
Worker in acute care hospital	0.49 (0.19–1.27)	0.47 (0.17–1.32)
Age, y, per 10 y increase	1.08 (0.76–1.54)	NA
Female sex	1.03 (0.30–3.56)	NA
Recipient of A(H1N1)pdm09 vaccine§	0.28 (0.03–2.28)¶	0.34 (0.04–2.85)
Weekly percentage of specimens yielding influenza per 5% increase	1.49 (1.28–1.73)#	1.36 (1.13–1.63)
Potential exposure conditions		
Hand-to-face habits**	3.09 (1.12–8.52)	NA
Child <18 y in household	3.13 (1.21–8.07)	NA
Contact with family member with ARI in prior wk	5.51 (1.81–16.76)	6.89 (2.17–21.84)
Contact with co-worker with ARI in prior wk	0.77 (0.10–6.16)	NA
Household crowding index >1††	1.99 (0.79–5.05)	NA
Public transit ≥8 trips per wk	0.62 (0.22–1.76)	NA

*Constant and time-dependent risk factors for symptomatic influenza infection (positive nasal swab specimen) in 732 primary contacts of the Influenza Cohort Study followed during June 2009–April 2010, Toronto, Ontario, Canada. OR, odds ratio; NA, not applicable; A(H1N1)pdm09: pandemic influenza A(H1N1) 2009 virus; ARI: acute respiratory illness.
†Adjusted for receipt of A(H1N1)pdm09 vaccine and weekly percentage of specimens yielding influenza.
‡Multivariable model including all variables with ORs listed below.
§Participants who had acquired A(H1N1)pdm09 ≤7 d after vaccination were considered unprotected.
¶Adjusted for weekly percentage of specimens yielding influenza only.
#Unadjusted.
**Defined as biting one's nails or cuticles, habitually putting one's fingers in his or her mouth or nose.
††Household crowding index is defined as number of persons per household divided by the number of bedrooms.

to those of Williams et al., who assessed serologically confirmed influenza during the 2007–08 influenza season in Berlin, Germany (*14*). They found no association between HCW status and influenza but demonstrated that the presence of children in the household and ownership of a car among participants with no children in the household were risk factors, whereas receipt of seasonal influenza vaccine was found to be protective. Similarly, Marshall et al. found no overall difference in influenza infection rates between hospital workers who did and did not have patient contact during the 2009 pandemic in Australia, but the authors identified exposure to children as a risk for influenza (*15*).

The results of this cohort study also add insight into occupational risk factors for influenza among persons who work in acute care hospitals. In contrast to a finding by Kawana et al. (*16*), neither our study nor those of Marshall et al. and Seto et al. detected an increased risk for influenza among workers who had direct patient care responsibilities (*17*). However, Marshall et al. indicated that working in an intensive care unit of a hospital was a risk factor for influenza, and wearing gloves while caring for patients who were on droplet precaution was protective. These findings are similar to ours in that exposure to aerosol-generating medical procedures, which are most often performed in intensive care units, was a risk factor for influenza, and adherence to hand hygiene, which may have an effect similar to appropriate glove use, was protective. Although collinearity of both putative risk and protective factors may continue to make it difficult to accurately identify risk factors for acquisition of influenza in health care settings, our data highlight the role of hand hygiene in the control of influenza infection (*18*), and of protective equipment use by persons who perform or assist with aerosol-generating medical procedures.

The mode of transmission of influenza remains a matter of ongoing debate. Although most experts believe that droplet and aerosol transmission are the most common modes of spread of influenza, our finding and that of Marshall et al. (*15*), as well as the evidence from the elementary school–based study by Talaat et al. that increasing hand hygiene adherence reduces the risk for infection with influenza, suggest that transmission by direct or indirect contact contributes substantially to influenza transmission (*18*). Appropriate hand hygiene practice should continue to be recommended to prevent influenza transmission.

Pandemic influenza vaccine became available in Canada at the peak of the second wave of the pandemic. This complicated our analysis in that the risk for influenza infection depended on differing times of receipt of influenza vaccine and on timing of the pandemic

Table 4. Characteristics of 563 health care workers in acute care hospitals during 2009 pandemic, Toronto, Ontario, Canada*

Characteristic	Influenza test status		
	No. negative or not ill, n = 550†	No. positive, n = 13‡	p value
Mean age, y (± SD)	42.2 (11.4)	42.5 (10.1)	0.91
Female sex	467/550 (84.9)	11/13 (84.6)	1.00
Occupation			
Nurse	180/539 (33.4)	3/13 (23.1)	0.55
Physician, physiotherapist, respiratory therapist	103/539 (19.1)	5/13 (38.5)	0.15
Other§	256/539 (47.5)	5/13 (38.5)	0.52
Potential exposure conditions			
Received A(H1N1)pdm09 vaccine¶	467/541 (86.3)	2/13 (15.4)	<0.001
Child <18 y in household	203/550 (36.9)	9/13 (69.2)	0.02
Child attending day care in household	74/542 (13.7)	3/13 (23.1)	0.40
Cares for >1 patient with ARI per week	141/539 (26.2)	5/12 (41.7)	0.32
Working in high-risk area#	227/461 (49.2)	7/11 (63.6)	0.35
Present during aerosol-generating medical procedure >1/wk**	66/521 (12.7)	5/13 (38.5)	0.02
Performs aerosol-generating medical procedure >1/wk	49/540 (9.1)	3/13 (23.1)	0.11
Years' experience, mean (± SD)	13.6 (11.4)	14.0 (9.3)	0.91
% adherence to hand hygiene, median (IQR)	95.0 (80.0–100)	77.5 (60.0–92.5)	0.02
Adherence to facial protection, %, median (IQR)	80 (50–99)	50 (30–75)	0.16
Hours worked per week, no. median (IQR)	40.0 (37.5–45.0)	37.5 (32.0–40.0)	0.22

*Data are no./total (%) unless otherwise specified. A(H1N1)pdm09, pandemic influenza A(H1N1) 2009 virus; ARI, acute respiratory illness; IQR, interquartile range.
†Participants who either did not report any illness or whose nasal swab samples tested negative for influenza.
‡All participants who tested positive were symptomatic.
§The distribution of other persons working in acute care hospitals was: administrative personnel: 30.4%; patient attendant/health care aide/service assistant: 0.4%; housekeeper/porter/central sterile supply/dispatch: 0.5%; medical imaging technologist/technician: 1.6%; pharmacist/pharmacy technician: 2.0%; ward clerk/unit coordinator: 1.4%; psychologist/social worker: 1.6%; laboratory technologist/technician: 4.7%; nutritionist/other food service staff: 1.1%; other: 3.4%.
¶Participants who had acquired A(H1N1)pdm09 ≤7 d of vaccination were considered unprotected.
#Emergency room, medical inpatient ward, intensive care unit, or pediatric ward.
**Aerosol-generating medical procedures are defined as any one of: administration of nebulized therapy or humidified oxygen at >40%, use of bag-valve mask, manual ventilation, non-invasive ventilation, open airway suctioning, bronchoscopy or other upper airway endoscopy, tracheostomy, endotracheal intubation, cardiopulmonary resuscitation, oscillatory ventilation, any procedure performed that involves manipulation of open ventilator tubing in a mechanically ventilated patient, sputum induction or other deliberate induction of coughing.

Table 5. Risk factors for symptomatic influenza infection in health care workers in acute care hospitals during 2009 pandemic, Toronto, Ontario, Canada*

Risk factor	OR (95% CI), adjusted†	OR (95% CI), multivariable‡
Age, y, per 10 y increase	0.99 (0.63–1.56)	NA
Female sex	1.79 (0.23–14.04)	NA
Potential exposure conditions		
Receipt of A(H1N1)pdm09 vaccine§	0.49 (0.07–3.67)¶	0.40 (0.04–3.99)
Weekly specimens yielding influenza, %, per 5% increase	1.56 (1.29–1.88) #	1.43 (1.17–1.73)
Child <18 y in household	3.33 (1.00–11.05)	NA
Contact with family member with ARI in prior week	7.26 (2.15–24.54)	7.86 (2.20–28.04)
Contact with co-worker with ARI in prior week	1.40 (0.16–12.40)	NA
Cared for patient with ARI in prior week	1.50 (0.44–5.14)	NA
Adherence to hand hygiene recommendations, per 10% increase	0.84 (0.73–0.98)	0.86 (0.74–0.99)
Adherence to facial protection recommendations, per 10% increase	0.92 (0.79–1.07)	NA
No. AGMP performed or assisted during previous week, per 10 procedures increase**	2.29 (1.26–4.17)	1.95 (1.10–3.48)

*Generalized estimating equation logistic regression analysis of constant and time-varying risk factors for influenza infection in 563 health care workers in acute care hospitals, Influenza Cohort Study, followed during June 2009–April 2010, OR, odds ratio; A(H1N1)pdm09: pandemic influenza A(H1N1) 2009 virus; NA, not applicable; ARI: acute respiratory illness; AGMP: aerosol-generating medical procedures.
†Adjusted for receipt of A(H1N1)pdm09 vaccine and weekly percentage of specimens yielding influenza.
‡Multivariable model including all variables with ORs listed below.
§Participants who had acquired A(H1N1)pdm09 ≤7 d after vaccination were considered unprotected.
¶Adjusted for weekly percentage of specimens yielding influenza only.
#Undadjusted.
**AGMP are defined as any one of the following: administration of nebulized therapy or humidified oxygen at >40%, use of bag-valve mask, manual ventilation, noninvasive ventilation, open airway suctioning, bronchoscopy or other upper airway endoscopy, tracheostomy, endotracheal intubation, cardiopulmonary resuscitation, oscillatory ventilation, any procedure performed that involves manipulation of open ventilator tubing in a mechanically ventilated patient, sputum induction or other deliberate induction of coughing; OR for being in the same room during AGMP (>1/week) 6.63 (95% CI 2.05–21.41); OR for participants performing AGMP (>1/week) 4.21 (1.12–15.76).

waves. We addressed these issues by using multivariable generalized estimating equation logistic regression for the analysis, which facilitated adjustment for timing of receipt of vaccine, and we accounted for the dynamics of the pandemic waves by incorporating weekly percentages of laboratory specimens that tested positive for influenza virus. We believe that our results are robust because 2 different approaches to adjust for changing risk over time led to the same results. Nevertheless, whether the relative percentage of positive specimens reflects the relative number of influenza cases in the community remains a matter of debate.

Our study has several limitations. It has a lack of power related to the small number of cases of symptomatic influenza during the second wave of the pandemic in this population of working adults. We attempted to minimize selection bias by using broad inclusion and limited exclusion criteria; nevertheless, the possibility of having access to rapid diagnosis and treatment during the second pandemic wave might have resulted in biased enrollment of participants who had a higher self-perceived risk for influenza infection, and perception of risk might differ between persons working in acute care hospitals and persons working in nonclinical settings. Similarly, generalizability may be hampered because participants in studies of influenza could differ from others in their attitudes toward vaccine acceptance and infection prevention practices. We tried to reduce the possibility of measurement bias in nasal swab collection by having a broad interpretation of respiratory illness because the interpretation of more detailed criteria for signs or symptoms of influenza infection (e.g., influenza-like illness) might differ between HCWs and non-HCWs, but differences might have remained. Although the self-collection of swab specimens occurred over 1–4 days after illness onset, it is unlikely that any cases would have been missed because previous studies have shown that A(H1N1)pdm09 remains readily detectable within this period (*19–21*). The study encompasses a selective sample of persons working in a limited number of acute care hospitals and other working adults with Internet access in a single geographic area during the 2009 influenza A(H1N1) pandemic. Although we deliberately selected controls likely to be at low risk for occupational exposure to influenza (e.g., not working in an occupation exposed to numerous children) in an effort not to miss an effect of the health care work environment, unmeasured biases in our control selection could have been present. In addition, our results may not be generalizable to seasonal influenza or to geopolitical areas where infection control practices in hospitals are different.

The yield of self- or parent-collected nasal swab specimens has been shown to be comparable to health care provider–collected nasopharyngeal aspirates from children and adults (*22–24*), but whether the yield of self-collected nasal swabs differs between HCWs and non-HCWs has not been assessed. There is evidence that microneutralization of antibody assays may demonstrate a greater sensitivity than HAI (*25*); as a result, we may have missed seroconversion by using the latter. Further seroconversions might have been missed by the delay between the first (upon enrollment) and the second

(April or May 2010) blood sampling caused by declining antibody titers over time. Recall bias might have played a role in that ill participants might have reported risk factors such as contact with sick people in the previous week more accurately than people who did not develop an illness. Finally, participating in the study may have reinforced awareness of the risk for influenza infection and thus may have raised adherence to protective measures.

We did not identify an increase in the risk for influenza among workers in acute care hospitals compared to office-based workers during the 2009 pandemic. However, our findings are limited by lack of power. Within an HCW group, we were able to identify activities that could help focus prevention. Increasing efforts to improve hand hygiene and the use of protective equipment during aerosol-generating medical procedures would further reduce the risk for influenza infection among HCWs.

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S.P.K. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Deaths Associated with Influenza Pandemic of 1918–19, Japan

Siddharth Chandra

Current estimates of deaths from the influenza pandemic of 1918–19 in Japan are based on vital records and range from 257,000 to 481,000. The resulting crude death rate range of 0.47%–0.88% is considerably lower than parallel and conservative worldwide estimates of 1.66%–2.77%. Because the accuracy of vital registration records for early 20th century Asia is questionable, to calculate the percentage of the population who died from the pandemic, we used alternative prefecture-level population count data for Japan in combination with estimation methods for panel data that were not available to earlier demographers. Our population loss estimates of 1.97–2.02 million are appreciably higher than the standing estimates, and they yield a crude rate of population loss of 3.62%–3.71%. This rate resolves a major puzzle about the pandemic by indicating that the experience of Japan was similar to that of other parts of Asia.

The influenza pandemic of 1918–19 caused unprecedented devastation (*1*); worldwide, it is estimated to have taken 25–100 million lives (*2*,*3*), exceeding the combined death toll of both world wars. One of the strangest aspects of the currently held wisdom about the pandemic is the curiously low death rate attributed to Japan compared with other countries in Asia. Official records for Japan put the death toll at 257,363 persons (*4*), resulting in a crude influenza-attributable death rate of 0.47%. Patterson and Pyle (*2*) reported 350,000 deaths, and Johnson and Mueller (*3*) cited a figure from Palmer and Rice (*5*) of 388,000 deaths. Given Japan's population of >54 million at the time (*6*), the influenza-attributable mortality rates (0.64%–0.71%) are remarkably low by Asian standards, although they are similar to the rates calculated for the United States, Canada, and western Europe (0.65%, 0.61%, and ≈0.48%, respectively) (*3*). Patterson and Pyle's (*2*) conservative estimate of a global rate of 1.66% and Johnson and Mueller's (*3*) substantial upward revision of that percentage to 2.77% suggest that the estimates for Japan, which are less than one quarter of the latter estimate, merit closer scrutiny. Although the epidemiologic approach used by Richard et al., which also uses death statistics reported by the Japanese health authorities, raises the estimate to 481,000 (or 0.88% of the population at the time) (*7*), even this estimate is extraordinarily lower than estimates from other parts of Asia.

As Taeuber argued in her classic book, *The Population of Japan*, Japan occupies a special place in demography (*8*). Worldwide it remains one of the largest economies (third in 2011) and one of the most populous countries (tenth in 2011). Yet, surprisingly, substantial knowledge gaps remain with regard to the influenza pandemic of 1918–19 in Japan, rendering it "a strangely neglected episode in modern Japanese history" (*4*, p. 389). For example, a search of Taeuber's work for the term "influenza" revealed only 1 mention of the influenza epidemic of 1918, in the context of speculation that it "may have led to reduced conceptions" (*8*, p. 233).

The few scholars who have studied the influenza pandemic in Japan have approached it from 1 of 3 broad perspectives: historical, epidemiologic, or demographic. The historical approach is exemplified by the works of Palmer and Rice, which provide a qualitative contextualization of aspects of the pandemic and its management in Japan (*4*,*5*,*9*,*10*). A second line of research is epidemiologic, within which 2 broad goals are pursued. The first goal is to produce estimates of major epidemiologic characteristics of the virus (*11–13*), and the second goal is to produce epidemiology-based estimates of mortality rates from the pandemic (*7*). The demographic approach is exemplified by Morita, Okazaki, Taeuber, and Yasukawa and Hirooka (*8*,*14–16*). Although these studies emphasize broader patterns of population growth in Japan, a few address the question of death rates during the pandemic. For example, Yasukawa and Hirooka (*16*) relied directly on official death statistics, including those from the pandemic, to produce estimates of the population in early 20th-century Japan. Unfortunately, the quantitative literature seems to have more or less accepted the official vital statistics on disease-specific deaths, feeding them (and therefore their inaccuracies) into otherwise technically refined estimates of population and population growth.

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A common characteristic of the above studies is their heavy reliance on official vital and health statistics of the time. Such data are widely recognized by demographers as being plagued by the often-severe problem of underreporting. Indeed, according to Johnson and Mueller, "it is generally accepted that recorded statistics of influenza morbidity and mortality are likely to be a significant understatement" (*3*, p. 108). For India, Davis estimated that the "amount of underregistration certainly exceeds 30 per cent at all times, and is probably nearer 50 per cent" (*17*, p. 34). For Indonesia, Gooszen advised that such data "should be regarded with a good deal of caution" (*18*, p. 32), and Nitisastro opined that "for the system of registering deaths, the quality of the results was poor" (*19*, p. 101). Japan is no exception to this pattern. According to Mosk, "we do not have a trustworthy picture of what happened to vital rates in the Tokugawa period…. The same can be said for the Meiji period" (*20*, p. 658), and Taeuber's assessment was that "the critical question is the accuracy of the records of vital events" (*8*, p. 50). The uncharacteristically low estimates of deaths from influenza in Japan provide a strong rationale for cross-checking the findings in the manner of Davis' classic study of India (*17*).

We therefore used recently developed statistical methods to estimate the loss of population in Japan from the influenza pandemic of 1918–19. We adopted an approach that intentionally avoids heavy reliance on vital registration data and is based instead on population count data for Japan of that period. By applying data for multiple prefectures over time to prefecture-level population statistics, we estimated population loss from the pandemic to be the difference between expected population (using the prepandemic trajectory) and observed population (using the postpandemic trajectory) (*17*,*21*,*22*). The new estimates are appreciably higher than the earlier estimates, bringing Japan's pandemic experience in line with that of other parts of Asia and resolving a major puzzle in the epidemiology of the 1918–19 pandemic.

Methods

Data-associated Issues

With regard to data, 3 issues should be considered. The first is the coverage of the population count data for Japan in the late 19th and early 20th centuries, described by Matsuda (*23*) and Taeuber (*8*). In 1871, the Imperial Japanese government passed a law, the *koseki-ho*, which required registration of households and persons in Japan. A major emphasis of the registrations was legal domicile, or *honseki* status. The first set of summations of these registers was made in 1898, after which they were computed every 5 years until 1918, for a total of 5 nationwide population counts derived directly from the registers (*8*,*24*–*26*). The number of persons who physically resided in different parts of Japan (de facto A-type population) was computed by adjusting the numbers of persons with *honseki* status downward to account for those who had *honseki* status but lived in other locations. These numbers were further adjusted to account for the discrepancy between numbers of registered persons who immigrated into the various prefectures, which always exceeded the numbers of persons who migrated out (de facto B-type population ([*27*]). Over time, through a process of learning by doing (habituation), the registration data became reasonably accurate (*8*).

For this study, we used data from the quinquennial (every 5 years) summations of 1898 and after. Given the de facto nature of the censuses of 1920 and after, we used the B-type population statistics for comparable data for 1918 and before (*6*). Because the population figures are based on repeated summations of records that were repeatedly updated, the count for a household was periodically revised upward or downward, and hitherto unreported births and deaths would have been more accurately captured by these revisions, even if they had not been reported in the annual vital registration records. Taeuber (*8*) provides evidence as follows: "Early publications of the Bureau of Statistics included a warning statement that the majority of the additions to the registers were the survivors of unrecorded births of earlier years," and "Failures to report deaths during the earlier years are evident in the accumulations of the aged in the successive reports." This phenomenon forms the basis for our reasoning that the quinquennial population count data are more accurate than the annual vital registration records.

The second data-associated issue is the change in the regime for population enumeration that began in 1920, when Japan conducted its first census of its de facto population. This census is widely regarded as having been accurate and yielded a population count of 55.96 million (*6*). After conducting the 1920 census, Japan conducted quinquennial censuses until the beginning of World War II. The problem with the timing of the change in the system is that quinquennial registration count totals are available up to 1918, and the quinquennial census counts started in 1920. Therefore, the break point in the system of population enumeration approximately coincides with the break point (1918) for which population loss is to be estimated. Any statistical estimation of change across such a break point must satisfactorily address discontinuity in the data collection system. Fortunately, earlier demographers and statisticians went to great lengths to splice the data across this break point, producing similar estimates. Taeuber (*8*), for example, demonstrated that a backward projection of population from 1920 through 1898 produces a 1898 population estimate that is remarkably similar to a forward projection of population from 1871 through 1898. The theme of splicing is also covered in the

works of Morita, Okazaki, and Yasukawa and Hirooka (*14–16*). Ohbuchi (*28*) compared the estimates of these authors, all of which are within 2% of each other for 1915 and 1920, and concluded that the estimates of Yasukawa and Hirooka, which are based on a reverse survival method, are the most reliable. Although the procedure used by Yasukawa and Hirooka is generally robust, its adjustment for the influenza pandemic fails because the official influenza death statistics of ≈178,000 for 1918–1920 were taken at face value and incorporated into estimates of life expectancy at birth (*16*). Therefore, inaccuracies in the official vital statistics of the period flow directly into the estimates of Yasukawa and Hirooka. When selecting the data for the analysis, therefore, we started with the observations of Yasukawa and Hirooka (*16*), who stated that by 1900, the most widely used population estimates of demographers (*14–16*) tend to converge and are close to the official population statistics. Next, because the official statistics are the only ones that contain published data at the prefectural level (*6*) (Morita, Okazaki, and Yasukawa and Hirooka [*14–16*] focus on producing Japan-wide data), we used the official statistics pertaining to the quinquennial population count (1918 and before) and census years (1920 and after).

The third data-related issue is the unreliability of the data before 1898 (*8,16*); therefore, we used the 1898 population count as the starting point in our analysis. To maintain balance of the dataset across the break point of 1918, we also limited our data to the censuses including and before 1935. The full dataset consists of observations for each of 47 prefectures for the population count for the years 1898, 1903, 1908, 1913, and 1918, and the census data for 1920, 1925, 1930, and 1935, for a total of 423 observations.

Data Analyses

Although for decades scholars have been intrigued by the subject of low mortality rates from the pandemic in Japan, the currently circulating estimates were produced before the development and mainstreaming of panel data estimation methods. The studies described above based population estimates on annual or quinquennial observations for all of Japan and used datasets that were small in terms of numbers of observations. Given the existence of a panel of prefectural data on population for 47 prefectures and multiple time points straddling the pandemic years, more recently developed panel data methods can be used to estimate a standard population growth process

Table 1. Population growth models and population loss estimates for Japan, 1903–1930 data*

Estimate	Model 1	2	3	4	5	6	7	8
Includes Kanto earthquake prefectures†	Yes	Yes	Yes	Yes	No	No	No	No
Includes Hokkaido outlier	Yes	Yes	No	No	Yes	Yes	No	No
Includes 1918 population count data	Yes	No	Yes	No	Yes	No	Yes	No
Intercept, γ_{00}	13.6120‡	13.5957‡	13.6197‡	13.6040‡	13.5799‡	13.5623‡	13.5876‡	13.5706‡
	0.0524	*0.0530*	*0.0530*	*0.0535*	*0.0534*	*0.0537*	*0.0541*	*0.0543*
Time trend, γ_{10}	0.0103‡	0.011‡	0.0095‡	0.0105‡	0.0098‡	0.0110‡	0.0089‡	0.0100‡
	0.0012	*0.0012*	*0.0009*	*0.0008*	*0.0012*	*0.0012*	*0.0009*	*0.0008*
Flu dummy, γ_{20}	–0.0344‡	–0.0477‡	–0.0364‡	–0.0492‡	–0.0374‡	–0.0518‡	–0.0397‡	–0.0536‡
	0.0055	*0.0070*	*0.0052*	*0.0069*	*0.0055*	*0.0067*	*0.0051*	*0.0067*
Flu dummy × time trend, γ_{30}	0.0006	–0.0004	0.0013§	0.0003	0.0002	–0.0010	0.0009	–0.0002
	0.0009	*0.0010*	*0.0006*	*0.0007*	*0.0009*	*0.0010*	*0.0006*	*0.0006*
No. observations	329	282	322	276	301	258	294	252
Hausman test statistic	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	p>0.9999	p>0.9999	p>0.9999	p>0.9999	p>0.9999	p>0.9999	p>0.9999	p>0.9999
Breusch-Pagan test statistic	924.89	653.01	921.41	652.71	848.22	599.17	847.85	601.55
	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001	p<0.0001
Population change from influenza, millions	–1.38	–1.97	–1.50	–2.02	–1.48	–2.12	–1.61	–2.17
Population change from influenza, %	–2.53	–3.62	–2.87	–3.87	–3.15	–4.51	–3.59	–4.85
Population change, 1918–19, millions	–0.66	–1.21	–0.89	–1.38	–0.90	–1.49	–1.13	–1.65
Population change, 1918–19, %	–1.20	–2.19	–1.69	–2.60	–1.89	–3.13	–2.51	–3.65
Annual population growth rate to pandemic, %	1.03	1.13	0.95	1.05	0.98	1.10	0.89	1.00
Annual population growth rate after pandemic, %	1.09	1.09	1.08	1.08	1.00	1.00	0.98	0.98

**Italics* indicate SE of the coefficient.
†Chiba, Kanagawa, Shizuoka, Tokyo.
‡p<0.01.
§p<0.05.

Table 2. Population growth models and population loss estimates for Japan, 1898–1935 data*

Estimate	Model 1	2	3	4	5	6	7	8
Includes Kanto earthquake prefectures†	Yes	Yes	Yes	Yes	No	No	No	No
Includes Hokkaido outlier	Yes	Yes	No	No	Yes	Yes	No	No
Includes 1918 population count data	Yes	No	Yes	No	Yes	No	Yes	No
Intercept, γ_{00}	13.6053‡	13.5971‡	13.6137‡	13.6059‡	13.5755‡	13.5673‡	13.5839‡	13.5762‡
	0.0523	*0.0526*	*0.0528*	*0.0530*	*0.0537*	*0.0539*	*0.0543*	*0.0545*
Time trend, γ_{10}	0.0106‡	0.0113‡	0.0097‡	0.0104‡	0.0100‡	0.0107‡	0.0091‡	0.0097‡
	0.0012	*0.0012*	*0.0009*	*0.0008*	*0.0012*	*0.0012*	*0.0008*	*0.0008*
Flu dummy, γ_{20}	–0.0355‡	–0.0464‡	–0.0373‡	–0.0476‡	–0.0379‡	–0.0486‡	–0.0399‡	–0.0501‡
	0.0053	*0.0060*	*0.0050*	*0.0060*	*0.0054*	*0.0062*	*0.0051*	*0.0062*
Flu dummy × time trend, γ_{30}	0.0002	–0.0005	0.0009	0.0002	–0.0002	–0.0009	0.0005	–0.0001
	0.0009	*0.0009*	*0.0006*	*0.0006*	*0.0009*	*0.0010*	*0.0006*	*0.0006*
No. observations	423	376	414	368	387	344	378	336
Hausman test statistic	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	p>0.9999	p>0.9999	p>0.9999	p>0.9999	p>0.9999	p>0.9999	p>0.9999	p>0.9999
Breusch-Pagan test statistic	1525.90	1171.30	1535.75	1183.05	1403.42	1078.01	1422.01	1097.53
	p<0.0001	p<0.0001	p<0.0001	p<0.0001	<0.0001	p<0.0001	p<0.0001	p<0.0001
Population change from Influenza, millions	–1.49	–2.02	–1.59	–2.05	–1.52	–1.98	–1.62	–2.01
Population change from Influenza, %	–2.72	–3.71	–3.03	–3.92	–3.23	–4.23	–3.62	–4.50
Population change, 1918 to 1919, millions	–0.75	–1.25	–0.96	–1.39	–0.93	–1.37	–1.14	–1.51
Population change, 1918 to 1919, %	–1.35	–2.26	–1.81	–2.63	–1.96	–2.88	–2.53	–3.34
Annual population growth rate to pandemic, %	1.06	1.13	0.97	1.04	1.00	1.07	0.91	0.97
Annual population growth rate after pandemic, %	1.08	1.08	1.06	1.06	0.98	0.98	0.96	0.96

*Italics indicate SE of the coefficient.
†Chiba, Kanagawa, Shizuoka, Tokyo.
‡p<0.01.

that explicitly builds in a break point for the influenza pandemic (*21*,*22*). By treating these 47 prefectures of Japan as individual units, each with its own set of observations, the panel data method leverages the large amount of additional information available at the prefectural level to generate a more robust picture of population change and the effect of the influenza pandemic on that process. This method is also flexible enough, given the large sample size, to accommodate prefecture-specific variation. In this manner, the method enables estimation of prefecture-specific growth processes, each with a prefecture-specific estimate of population loss from the pandemic, while still leveraging the entire set of observations to create an aggregate estimate for Japan. This method is implemented by running a regression of the logarithm of population on a linear time trend while allowing for a 1-time (downward) shift in that time trend during 1918–19 to capture influenza-attributable population loss. Details of this method are provided in the online Technical Appendix (wwwnc.cdc.gov/EID/article/19/4/12-0103-Techapp1.pdf).

To examine the robustness of the estimates, we conducted a variety of sensitivity analyses. First, to control for the possible inaccuracy of the 1898 data and for the effects of outliers in time (the 1898 and 1935 data), we estimated models without these 2 time points. Second, we estimated models without the 4 prefectures that were most affected by the devastating Kanto earthquake of 1923: Chiba, Kanagawa, Shizuoka, and Tokyo. Third, because the 1918 population count was reported as of December of that year (i.e., the year of the pandemic), thereby introducing the possibility of contamination in the growth rate estimate for the prepandemic trajectory, we estimated models without the 1918 data. Finally, given the atypical population dynamic in Hokkaido, a frontier region in the early 20th century to which a large and prolonged wave of migration was in progress, we estimated models without data for Hokkaido. These sensitivity exercises yielded a total of 16 possible permutations of the model. Additional sensitivity analyses involved using the alternative A-type statistics (*6*) and dropping the data for 1898 (i.e., using 1903 as the earliest year) to account for the above-mentioned habituation process.

Results

Tables 1 and 2 contain the parameter estimates for the 16 models. Without exception, the models show the significant negative effect of the influenza pandemic on Japan's population (via the flu dummy described in the

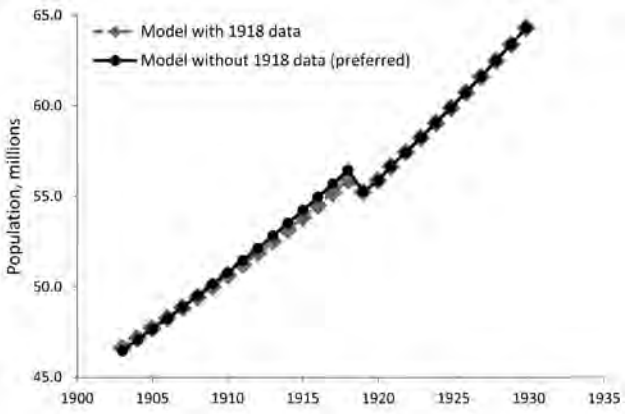

Figure. Effect of including 1918 data on estimated population of Japan. Data cover 1903–1930 and include observations for Hokkaido and the prefectures affected by the Kanto earthquake of 1923 (Chiba, Kanagawa, Shizuoka, and Tokyo).

online Technical Appendix); the calculated population loss ranged from 1.38 to 2.05 million persons. The range of estimated population growth rates across the models is 0.89%–1.13% per year, which is in line with the summary of estimates presented by Ohbuchi (*28*). In all but 1 model, there is no appreciable difference in the rates of population growth before and after the pandemic. As predicted, the inclusion of the population count data for 1918, which already reflect some but not all deaths from the pandemic, pulls the prepandemic population growth trajectory down (Figure), yielding substantially lower estimates of death and population loss than corresponding models that did not include those data (Tables 1, 2). For this reason, the models that exclude the 1918 data are preferred to the models that include the 1918 data.

Table 3 demonstrates that the models that control for other phenomena, including the Kanto earthquake of 1923, the 1898 and 1930 data, and the Hokkaido outlier, generate ranges of estimates that are similar to each other. Use of the alternative but less preferred A-type statistics (*6*) greatly increased the estimates of the number of deaths, thereby strengthening our conclusions.

The only control that yielded distinct estimates conditional on its inclusion was the 1918 data control; the ranges of estimates for models that include the 1918 data (−1.38 to −1.61 million and −1.49 to −1.62 million) do not overlap with the ranges of estimates for models that exclude the 1918 data (−1.97 to −2.17 million and −1.98 to −2.05 million). Because other controls seem to have no material effect on the results, the final models selected are the ones in which the 1918 data are dropped but none of the other controls are implemented (i.e., the models in the second column of Tables 1 and 2). The estimated population loss is therefore 1.97 or 2.02 million persons, which translates to a drop in population of 3.62% or 3.71%.

Discussion

For nearly a century, Japan's experience during the influenza pandemic of 1918–19 has been viewed as an anomaly within the broader Asian experience. In stark contrast with significantly higher estimates for deaths in Asia and globally, which themselves are often conservative, the standing mortality rates for Japan, based heavily on vital registration data, are <1%. There is, however, substantial reason to believe that vital registration data for the early 20th century in the most densely populated parts of Asia, including British India (*17*), the Dutch East Indies (*19*), and Japan (*8*,*20*), are inaccurate, suggesting the need for verification of mortality rates by using the Davis method (*17*), which is based on population count or census data. The key result of this study is that when these alternative population counts and census data are used, the experience of Japan conforms more closely to that of the rest of Asia; in Japan, rates of population loss approach 4% and an actual loss of ≈2 million. These estimates are similar to those for India (*17*,*22*). This result has implications for the large bodies of work on the epidemiology of the influenza pandemic of 1918–19 and, more broadly, the demographic history of Japan. Even adjusting for the possibility that a brief decline in fertility partly explains the population loss estimated in this study, the number of deaths in Japan were in all probability much higher than previously believed.

The results of this study come from using an alternative data source rather than vital registration data. Although the alternative data source is vulnerable to any inaccuracies inherent in the population counts and censuses of Japan, it nevertheless provides a way to confirm or contradict prior results that were based on vital registration data in the manner of Davis (*17*) and Chandra et al. (*22*) for India and Chandra (*21*) for Indonesia. Given the relatively reliable nature of population count and census data in comparison with vital registration data, however, the inaccuracies in the above analysis, in percentage terms, are probably smaller for population count and census data than for vital registration data.

A second possible limitation of the family of models estimated above is the implicit assumption of constant population growth rates for the periods before and after the pandemic. The analyses of Japanese demographers suggest some variation in birth and death rates during this period (*29*). Yet because we assumed stable population growth (derived from the differential between birth and death rates, with adjustment for migration), the models are tenable in view of the findings of these demographers of fairly stable population growth rates in Japan between 1900 and 1920 (*28*). The finding of population growth in the models (Tables 1, 2) that lies within the range of earlier estimates is further cause for confidence in these models.

Table 3. Sensitivity analysis of influenza-induced change estimates for Japan

	Ranges for estimated population change from influenza (millions)							
	1903–1930 data				1898–1935 data			
	Included		Excluded		Included		Excluded	
Controls for models	Low	High	Low	High	Low	High	Low	High
Kanto earthquake prefectures*	–1.38	–2.02	–1.48	–2.17	–1.49	–2.05	–1.52	–2.01
Hokkaido outlier	–1.38	–2.12	–1.50	–2.17	–1.49	–2.02	–1.59	–2.05
1918 population count data	–1.38	–1.61	–1.97	–2.17	–1.49	–1.62	–1.98	–2.05

*Chiba, Kanagawa, Shizuoka, Tokyo.

Statistical by-products of this study include the substantial upward revision of the toll of the pandemic and the information about annual population estimates for Japan. The higher number of deaths should affect worldwide estimates of deaths from the pandemic published in studies, such as those by Patterson and Pyle (*2*) and Johnson and Mueller (*3*), and estimates about the epidemiologic characteristics of the disease in Japan that depend on those data. The annual population estimates for Japan should advance the rich literature for Japan as a whole and for the 47 prefectures by generating new estimates that explicitly account for the effect of the pandemic. Although the estimates for years distant from the influenza pandemic are similar to those produced by demographers, including Morita, Okazaki, and Yasukawa and Hirooka, they depart substantially from these estimates for 1915–1920, with implications for the earlier published works that have used these data.

Given the virulence of the influenza A(H1N1) virus that caused the disease and the continued worry caused by the possibility of its reemergence (*1*), this study dispels the myth that Japan was spared the ravages of the influenza pandemic of 1918–19. Japan is not an exception to be studied for possible solutions or measures that might ameliorate the effects of such an epidemic in the future. Rather, its experience is typical of that of other Asian countries for which we have more reliable estimates.

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Methicillin-Resistant *Staphylococcus aureus* Colonization of the Groin and Risk for Clinical Infection among HIV-infected Adults

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Data on the interaction between methicillin-resistant *Staphylococcus aureus* (MRSA) colonization and clinical infection are limited. During 2007–2008, we enrolled HIV-infected adults in Atlanta, Georgia, USA, in a prospective cohort study. Nares and groin swab specimens were cultured for *S. aureus* at enrollment and after 6 and 12 months. MRSA colonization was detected in 13%–15% of HIV-infected participants (n = 600, 98% male) at baseline, 6 months, and 12 months. MRSA colonization was detected in the nares only (41%), groin only (21%), and at both sites (38%). Over a median of 2.1 years of follow-up, 29 MRSA clinical infections occurred in 25 participants. In multivariate analysis, MRSA clinical infection was significantly associated with MRSA colonization of the groin (adjusted risk ratio 4.8) and a history of MRSA infection (adjusted risk ratio 3.1). MRSA prevention strategies that can effectively prevent or eliminate groin colonization are likely necessary to reduce clinical infections in this population.

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections are a substantial cause of illness and a major public health problem (*1*). Although MRSA was traditionally considered a health care–associated pathogen, it has emerged worldwide as a notable cause of community-associated skin and soft tissue infections (*2*). In the United States, MRSA pulsed-field gel electrophoresis (PFGE) type USA300 strains have caused most community-associated MRSA infections (*3*). High rates of community-associated (*4*–*6*) and health care–associated MRSA infections have also been described among HIV-infected persons (*7*), although the underlying basis for this association is unknown. Proposed mechanisms include immune dysfunction (*5*,*7*,*8*), behavioral risk factors (*9*), and increased exposure to the health care system (*10*). The prevalence of MRSA colonization among HIV-infected persons is also high (10%–17%) (*11*,*12*), compared with that in the general US population (0.8%–1.5%) (*13*,*14*). Colonization with *S. aureus* is a risk factor for subsequent clinical infection (*15*,*16*), and the site of colonization may also be an key risk factor (*17*). For example, although the anterior nares is considered the primary reservoir of *S. aureus* (*18*), MRSA PFGE type USA300 might preferentially colonize the buttocks, genitals, and perineum (*17*), leading to more infections in these anatomical areas. Improving our understanding of the interaction between MRSA colonization and clinical infection among persons with HIV is necessary so that effective prevention strategies can be developed for this population.

Methods

Study Design

Study participants were recruited from the Atlanta Veterans Affairs Medical Center (Atlanta, GA, USA) HIV clinic, which provides medical care to ≈1,400 HIV-infected veterans and is the largest Veterans Affairs Medical Center HIV clinic in the United States. This study was approved by institutional review boards for Emory University and the Centers for Disease Control and Prevention (CDC) and the Veterans Affairs Research and Development Committee. Eligible participants were HIV-infected, ≥18 years of age, receiving outpatient medical care at the Atlanta Veterans Affairs Medical Center HIV clinic, and competent to provide informed consent. All eligible participants who attended the clinic from September 2007

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through April 2008 were invited to participate in the study. Participants completed study visits at enrollment and then at 6 and 12 months.

At enrollment, data on patients' demographic characteristics, medical history, and antimicrobial drug use within the past 12 months, and microbiologic data on previous *S. aureus* infections were obtained from electronic medical records. Participants also completed a questionnaire at enrollment and at 12 months that focused on their living situation, self-reported history of skin infections, personal hygiene, sexual behavior, and drug use over the past 12 months.

Microbiologic Procedures

At each study visit, specimens for *S. aureus* culture were collected from the anterior nares and the groin by using sterile rayon swabs and placed in liquid Stuart's transport media (Becton Dickinson, Sparks, MD, USA). Study staff collected specimens from the anterior nares, and participants were instructed (using a diagram of the human body) to collect specimens from the groin by swabbing in the skin folds between the thigh and genital area. Swabs were plated on mannitol salt agar (Becton Dickinson) and CHROMagar MRSA (Becton Dickinson) and then placed in 5 mL of trypticase soy broth with 6.5% sodium chloride (Becton Dickinson) as described (*19*,*20*). At each study visit, participants were classified as MRSA colonized if MRSA was detected from either the nares or groin culture. Participants were classified as colonized with methicillin-susceptible *S. aureus* (MSSA) if MSSA was detected and MRSA was not detected. Participants colonized with both MSSA and MRSA (regardless of site) were classified as MRSA colonized.

All MRSA isolates were genotyped by PFGE with *Sma*I (New England Biolabs, Beverly, MA, USA) as described (*13*,*19*,*20*). PFGE patterns were analyzed with BioNumerics Software v 5.10 (Applied Maths, Austin, TX, USA) and were assigned to USA pulsed-field types by using Dice coefficients and 80% relatedness. USA500, Iberian, and Archaic PFGE types were grouped together as USA500/Iberian because they are closely related and difficult to separate by PFGE (*21*). PCR was used to screen for staphylococcal cassette chromosome *mec* type and to detect Panton-Valentine leukocidin genes for all isolates (*22*). USA300 was defined as an isolate with a USA300 PFGE pattern that was positive for Panton-Valnetine leukocidin genes and contained staphylococcal cassette chromosome *mec* type IVa.

Prospective Monitoring for Incident MRSA Clinical Infections

Electronic medical and microbiology records were prospectively monitored for incident MRSA clinical infections for 24 months. Participants were classified with a MRSA clinical infection if a clinical infection was documented in the medical record and MRSA was isolated from the culture. Participants with a MRSA clinical infection completed a supplemental questionnaire that focused on the signs and symptoms of their infection and its clinical course. We defined a skin and soft tissue infection in the groin as an infection that involved the buttocks, perineum, genitals, anus, or proximal thigh.

Statistical Methods

The primary analysis compared participants in whom a MRSA clinical infection developed with those in whom a MRSA clinical infection did not develop. All analyses were performed by using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). The Wilcoxon rank-sum test (continuous variables) and the χ^2 and Fisher exact tests (categorical variables) were used to test for differences in clinical, demographic, and behavioral variables among participants with and without MRSA clinical infection. Statistical significance was indicated by a p value <0.05. By using a multivariate log-linked binomial regression model (Proc Genmod; SAS Institute Inc.) (*23*), adjusted risk ratios (aRRs), and 95% CIs were calculated to identify variables associated independently with the development of MRSA clinical infection. All statistically significant ($p<0.05$) variables in univariate analysis (unadjusted RR) were included in a multivariate model (*24*), and variables with $p>0.2$ in the adjusted model were dropped sequentially to create a parsimonious model that was examined for goodness of fit after each step. We also evaluated variables in the final parsimonious model with Kaplan-Meier survival methods with corresponding log-rank tests and Cox proportional hazards models (Proc PHREG; SAS Institute Inc.) of time to MRSA clinical infection.

Results

We enrolled 600 HIV-infected veterans, most of whom (98%) were male with a median age of 52 years (interquartile range [IQR] 45–59 years). Four hundred forty-one (74%) participants were non-Hispanic Blacks, and 315 (53%) were men who had sex with men. The median most recent CD4 cell count was 416 cells/μL (IQR 250–579 cells/μL), and 474 (79%) participants were receiving antiretroviral therapy. MRSA colonization was detected in 79 (13%) of 600 participants at baseline, in 66 (13%) of 502 at 6 months, and in 62 (15%) of 426 participants at 12 months (Table 1). In addition, MRSA colonization with 2 distinct MRSA strains was detected in 2 participants at baseline, in 1 participant at 6 months, and in 2 participants at 12 months, resulting in a total of 81, 67, and 64 isolates collected at each of the 3 time points, respectively. MSSA was detected in 180 (30%) participants

Table 1. Prevalence of *Staphylococcus aureus* colonization among HIV-infected adults, Atlanta, Georgia, USA, 2007–2009*

S. aureus colonization status†	Participants, no. (%)		
	At enrollment, n = 600	At 6-mo visit, n = 502	At 12-mo visit, n = 426
MRSA	79‡ (13)	66‡ (13)	62‡ (15)
MSSA	180 (30)	156 (31)	118 (28)
No *S. aureus*	341 (57)	280 (56)	246 (58)

*MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus;* PFGE, pulsed-field gel electrophoresis.
†Obtained from nares and groin swab specimens.
‡MRSA and MSSA co-colonization was detected in 11 participants at baseline, 10 participants at 6 mo, and 9 participants at 12 mo. For analysis these participants were classified as MRSA colonized. In addition, MRSA colonization with 2 distinct MRSA PFGE patterns was detected in 2 participants at baseline, 1 participant at 6 mo, and 2 participants at 12 mo.

at baseline, 156 (31%) at 6 months, and 118 (28%) at 12 months (Table 1). USA500/Iberian (n = 112, 53%) and USA300 (n = 71, 33%) were the most common colonizing MRSA PFGE types identified. USA100 (n = 14, 7%) and other PFGE types (n = 15, 7%) were uncommon. Among MRSA-colonized participants, MRSA was detected solely in the nares of 87 (41%) participants, in both the nares and groin of 81 (38%), and only in the groin for 44 (21%) participants. USA300 accounted for 20 (23%) isolates detected solely in the nares, 29 (36%) detected in the nares and the groin, and 22 (50%) detected solely in the groin (Figure 1). Compared with other PFGE types, USA300 was more likely to be detected solely in the groin (RR = 1.9; 95% CI 1.2–3.3; p = 0.02). Including groin cultures increased the overall detection of MRSA by 26% and of USA300 by 44% compared with nasal culture alone.

Over a median of 2.1 years of follow-up, 29 MRSA clinical infections occurred in 25 participants (2.5 infections/100 person-years). Skin and soft tissue infections (n = 24) were the most common, followed by pneumonia (n = 3) and bacteremia (n = 2). Three (13%) of the skin and soft tissue infections required hospitalization, and 13 (54%) of 24 skin and soft tissue infections occurred in the groin. Of the 25 participants in whom MRSA clinical infection developed, MRSA colonization was detected at baseline in the groin only or groin and nares in 12 (48%) of 25 participants in whom a MRSA clinical infection developed, compared with 37 (6%) of 575 participants in whom an infection did not develop (p<0.0001). MRSA colonization was also detected at a study visit (baseline, 6 months, or 12 months) preceding clinical infection in 17 (68%) of 25 participants. Among clinical isolates available for PFGE typing from an initial MRSA clinical infection (n = 22), USA300 (n = 14, 64%) was the most common and was identified in 9 (69%) of 13 skin and soft tissue infections that occurred in the groin (Table 2). USA500/Iberian (n = 8, 36%) was also common and was identified in all of the pneumonia and bacteremia infections. In patients with preceding colonization, the PFGE type of the clinical isolate and preceding colonizing isolate always matched (n = 17/17).

In univariate analysis, factors associated with an increased risk of developing MRSA clinical infection included MRSA colonization detected in the groin at baseline, a lower CD4 cell count, a previous history of an abscess, a medical history of MRSA clinical infection, renal insufficiency, a history of syphilis, the use of certain antistaphylococcal agents in the past 12 months, contact with a prison or jail, and certain hygienic factors (Table 3, Appendix, wwwnc.cdc.gov/EID/article/19/4/12-1353-T3.htm). A suppressed HIV viral load (<400 copies/mL) and use of antiretroviral therapy were associated with a lower risk for development of MRSA clinical infection. Of note, no MRSA clinical infections developed in the 30 participants with MRSA colonization that was detected solely in the nares at baseline. In multivariate analysis, MRSA colonization detected in the groin at baseline (aRR 4.8 95% CI 2.1–10.8) and a medical history of MRSA clinical infection (aRR 3.1 95% CI 1.4–7.3) were the only 2 factors that remained significantly associated with an increased risk

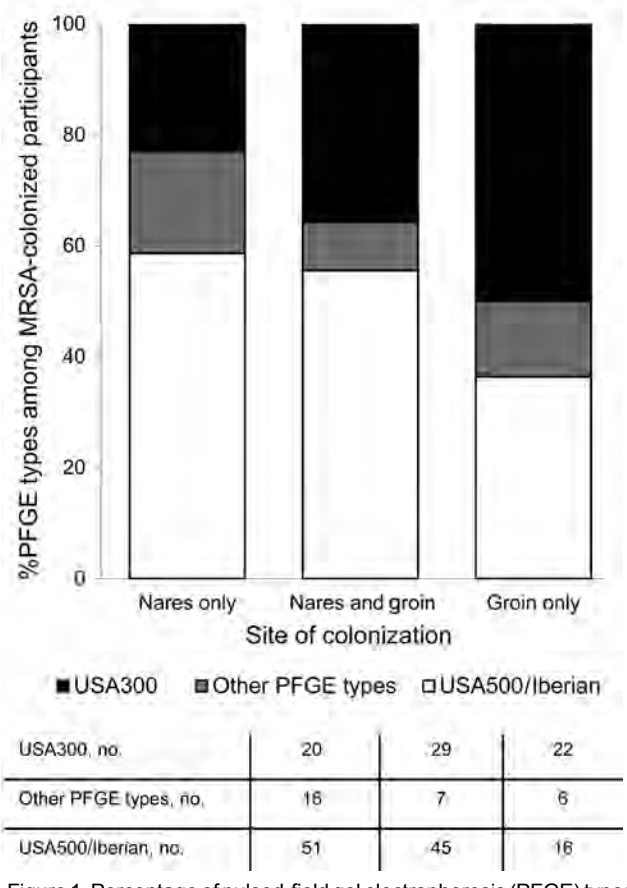

Figure 1. Percentage of pulsed-field gel electrophoresis (PFGE) types by anatomic site of detection in methicillin-resistant *Staphylococcus aureus* (MRSA)–colonized HIV-infected adults (n = 212 MRSA colonizing isolates; 3 study visits aggregated), Atlanta, Georgia, USA, 2007–2009. Other PFGE types: USA100 (n = 14), USA600 (n = 1), USA700 (n = 4), USA800 (n = 7), USA1000 (n = 3).

Table 2. Location of infection and preceding colonization status of 25 HIV-infected adults with MRSA clinical infection, Atlanta, Georgia, USA, 2007–2009*

Infection/ participant	Infection location	Clinical infection PFGE type	Colonization status preceding clinical infection	Colonizing PFGE type	Second clinical infection
Skin and soft tissue infections in the groin				–	
VA1	Buttock	USA300	None		
VA2	Buttock	USA300	Nares and groin	USA300	
VA3	Buttock	USA300	None	–	
VA4	Perianal	USA300	None	–	
VA5	Perianal	USA300	Nares and groin	USA300	
VA6	Pubic area	USA300	Nares and groin	USA300	
VA7	Scrotum	USA300	None	–	
VA8	Thigh	USA300	None	–	
VA9	Hip	USA500/Iberian	Nares and groin	USA500/Iberian	
VA10	Rectum	USA500/Iberian	Nares and groin	USA300 and USA500/Iberian	1 mo later: USA500/Iberian rectal infection
VA11	Buttock	No specimen	Groin only	USA300	
VA12	Buttock	No specimen	Nares and groin	USA300	
Skin and soft tissue infections outside of the groin					
VA13	Axilla	USA300	Nares and groin	USA300	
VA14	Axilla	USA300	Groin only	USA300	
VA15	Lip	USA300	None	–	
VA16	Lower extremity	USA300	None	–	
VA17	Lower extremity	USA300	Nares and groin	USA300 and USA100	
VA18	Scalp	USA300	Groin only	USA300	
VA19	Back	USA500/Iberian	Nares and groin	USA500/Iberian	
VA20	Scalp	USA500/Iberian	Nares and groin	USA500/Iberian	12 mo later: USA500/Iberian decubitus ulcer infection
VA21	Scalp	No specimen	Nares and groin	USA300	
Invasive clinical infections					
VA22	Bloodstream	USA500/Iberian	Nares and groin	USA500/Iberian	
VA23	Bloodstream	USA500/Iberian	Nares and groin	USA500/Iberian	
VA24	Lung	USA500/Iberian	Nares and groin	USA500/Iberian	6 mo later: USA500/Iberian pneumonia
VA25	Lung	USA500/Iberian	None	–	2 mo later: USA500/Iberian infection on foot

*MRSA, methicillin-resistant *Staphylococcus aureus*; PFGE, pulsed-field gel electrophoresis; –, not applicable because the participant was not colonized before their clinical infection.

for development of MRSA clinical infection (online Table 3, appendix). MRSA colonization detected in the groin included participants with MRSA colonization detected only in the groin (n = 14; aRR 6.6) and participants with MRSA colonization detected in the nares and groin (n = 35; aRR 4.2). This analysis was repeated by using a multiple-predictor Cox proportional hazards model to account for time to MRSA clinical infection and MRSA colonization detected in the groin at baseline (adjusted hazard ratio 5.9; 95% CI 2.5–13.9) and a medical history of MRSA clinical infection (aHR 4.0; 95% CI 1.6–9.6) were significant predictors of time to MRSA clinical infection. Among the 79 participants with MRSA colonization at baseline, USA300 colonization was associated with a nonsignificant but increased risk of developing MRSA clinical infection, compared with other PFGE types (RR 2.1; 95% CI 0.7–5.9). In a separate analysis, MSSA colonization was not associated with developing a MRSA clinical infection (RR 0.8; 95% CI 0.3–2.7).

In a subanalysis of MRSA colonization in 383 HIV-infected adults from whom samples were cultured at all 3 visits, MRSA colonization was detected in 48 (13%) participants at baseline, in 52 (14%) at 6 months, and in 50 (13%) at 12 months. Approximately equal numbers of participants became colonized with MRSA or were no longer colonized at each sequential study visit to maintain this stable colonization prevalence (Figure 2). On a percentage basis at each sequential study visit, 21%–31% of MRSA-colonized participants were no longer colonized (without treatment) and 4%–6% of previously uncolonized participants became colonized with MRSA. Over 12 months, MRSA colonization was persistent (detected at all 3 visits) in 26 (7%) participants and intermittent (detected in 1 or 2 visits) in 54 (14%) participants (Figure 2). The PFGE type remained stable in 23 (88%) of 26 participants with persistent colonization and in 16 (89%) of 18 participants with intermittent colonization at 2 visits. Swab specimens from participants with persistent colonization (n = 78 MRSA isolates from 26 participants) were more likely to yield heavy growth of MRSA (growth detected on direct agar plating without broth enrichment) than were isolates from participants with intermittent colonization (n = 72 MRSA isolates from 54 participants)

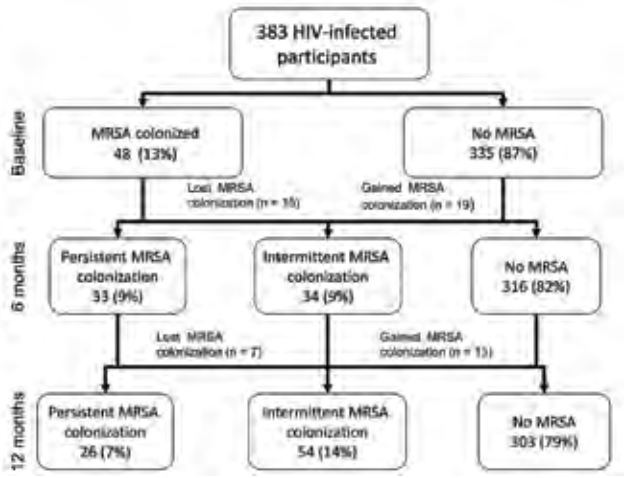

Figure 2. Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) recovered from nares and groin swabs of HIV-infected adults at each study visit among participants who had specimens cultured at all 3 visits (n = 383). Atlanta, Georgia, USA, 2007–2009.

[91% vs. 75%; p = 0.009]. PFGE type (USA300 vs. other PFGE types) was not significantly associated with persistent vs. intermittent colonization (p = 0.27).

Discussion

MRSA clinical infections (mainly skin and soft tissue infections) were common among HIV-infected adults in this study. The prevalence of MRSA colonization was also high at each study visit (13%–15%), and MRSA colonization in the groin was a risk factor for developing a MRSA clinical infection. MRSA PFGE types USA300 and USA500/Iberian were common causes of colonization and clinical infection. USA300 more commonly caused colonization of the groin and clinical skin and soft-tissue infection in the groin. MRSA prevention strategies with HIV-infected adults that can effectively address colonization at this anatomical site are likely necessary to reduce MRSA clinical infections in this population.

HIV-infected persons have been found to have 6× the risk for community-associated MRSA skin and soft-tissue infections than HIV-negative patients (*25*) and an increased odds of having community-acquired *S. aureus* bacteremia (*26*). In this study, MRSA colonization in the groin and a medical history of MRSA clinical infection were risk factors for clinical infection. Because, in our study, most skin and soft tissue infections occurred in the groin, colonization of the groin may have directly precipitated clinical infection in this anatomical area. In a previous analysis, we demonstrated that MRSA colonization was also associated with a medical history of MRSA clinical infection, contact with jails and prisons, and correlates of risky sexual behavior (i.e., rarely or never using condoms) (*20*). In addition, in this analysis, adjusting for MRSA colonization in the groin diminished the association between MRSA clinical infection and risk factors for exposure to MRSA (e.g., contact with jails and prisons) and risk factors related to hygiene (e.g., shaving the groin, genital, or buttock area). These findings suggest that MRSA colonization in the groin may also be a marker of more frequent exposure to MRSA in the environment or poor hygiene or an indicator of immunologic dysfunction (i.e., impaired neutrophil function [*27*]) that in turn increases a person's susceptibility to clinical infection.

Prior studies have demonstrated that USA300 causes most community-associated MRSA infections in the United States, whereas USA500/Iberian clones are associated with health care–associated MRSA infections (*1*). This epidemiology, however, is changing (*28*), and participants in this study had risk factors for both community-associated and outpatient health care–associated MRSA exposures. In this study, USA300 caused most skin and soft tissue infections and was more likely colonize the groin only. Other PFGE types, however, also caused both clinical infections and groin colonization, and PFGE type (USA300 vs. other PFGE types) was not independently associated with risk for MRSA clinical infection. These findings suggest that the presence of MRSA colonization in the groin is more useful clinical knowledge than identifying the PFGE type causing colonization (which is rarely determined in clinical practice anyway).

The association of MRSA colonization and the development of clinical infection in this study suggest that MRSA decolonization with topical or systemic treatment may be an effective method to prevent clinical infections in this population. A randomized clinical trial of adult hospitalized surgical patients found that using intranasal mupirocin and chlorhexidine gluconate soap total-body wash substantially reduced the rate of health care–associated *S. aureus* infection by 58% in patients who were nasal carriers of *S. aureus* (*29*). Although several randomized controlled trials have demonstrated that MRSA colonization can be eliminated from the groin (*30*), and short-term clinical benefits of *S. aureus* decolonization have been demonstrated in the hospitalized setting, data have not been available to support decolonization as a method of preventing MRSA clinical infections in a community or outpatient setting (*31*). In this study, we observed that although MRSA colonization was frequent, it also fluctuated considerably over time. MRSA colonization spontaneously resolved in approximately half of participants over 12 months, but new MRSA colonization was detected in as many previously uncolonized participants. A MRSA decolonization program would therefore treat a substantial number of persons whose MRSA colonization would have resolved spontaneously and would require ongoing screening to identify new colonization. The substantial fluctuations in MRSA colonization status in this study suggest that strategies that emphasize hygiene

and avoidance of potential MRSA exposures might be more effective at preventing MRSA clinical infections in this setting than decolonization, but this hypothesis should be tested in a clinical study that includes decolonization of MRSA from the groin as an intervention.

Our study had several limitations. First, our study population was 98% male and our findings are not generalizable to HIV-infected women. Second, the MRSA epidemic in the United States continues to evolve (*7*), and new risk factors for MRSA infection in HIV-infected adults may emerge that were not significant in this study. In addition, in our study, some risk factors for community-associated MRSA clinical infections, such as methamphetamine use (*9*) and close contact with someone with a skin infection, were not associated with MRSA clinical infection. These differences might be explained by low frequencies of certain risk factors (i.e., methamphetamine use) in our study population or a social desirability bias may have limited the full disclosure of drug use and sexual and hygienic behavior. Third, we evaluated 45 variables in univariate analysis and 16 variables in the initial multivariate model before creating a final parsimonious model with 6 variables. Although evaluating an extensive list of potential risk factors for MRSA clinical infection had some advantages, the extensive list also increased variance in the initial multivariate model. Fourth, the optimal sampling (i.e., which sites to swab and how to collect the specimen) and microbiologic techniques to evaluate MRSA colonization in the groin have not been established. Although we used microbiologic techniques that have been demonstrated to improve MRSA detection (*19*), we may have underestimated the true prevalence of groin colonization. Finally, participants may have had MRSA clinical infections that were not cultured, and these infections would not have been captured by our electronic monitoring of microbiologic records. Therefore, we might have underestimated the true incidence of MRSA clinical infections in this population.

In this study of HIV-infected adults, MRSA clinical infections were common and associated with MRSA colonization in the groin and a medical history of MRSA clinical infection. MRSA PFGE types USA300 and USA500/Iberian contributed to clinical infections, and participants had risk factors for both community-associated and health care–associated MRSA exposures. Given this high incidence of MRSA clinical infections, both community-associated and hospital-associated MRSA prevention strategies should be emphasized in HIV-infected adults in settings with high rates of MRSA clinical infections. Current community-associated MRSA prevention strategies include keeping cuts and scrapes clean and covered; practicing good hand hygiene; avoiding shared personal items, such as towels and razors; and decolonization in certain situations (*31*). Given the frequency of MRSA colonization in the groin and its association with clinical infection, MRSA prevention strategies (both hygienic practices and decolonization treatments) with HIV-infected adults should be used to prevent or eliminate colonization at this anatomic site to reduce MRSA clinical infections in this population.

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Feline Origin of Rotavirus Strain, Tunisia, 2008

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In Tunisia in 2008, an unusual G6P[9] rotavirus, RVA/human-wt/TUN/17237/2008/G6P[9], rarely found in humans, was detected in a child. To determine the origin of this strain, we conducted phylogenetic analyses and found a unique genotype constellation resembling rotaviruses belonging to the feline BA222-like genotype constellation. The strain probably resulted from direct cat-to-human transmission.

Group A rotaviruses (RVAs) are a leading cause of severe acute gastroenteritis in infants and young children. An infectious RVA virion is a triple-layered icosahedral particle that contains 11 segments of double-stranded RNA (*1*). The outer protein layer is formed by virus capsid protein (VP) 4 (P antigen) and VP7 (G antigen), each of which is used for binomial nomenclature (*1*). At least 27 G genotypes and 35 P genotypes have been identified (*2*). Globally, only 6 G/P-genotype combinations are of epidemiologic relevance to humans: G1P[8], G3P[8], G4P[8], G9P[8], and G12P[8], which are typically found in combination with a Wa-like genotype constellation (I1-R1-C1-M1-A1-N1-T1-E1-H1), and G2P[4], which is found in combination with a DS-1-like genotype constellation (I2-R2-C2-M2-A2-N2-T2-E2-H2) (*3*).

Certain G genotypes rarely encountered in humans are commonly associated with RVA strains from animals (*4*). For example, G6 RVA strains are occasionally detected in humans but are a common genotype in cattle (*4*). Complete genomes have been determined for 11 human G6 RVA strains: 7 G6P[14], 2 G6P[9], 1 human–animal reassortant G6P[6], and 1 unique G6P[11] (*5*–*9*).

The P[9] genotype is commonly associated with the G3 or G6 genotype and is believed to be typical for feline and canine RVA strains (*4*). A few G3P[9] and G3P[3] RVA strains have been detected in humans, and they are believed to be the result of direct interspecies transmission from cats or dogs to humans, possibly in combination with reassortment (*10*–*13*).

Previously, 2 genotype constellations among feline and canine RVA strains, cat97-like and AU-1-like, were described (*13*). The genotype constellations were G3-P[3]-I3-R3-C2-M3-A9-N2-T3-E3-H6 and G3-P[9]-I3-R3-C3-M3-A3-N3-T3-E3-H3, respectively. Recently, the complete genomes of a feline strain (RVA/cat-wt/ITA/BA222/2005/G3P[9]) and 2 feline-like human RVA strains (RVA/human-wt/ITA/PAI58/1996/G3P[9] and RVA/human-wt/ITA/PAH136/1996/G3P[9]) were shown to possess a distinct genotype constellation, G3-P[9]-I2-R2-C2-M2-A3-N1/N2-T3/T6-E2-H3 (*11*), representing a tentative third feline genotype constellation (BA222-like). This tentative third feline BA222-like genotype constellation is an intriguing genotype mosaic, sometimes possessing Wa-like nonstructural protein (NSP) 2 or NSP3 gene segments and partially resembling the genotype constellation found in RVA strains from cattle and other artiodactyla (*5*,*7*,*10*,*11*). Full-genome sequences of unusual human RVA strains are being analyzed to detect interspecies transmission, reassortment, and evolutionary relationships between human and animal RVAs (*10*,*11*).

In 2008, during continuous surveillance for human RVA in Tunisia, we identified an unusual G6P[9] strain in an 8-month-old hospitalized child (*14*). To understand the evolution and origin of this unusual strain, RVA/human-wt/TUN/17237/2008/G6P[9] (hereafter referred to as strain 17237), we conducted phylogenetic analyses.

The Study

The full-length genome sequence of the virus was determined as described (*5*). Primers used for all 11 segments are shown in online Technical Appendix Table 1 (wwwnc.cdc.gov/EID/article/19/4/12-1383-Techapp1.pdf). Multiple sequence alignments and phylogenetic analyses were conducted by using MEGA version 5.05 (www.megasoftware.net). Sequences were deposited in GenBank (accession nos. JX271001–JX271011).

Strain 17237 possessed the unique genotype constellation G6-P[9]-I2-R2-C2-M2-A3-N1-T6-E2-H3. This constellation was compared with that of the human G6P[9] strain Se584, feline/canine-like human RVA strains (KF17, PAH136, PAI58, and 0537), and several animal strains (Table). Strain 17237 shared the same combination of genotypes with human RVA strain PAH136 (*10*) except for VP7 (strain 17237 contained G6 instead G3). Overall, strain 17237 shared 8–10 genotypes with RVA strains possessing the BA222-like genotype constellation and 8–9 genotypes with several bovine or bovine-like RVA strains.

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Table. Comparison of genomic constellation of group A rotavirus strain RVA/human-wt/TUN/17237/2008/G6P[9] from Tunisia with reference strains*

Strain	Genotype constellation	VP7	VP4	VP6	VP1	VP2	VP3	NSP1	NSP2	NSP3	NSP4	NSP5
RVA/human-wt/TUN/17237/2008/G6P[9]	BA222-like	**G6**	**P[9]**	**I2**	**R2**	**C2**	**M2**	**A3**	**N1**	**T6**	**E2**	**H3**
RVA/human-wt/ITA/PAH136/1996/G3P[9]	BA222-like	G3	**P[9]**	**I2**	**R2**	**C2**	**M2**	**A3**	**N1**	**T6**	**E2**	**H3**
RVA/cat-wt/ITA/BA222/2005/G3P[9]	BA222-like	G3	**P[9]**	**I2**	**R2**	**C2**	**M2**	**A3**	**N1**	T3	**E2**	**H3**
RVA/human-wt/ITA/PAI58/1996/G3P[9]	BA222-like	G3	**P[9]**	**I2**	**R2**	**C2**	**M2**	**A3**	N2	**T6**	**E2**	**H3**
RVA/human-tc/USA/Se584/1998/G6P[9]	BA222-like	**G6**	**P[9]**	**I2**	**R2**	**C2**	**M2**	**A3**	N2	T1	**E2**	**H3**
RVA/human-wt/JAP/KF17/2009/G6P[9]	BA222-like	**G6**	**P[9]**	**I2**	**R2**	**C2**	**M2**	**A3**	N2	T3	E3	**H3**
RVA/human-wt/USA/0537/2002/G3P[9]	BA222-like	G3	**P[9]**	**I2**	**R2**	**C2**	**M2**	**A3**	N2	T1	**E2**	**H3**
RVA/cat-tc/AUS/Cat2/1984/G3P[9]	BA222-like /cat97-like	G3	**P[9]**	I3	R3	**C2**	M3	**A3**	**N1**	**T6**	E3	**H3**
RVA/human-tc/ITA/PA169/1988/G6P[14]	Bovine-like	**G6**	P[14]	**I2**	**R2**	**C2**	**M2**	**A3**	N2	**T6**	**E2**	**H3**
RVA/human-wt/BEL/B10925/1997/G6P[14]	Bovine-like	**G6**	P[14]	**I2**	**R2**	**C2**	**M2**	**A3**	N2	**T6**	**E2**	**H3**
RVA/human-wt/ITA/111–05–27/2005/G6P[14]	Bovine-like	**G6**	P[14]	**I2**	**R2**	**C2**	**M2**	**A3**	N2	**T6**	**E2**	**H3**
RVA/cow-tc/FRA/RF/1982/G6P[1]	Bovine	**G6**	P[1]	**I2**	**R2**	**C2**	**M2**	**A3**	N2	**T6**	**E2**	**H3**
RVA/cow-tc/VEN/BRV033/1990/G6P6[1]	Bovine	**G6**	P[1]	**I2**	**R2**	**C2**	**M2**	**A3**	N2	**T6**	**E2**	**H3**
RVA/cow-tc/USA/WC3/1981/G6P[5]	Bovine	**G6**	P[5]	**I2**	**R2**	**C2**	**M2**	**A3**	N2	**T6**	**E2**	**H3**
RVA/cow-tc/KOR/KJ19–2/2004/G6P[7]	Bovine	**G6**	P[7]	**I2**	**R2**	**C2**	**M2**	**A3**	N2	**T6**	**E2**	**H3**
RVA/rhesus-tc/USA/PTRV/1990/G8P[1]	Bovine-like	G8	P[1]	**I2**	**R2**	**C2**	**M2**	**A3**	N2	**T6**	**E2**	**H3**
RVA/human-tc/KEN/B12/1987/G8P[1]	Bovine-like	G8	P[1]	**I2**	**R2**	**C2**	**M2**	**A3**	N2	**T6**	**E2**	**H3**
RVA/human-tc/USA/DS-1/1976/G2P[4]	DS-1-like	G2	P[4]	**I2**	**R2**	**C2**	**M2**	A2	N2	T2	**E2**	H2
RVA/human-tc/JPN/AU-1/1982/G3P3[9]	AU-1-like	G3	**P[9]**	I3	R3	C3	M3	**A3**	N3	T3	E3	**H3**
RVA/human-tc/USA/Wa/1974/G1P1A[8]	Wa-like	G1	P[8]	I1	R1	C1	M1	A1	**N1**	T1	E1	H1

*__Boldface__ indicates genotypes that are identical to group A rotavirus RVA/human-wt/TUN/17237/2008/G6P[9]. VP, virus capsid protein; NSP, nonstructural protein.

Phylogenetic analyses showed that all 11 genome segments of strain 17237 were most closely related to strains of either feline-like human or feline origin (Figures 1, 2). Strain 17237 clustered most closely with RVA/human-wt/ITA/PA43/2003/G6P[9], RVA/human-wt/JAP/KF17/2009/G6P[9], and RVA/human-wt/BEL/B1711/2002/G6P[6] strains, all of which are suspected to have at least a partial animal (bovine-like or feline-like) origin (*6*,*7*). The P[9] genome segment was most closely related to RVA strains RVA/human-wt/RUS/Nov10-N507/2010/G3P[9], BA222, and KF17. The VP1, VP6, NSP2, and NSP4 genome segments of strain 17237 were closely related to BA222, clustering in the R2, I2, N1, and E2 genotypes, respectively. This G3P[9] feline RVA strain BA222 is believed to have a common origin with animal RVA strains and RVA strains that are zoonotically transmissible to humans (*11*). The NSP2 gene segment of strain 17237 clustered in the N1 genotype and was distantly related to typical human Wa-like RVA strains. The VP2, NSP3, and NSP5 gene segments were closely related to RVA/human-wt/ITA/PAI58/1996/G3P[9]. The VP3, NSP1, and NSP3 genome segments clustered closely with RVA/human-wt/ITA/PAH136/1996/G3P[9]. NSP1 and NSP5 clustered closely with RVA/human-wt/USA/0537/2002/G3P[9]. These 3 human strains (PAI58–96, PAH136–96, and 0537) are believed to be of feline origin and possess a BA222-like genotype constellation.

Conclusions

The genome constellation of strain 17237 is similar to that of strains belonging to the tentative feline BA222-like genotype constellation (Table). It has been speculated that several of these BA222-like RVA strains resulted from multiple reassortment events among RVA strains

originating from different hosts (cattle, other ruminants, humans, cats, dogs) (*10*,*11*). However, a recent article speculates that this genotype constellation, although reminiscent to bovine-like RVA strains, might represent a true feline genotype constellation (*12*).

Our results support this hypothesis in 2 ways. The first source of support comes from the fact that BA222-like RVA strains have been detected on several continents: Europe (Italy), North America (United States), Asia (Japan), and now Africa (Tunisia) (*7*,*10*). RVA strains with this BA222-like genotype constellation are much more likely to circulate in a certain host species rather than result from distinct multiple reassortment events in each of the above-mentioned countries. The second source of support comes from the fact that our phylogenetic analyses confirmed that each of the 11 gene segments

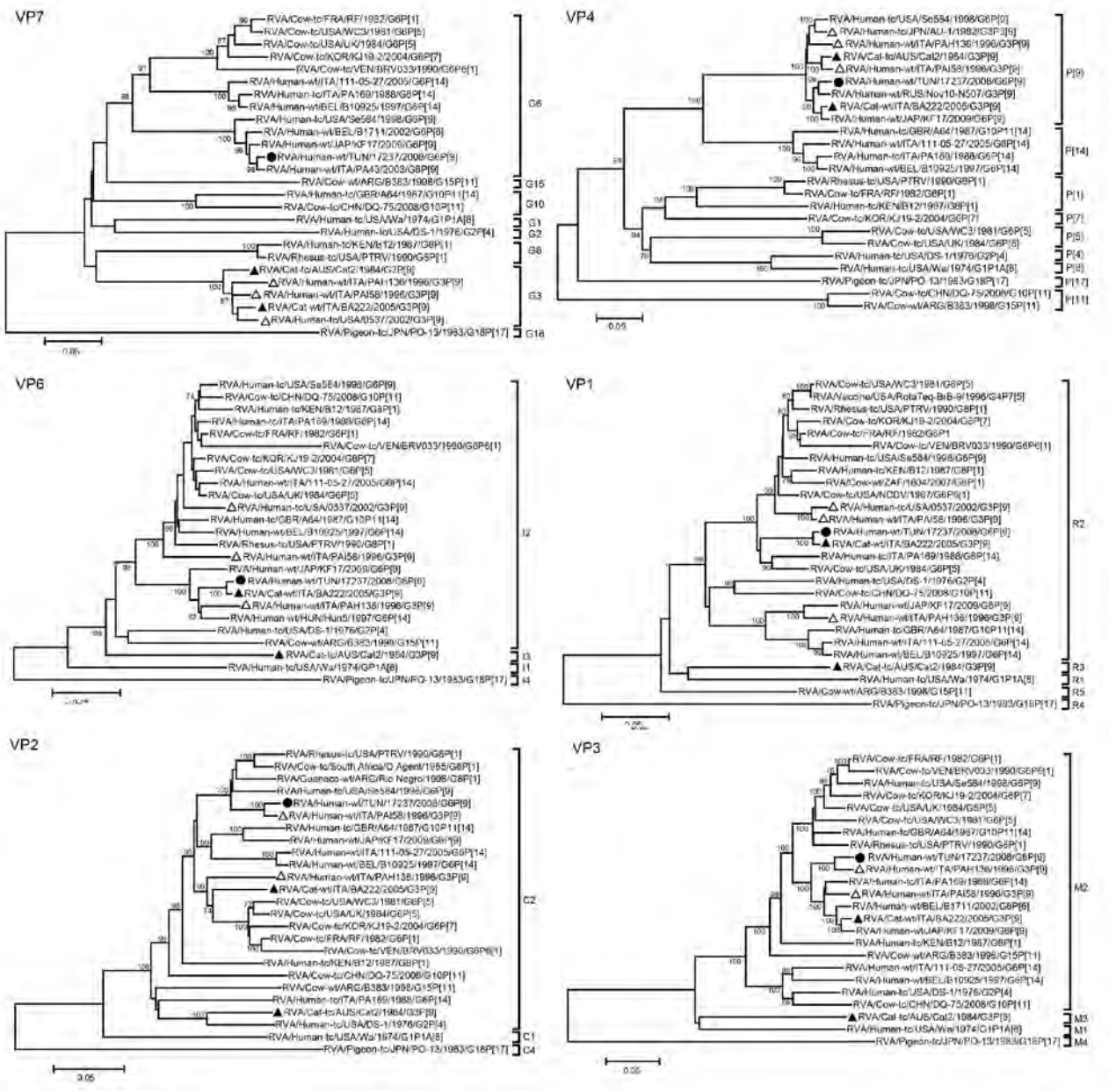

Figure 1. Phylogenetic trees of the full-length nucleotide sequences of the group A rotavirus (RVA) virus capsid protein (VP) 7, VP4, VP6, VP1, VP2, and VP3 genes. Phylogenetic trees were constructed by using the neighbor-joining method with the Kimura 2-parameter method. Bootstrap values (1,000 replicates) >70% are shown. Filled circles indicate strain RVA/human-wt/TUN/17237/2008/G6P[9] from Tunisia; filled triangles indicate feline RVA strains; and open triangles indicate feline/canine-like human RVA strains. GenBank accession numbers of the sequences of reference strains are shown in online Technical Appendix Table 2 (wwwnc.cdc.gov/EID/article/19/4/12-1383-Techapp1.pdf). Scale bars indicate nucleotide substitutions per site.

of strain 17237 was more closely related to BA222-like RVA strains than to bovine or bovine-like RVA strains. This finding strengthens the hypothesis that each of the BA222-like RVA strains did not result from individual multiple reassortment events but rather that this genotype constellation now circulates (most likely in cats) around the world and might have resulted from >1 reassortment events in the more distant past.

To further support or refute this hypothesis, more complete genomes must be determined from RVA strains from cats and dogs. Moreover, because P[9] is believed to be typical for feline/canine RVA strains, it would be intriguing to determine whether this strain could persist in the human population and could become competitive with already established P genotypes in humans. The recently emerged human G9 RVA strain is believed to have

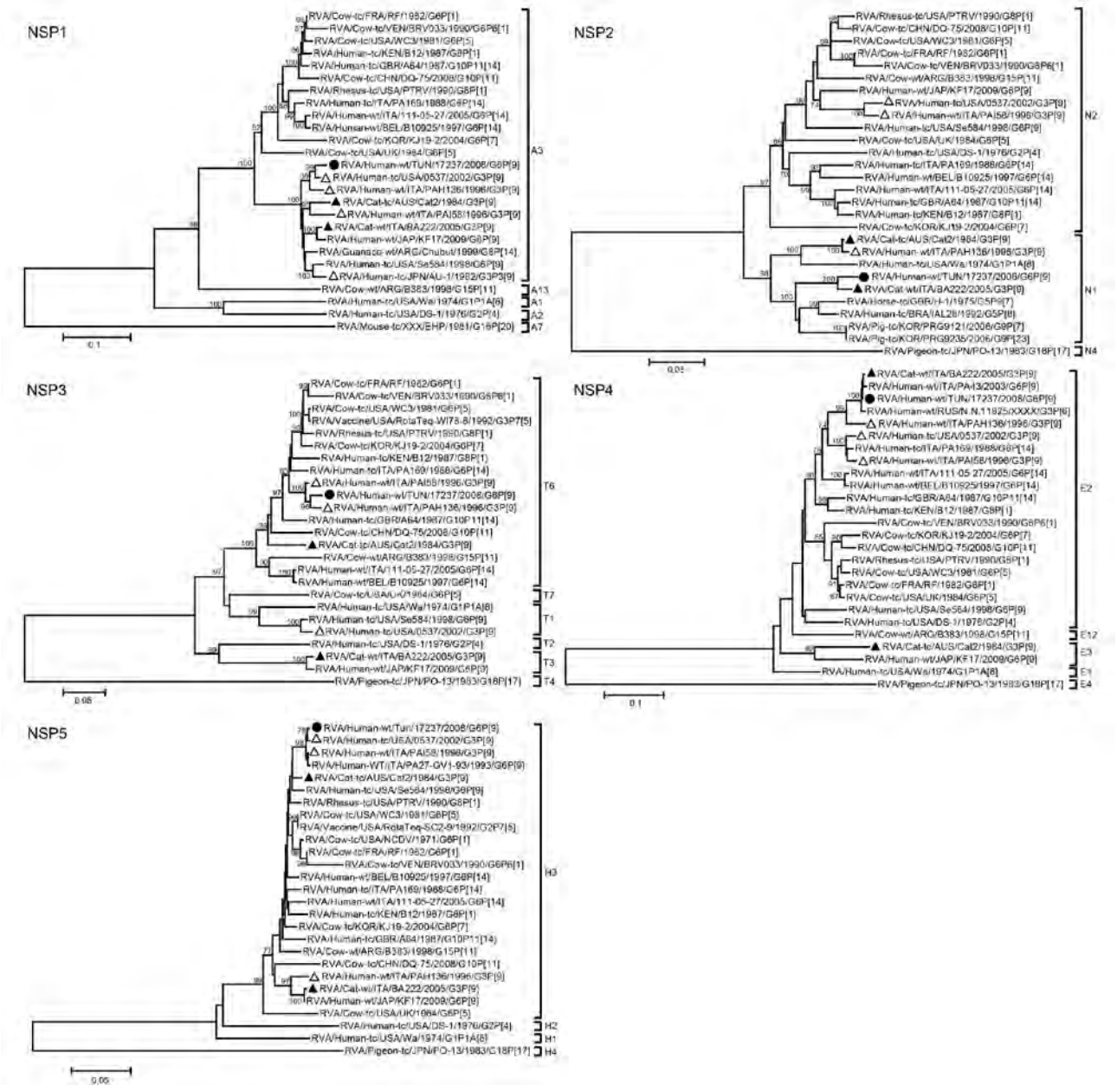

Figure 2. Phylogenetic trees of the full-length nucleotide sequences of the group A rotavirus (RVA) nonstructural protein (NSP) genes. Phylogenetic trees were constructed by using the neighbor-joining method with the Kimura 2-parameter method. Bootstrap values (1,000 replicates) >70% are shown. Filled circle indicates strain RVA/human-wt/TUN/17237/2008/G6P[9] from Tunisia, filled triangles indicate the feline RVA strains, and open triangles indicate the feline/canine-like human RVA strains. GenBank accession numbers of the sequences of reference strains are shown in online Technical Appendix Table 2 (wwwnc.cdc.gov/EID/article/19/4/12-1383-Techapp1.pdf). Scale bars indicate nucleotide substitutions per site.

originated from pigs and to have become established in the human population as the fifth major human RVA genotype, after multiple genome reassortment events with typical human Wa-like RVA strains (*15*).

The unusual G6P[9] RVA strain 17237 most likely resulted from direct interspecies transmission from a cat to a human. Interspecies transmission increases potential for spread of unusual and uncommon RVA strains. The findings of this study highlight the need for continuous monitoring of RVA strains and timely recognition of novel or rare genotypes. Continued surveillance of RVA strains in industrialized and developing countries, and in humans and animals, will provide more insights into interspecies transmission processes of RVAs. In turn, this information could help determine how the introduction of novel genes might affect the evolution of the RVA populations that infect humans.

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Tick-borne Encephalitis Virus in Horses, Austria, 2011

James O. Rushton, Sylvie Lecollinet, Zdenek Hubálek, Petra Svobodová, Helga Lussy, and Norbert Nowotny

An unexpectedly high infection rate (26.1%) of tick-borne encephalitis virus (TBEV) was identified in a herd of 257 horses of the same breed distributed among 3 federal states in Austria. Young age ($p<0.001$) and male sex ($p = 0.001$) were positively associated with infection.

Tick-borne encephalitis (TBE), which is caused by tick-borne encephalitis virus (TBEV), is a potentially fatal disease of the central nervous system, mainly in humans, but also in monkeys, dogs (*1*), and horses. Ruminants such as goats, sheep, and cattle are considered to be sporadically infected subclinically. However, they might be the source of disease in humans who consume nonpasteurized milk and milk products (*2*). TBEV-associated central nervous system disease in ruminants is rare (*3*).

TBEV occurs in natural foci and is endemic to many countries in Europe and parts of central and eastern Asia (*4*). The principal vectors for transmission are ticks of the genus *Ixodes*. Although TBEV in humans has been studied extensively, there are only a limited number of reports on TBEV in animals (*5*), especially horses. Only 2 reports were found in the German literature on the epidemiology of TBEV infection in horses (*6,7*), and 1 case report was found on clinical symptoms of TBE in a mare (*8*). The purpose of this study was to determine the status of TBEV infection in a large population of a single horse breed in Austria.

The Study

Serum samples from 257 horses of the same breed that were distributed among 3 federal states in Austria were obtained in April 2011 and screened by using a commercial ELISA (ID Screen West Nile Competition ELISA Kit; IDvet, Montpellier, France) for antibodies against flaviviruses. ELISA-positive serum samples were further investigated by using virus-specific neutralization assays for the 3 flaviviruses circulating in Austria (West Nile virus [WNV], Usutu virus [USUV], and TBEV). Neutralization assays were conducted independently by 2 laboratories (*9,10*). Results were analyzed by using SPSS version 17 software (SPSS IBM, Armonk, NY, USA). Associations of sex, age, and location with positive results were tested by using 1-way analysis of variance and tested for significance by using the χ^2 test. Differences in age between horses positive or negative for flaviviruses were determined by using the Student *t*-test. A p value <0.05 was considered significant for all analyses.

The study comprised 113 (44.0%) mares, 139 (54.0%) stallions, and 5 (2.0%) geldings. The mean ± SD age of horses was 8.1 ± 6.3 years (range 1–32 years). A total of 154 (59.9%) horses were boarded in Styria, 66 (25.7%) in Vienna, and 37 (14.4%) in Lower Austria and kept in various types of housing. All 3 locations are considered areas to which WNV, USUV, and TBEV are endemic. The animals were free from clinical symptoms associated with flavivirus infections. None of the horses were vaccinated with WNV and TBEV vaccines (TBEV vaccines are not licensed for use in horses).

Sixty-seven (26.1%) horses were positive for antibodies against flaviviruses by ELISA, and all 67 were positive for TBEV by virus-specific neutralization tests (Table, Appendix, wwwnc.cdc.gov/EID/article/19/4/12-1450-T1.htm). Positive results were distributed among 17 mares, 49 stallions, and 1 gelding. The difference in results between sexes was significant ($p = 0.001$) (Figure). The mean ± SD ages of horses positive and negative for TBEV antibodies were 5.9 ± 4.2 and 8.9 ± 6.7 years, respectively ($p<0.001$) (Figure). Thirty-seven positive horses were kept in Styria, 16 in Vienna, and 14 in Lower Austria. The difference in positive results for horses at the 3 locations was not significant. Low-level cross-reactivity for WNV and USUV was observed in 9 animals (Table).

Conclusions

The main findings of our study were a comparatively high seropositivity rate of 26.1%, a higher prevalence of TBEV-specific antibodies in younger horses, and a higher prevalence of TBEV-specific antibodies in stallions. We expected the horses to have subclinical infections with WNV lineage 2, which was introduced recently into central Europe (*11*), including Austria (*12*). However, it is well known that WNV IgG ELISAs show cross-reactivity with other flaviviruses, necessitating the use of virus-specific neutralization assays for identification of an etiologic flavivirus.

The population in our study had a 2-fold higher infection rate than that observed in a similar study in Austria in 1999 in a population of 468 horses (*6*). A partial explanation for this

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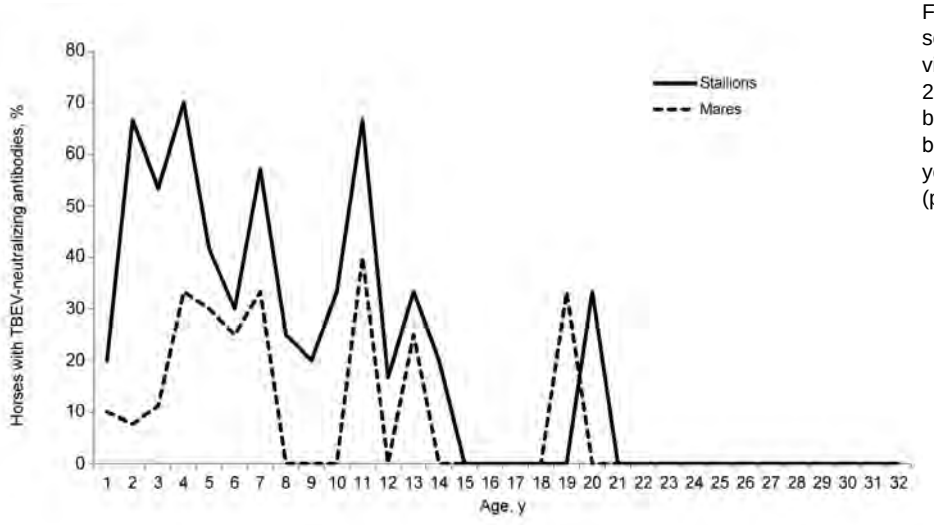

Figure. Percentages of horses seropositive for tick-borne encephalitis virus (TBEV) by age and sex, Austria, 2011. Geldings were excluded for better illustration. The difference between groups was significant for young age ($p<0.001$) and male sex ($p = 0.001$).

difference might be yearly fluctuating TBEV prevalence, as measured by diagnosed human infections (http://zecken.at/fsme/fsme-faelle-in-oesterreich/). In 1999, the lowest number (41) of human TBE cases was recorded in Austria (87, 79, 63, and 113 human cases were diagnosed in 2008, 2009, 2010, and 2011, respectively). A study in Germany in 2006 identified 2.9% of 240 horses with TBEV neutralizing antibodies (*7*). A more recent update on TBEV seropositivity in other animal species showed prevalence rates of 26.5% in cattle and 7.0% in sheep (*10*). This study did not detect antibodies against TBEV in 40 horses. A study on the prevalence of TBEV in dogs in Austria reported that 131 (24.0%) of 545 dogs examined had antibodies against TBEV (*13*).

We did not observe any influence of location on the likelihood of TBEV seropositivity. All 3 locations are within TBEV-endemic areas (http://zecken.at/fsme/verbreitungsgebiete/). However, none of the seropositive horses showed any clinical symptoms of an arbovirus infection at any time.

Regarding age distribution, a higher proportion of younger horses (mean age 5.9 years) had antibodies against TBEV than older horses (e.g., none of the horses 15–18 and 21–32 years of age had antibodies against TBEV). Older horses were in the same pastures as young horses. This finding contrasts with results of epidemiologic studies in cattle, in which animals ≤3 years of age showed a lower prevalence of antibodies against TBEV than did older animals (*10*). .

Tick exposure has always been high in the investigated areas, as reported in a study conducted >10 years ago, in which 52%–93% of horses were positive for antibodies against *Borrelia afzelii* by immunoblotting. Most of these horses had already been infected during their first year of age and were subsequently reinfected (*14*). Thus, older horses in our study might have been infected at a young age and showed a subsequent decrease in neutralizing antibodies below the detection limit.

The reason for the high number of seropositive stallions is unclear because stallions in the study were distributed among all 3 locations, and 33 (67.3%) of 49 were boarded in boxes (individual stable compartments that limit contact with other members of the population). Mares were kept exclusively in 1 TBEV-endemic location, mainly in pastures. However, stallions were more frequently transferred to other regions (e.g., for mating), where they might have been infected because of potentially higher tick infestation rates. It is also possible that for unknown biological reasons males are more frequently affected by ticks than females, as suggested by Perkins et al. (*15*) in a study on the yellow-necked mouse (*Apodemus flavicollis*).We plan to conduct further experiments to elucidate why ticks seem to be more attracted to male hosts than female hosts.

We observed comparatively low antibody prevalence in yearlings of both sexes, which was probably caused by decreasing, but still protective, maternally transmitted immunity. Seropositivity peaks in both sexes at 4, 7, 11, 13, and 19–20 years of age indicated infections and subsequent reinfections in certain years with higher TBEV activity (Figure).

Our study suggests that horses are prone to TBEV infection. However, they remain mostly asymptomatic. Thus, horses may be considered sentinel hosts for monitoring the spread of TBEV.

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Hepatitis Virus in Long-Fingered Bats, Myanmar

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During an analysis of the virome of bats from Myanmar, a large number of reads were annotated to orthohepadnaviruses. We present the full genome sequence and a morphological analysis of an orthohepadnavirus circulating in bats. This virus is substantially different from currently known members of the genus *Orthohepadnavirus* and represents a new species.

The family *Hepadnaviridae* comprises 2 genera (*Orthohepadnavirus* and *Avihepadnavirus*), and viruses classified within these genera have a narrow host range. The genus *Orthohepadnavirus* consists of pathogens that infect mammals, and it currently contains 4 species: *hepatitis B virus*, *woodchuck hepatitis virus*, *ground squirrel hepatitis virus*, and *woolly monkey hepatitis B virus*. The genus *Avihepadnavirus* contains 2 avian species: *duck hepatitis B virus* and *heron hepatitis B virus* (*1*). Hepadnaviruses mainly infect the liver cells of their hosts and, in humans, cause hepatitis B, cirrhosis, and hepatocellular carcinoma (*2*). Approximately 2 billion persons worldwide are infected with hepatitis B virus (HBV), and 600,000 persons die every year from the consequences of hepatitis B (*3*).

Bats are associated with an increasing number of emerging and reemerging viruses, many of which pose major threats to public health (*4*). We conducted a viral metagenomic analysis of 6 species of bats from Myanmar. The analysis revealed a large number of viral contigs annotated to orthohepadnavirus with <70% nt identity (B. He, unpub. data), suggesting the presence of orthohepadnaviruses in these animals. We describe the virus by full genomic analysis and morphologic observation.

The Study

We purchased 853 freshly killed insectivorous bats in Sedon and Wutao Counties in southeastern Kachin State, Myanmar; the counties are adjacent to Yunnan Province, People's Republic of China. The bats covered 6 species: *Miniopterus fuliginosus* (n = 640), *Hipposideros armiger* (n = 8), *Rhinolophus ferrumequinum* (n = 176), *Myotis chinensis* (n = 11), *Megaderma lyra* (n = 6), and *Hipposideros fulvus* (n = 12). All bat tissue samples were subjected to viral metagenomic analysis (unpublished data). The sampling of bats for this study was approved by the Administrative Committee on Animal Welfare of the Institute of Military Veterinary, Academy of Military Medical Sciences, China.

We used PCR to further study the prevalence of orthohepadnavirus in the 6 bat species; the condition of the samples made serologic assay and pathology impracticable. Viral DNA was extracted from liver tissue of each of the 853 bats by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). To detect virus in the samples, we conducted PCR by using the TaKaRa PCR Kit (TaKaRa, Dalian, China) with a pair of degenerate pan-orthohepadnavirus primers (sequences available upon request). The PCR reaction was as follows: 45 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 40 s, and a final extension at 72°C for 7 min. Positive results were obtained for 22 long-fingered bats (*Miniopterus fuliginosus*). Of these bats, 2.19% (7/320) were from Sedon County and 4.69% (15/320) from Wutao County; the viruses they harbored shared >98% nt identity. No other species had positive amplification results, indicating that *M. fuliginosus* was the most likely species to harbor orthohepadnaviruses.

Of the 22 positive samples, 3 were randomly selected for full genome amplification: M086 from Sedon County and 776 and M005 from Wutao County. PCR was conducted by using the PCR protocol defined above with high-fidelity *Pfu* DNA polymerase (Promega, Madison, WI, USA) and 4 pairs of specific primers (sequences available upon request). Four overlapping amplicons were obtained, sequenced in both directions, and assembled into the full genomic sequence by using SeqMan, version 7.1.0 (DNASTAR, Madison, WI, USA). All 3 full genomes (GenBank accession nos. JX941466– JX941468) were 3,230 nt in length, which is close to the size of primate hepatitis viruses (≈3,200 nt) but smaller than rodent hepatitis viruses (≈3,300 nt). We analyzed the genome structure by using Vector NTI Advance 10 (Invitrogen, Carlsbad, CA). The results showed that the bat hepatitis viruses (BtHVs) contained the same circular and compact genomic structure as other orthohepadnaviruses, comprising 4 open reading frames encoding the multifunctional Pol, preS1/preS2/S, preC/C, and X proteins in the same direction (Figure 1, panel A).

Genomic sequence comparison and phylogenetic analysis based on amino acids of the *pol* gene (2,562 bp) were constructed with ClustalW version 2.0 (www.clustal.org/)

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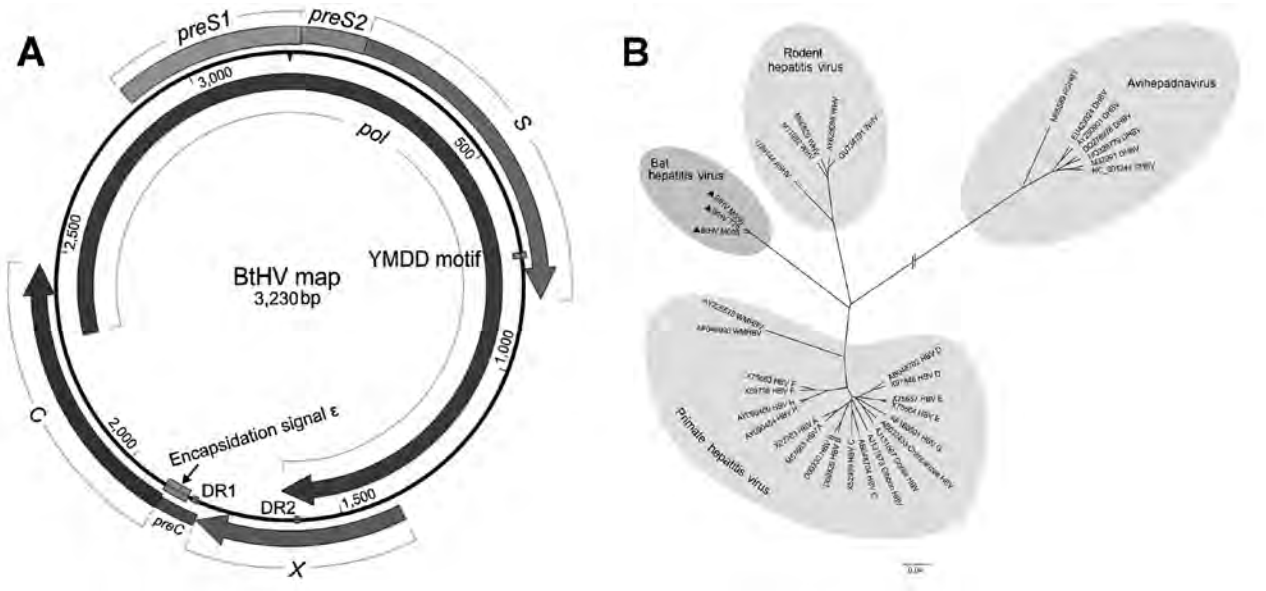

Figure 1. Predicted schematic representation of the bat hepatitis virus (BtHV) genome and its phylogenetic relationship with other hepadnaviruses. A) Genomic structural map of BtHV. Boxes and arrows represent the open reading frames encoding the main proteins: *pol* gene (2,305–1,636), *preS1/S2* and *S* gene (2,864–833), *preC/C* gene (1,815–2,468) and *X* gene (1,378–1,812). Two 12-nt direct repeat sequences (DR1 from 1,825 to 1,836 and DR2 from 1,594 to 1,605), the encapsidation signal e (1,848–1,903), and YMDD domain (734–745) are also depicted in the map. B) Phylogenetic analysis of BtHVs and other hepadnaviruses based on amino acid sequences of *pol* genes. Representatives of hepadnavirus species belonging to *Orthohepadnavirus* and *Avihepadnavirus* genera were used; their GenBank accession nos. are shown in the trees. The different genotypes of human hepatitis B virus are also included. The 3 BtHV isolates are identified by black triangles. Scale bar indicates nucleotide substitutions per site.

and MEGA5 (*5*). Phylogenetic tree analysis showed that previously described orthohepadnaviruses formed 2 clusters, primate hepatitis viruses and rodent hepatitis viruses, whereas the 3 newly identified BtHVs formed an independent cluster within the *Orthohepadnavirus* genus (Figure 1, panel B). Sequence comparison showed that the full genomes of the BtHVs were 63.1%–65.3% and 33.9%–34.8% identical to members of the *Orthohepadnavirus* and *Avihepadnavirus* genera, respectively. Similar low identities were also observed separately in the 4 genes of the BtHVs (Table). These results support the classification of the BtHVs within the *Orthohepadnavirus* genus, being distantly related to current species and likely to form a new species designated as BtHV.

Hepadnaviruses have not been grown in any available in vitro cell system; thus, we did not attempt to isolate BtHV in cell culture. To detect the presence of virus particles, we used pooled liver tissues from the 3 bats that were randomly selected for full genome amplification. We homogenized the pooled tissues in SM buffer (50 mM Tris, 10 mM $MgSO_4$, 0.1M NaCl; pH7.5), followed by clarification by low-speed centrifugation to remove cell debris. We then passed the pooled sample through a 0.22-μm syringe filter (Millipore, Carrigtwohill, Ireland). Polyethylene glycol 6000 was added, and the resulting precipitate was sedimented at 12,000 × *g* in a desktop centrifuge (Eppendorf, Hamburg, Germany) for 40 min at 4°C. The pellet was resuspended and examined after negative staining in a

Table. Gene lengths and percentage identity between bat orthohepadnavirus and other hepadnaviruses*

	pol gene				*preS1/preS2/S* gene				*preC/C* gene				*X* gene			
Virus†	nt	% ID	aa	% ID	nt	% ID	aa	% ID	nt	% ID	aa	% ID	nt	% ID	aa	% ID
BtHV776	2562	–	853	–	1200	–	399	–	654	–	217	–	435	–	144	–
HBV	2532	63	843	57	1203	63	400	59	639	65	212	66	465	61	154	49
WMHBV	2508	63	835	55	1176	64	391	60	636	65	211	63	459	66	152	50
WHV	2640	66	879	56	1281	66	426	51	678	69	225	71	426	67	141	44
ASHV	2634	67	877	53	1284	67	427	52	654	68	217	71	417	69	138	52
DHBV	2526	41	841	30	1104	43	367	30	888	42	295	22	NA	–	NA	–

*nt, nucleotide length; % ID, percentage identity of nt and amino acid sequence between BtHV and other viruses; aa, amino acid length; BtHV, bat hepatitis virus; –, not applicable; HBV, hepatitis B virus; WMHBV, woolly monkey HBV; WHV, woodchuck hepatitis virus; ASHV, arctic squirrel hepatitis virus; DHBV, duck HBV; NA, not available.
†GenBank accession nos. for HBV, WMHBV, WHV, ASHV, and DHBV are D00329, AF046996, AY344076, U29144, and EU429324, respectively.

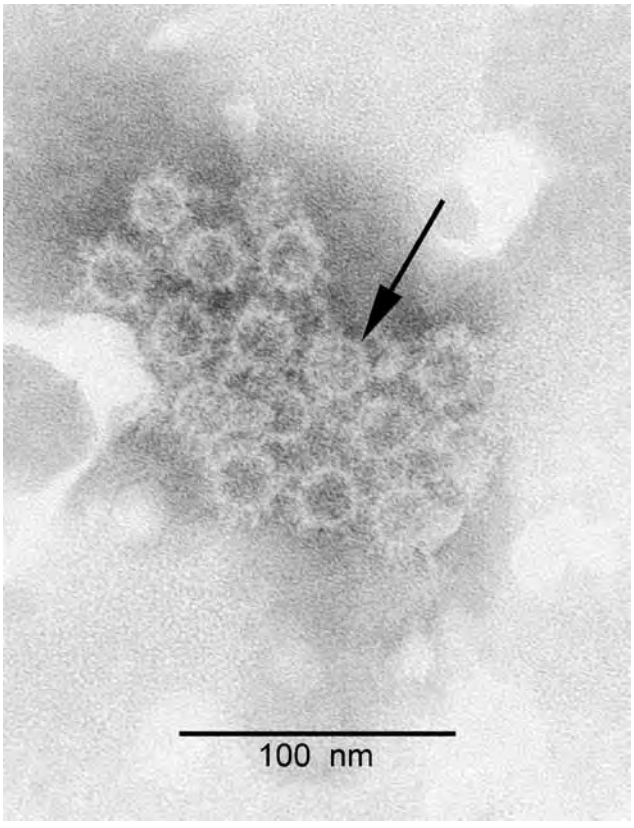

Figure 2. Electron microscopy of negative-stained orthohepadnavirus particles (arrow) from a bat. Clumps of Australia antigen–like particles are seen.

JEM-1200 EXII transmission electron microscope (JEOL, Tokyo, Japan). Numerous spherical particles of ≈20 nm diameter were observed (Figure 2). The particles were morphologically similar to the Australia antigens of HBV, the most abundant viral component found in HBV-infected humans and animals and also known as surface protein or S antigen (*6*,*7*). PCR amplification of DNA extracted from the virus pellet revealed the full genome of the BtHV, with the expected size of ≈3200 bp (image not shown).

Conclusions

Our observations provide strong evidence for the circulation of orthohepadnaviruses in at least 1 species of bats, *M. fuliginosus*, in Myanmar. These bats have a wide distribution (*8*), and increasing numbers of viruses, including coronaviruses and betaherpesviruses, are being isolated from them (*9*,*10*). Of the 6 bat species we sampled, only *M. fuliginosus* was positive for BtHV. The prevalence of BtHV-positive bats in the 2 counties from which we obtained bats, was 2.2% and 4.7%, respectively, indicating that this species is likely a natural reservoir host of BtHV. The lack of detection of BtHV in bats from the other 5 species may be due to the limited numbers of bats sampled (although no evidence of hepadnavirus was found in any of the 176 *R. ferrumequinum* bats) or to a narrow host range of the virus. Further study is required to determine the tropism and prevalence of BtHVs in other bat species.

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Hand, Foot, and Mouth Disease Caused by Coxsackievirus A6, Thailand, 2012

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In Thailand, hand, foot, and mouth disease (HFMD) is usually caused by enterovirus 71 or coxsackievirus A16. To determine the cause of a large outbreak of HFMD in Thailand during June–August 2012, we examined patient specimens. Coxsackievirus A6 was the causative agent. To improve prevention and control, causes of HFMD should be monitored.

Coxsackievirus A6 (CAV6) is 1 of 10 genotypes within the family *Picornaviridae*, genus *Enterovirus*, species *Human enterovirus A*. Other genotypes include coxsackievirus A16 (CAV16) and enterovirus 71 (EV71). Although CAV6 is commonly associated with hand, foot, and mouth disease (HFMD) and herpangina (*1*,*2*), it has not been of concern until the recent global outbreaks of HFMD (*3*–*6*).

In Thailand, the viruses predominately associated with HFMD have been EV71 and CAV16 (*7*,*8*); to our knowledge, CAV6 has not been implicated. In 2012, extensive outbreaks of HFMD occurred in Thailand. To determine the pattern, causative agents, and clinical manifestations of HFMD in this 2012 outbreak, we analyzed specimens from patients. This study was approved by the institutional review board of the Faculty of Medicine, Chulalongkorn University; the requirement for written informed consent was waived because the samples were analyzed anonymously.

The Study

In Thailand, HFMD usually occurs during the rainy season (June–August); average incidence during 2007–2011 was 20.2 cases per 100,000 population (*9*,*10*). In 2012, an extensive outbreak of HFMD occurred; the incidence rate was 3-fold higher than the average incidence rate of 58.15 cases per 100,000 population or >36.000 cases; the 2012 outbreak included 2 fatal cases of EV71 encephalitis (*11*). In this outbreak, 2 clinical patterns were observed, and 2 case definitions were applied. Suspected HFMD cases were defined as painful blisters in the oropharynx and blisters on the palms, soles, knees, elbows, and/or buttocks. Suspected herpangina cases were defined as painful blisters in the mouth only, predominantly on the soft palate. Suspected HFMD and herpangina cases were virologically confirmed if samples were positive for viral RNA by nested PCR.

During January–October 2012, a total of 847 samples were collected from 825 patients with suspected cases. Among those 825 patients, the diagnosis was HFMD for 672 (81.4%) and herpangina for 153 (18.6%). Patients' ages ranged from 1 month to 38 years. The samples were collected from hospitalized patients and outpatients who had a clinical diagnosis of HFMD or herpangina and who came from different parts of Thailand: Bangkok, 566 cases; Khonkaen, 252 cases; Suphanburi, 4 cases; and Saraburi, Rayong, and Chantaburi, 1 case each (Figure 1). Of the 847 samples, 695 were rectal swabs, 73 fecal, 39 throat swabs, 20 serum, 9 vesicle fluid, 7 nasal swabs, 3 cerebrospinal fluid, and 1 saliva. All samples, other than stool samples, were collected in virus transport media modified according to recommendations by the World Health Organization (*12*). Fecal samples were diluted 1:10 with phosphate-buffered saline and centrifuged, and the supernatant was collected for testing. Viral RNA was extracted from 200-μL samples

Figure 1. Location of sample collection sites during outbreak of hand, foot, and mouth disease, Thailand, January–October 2012.

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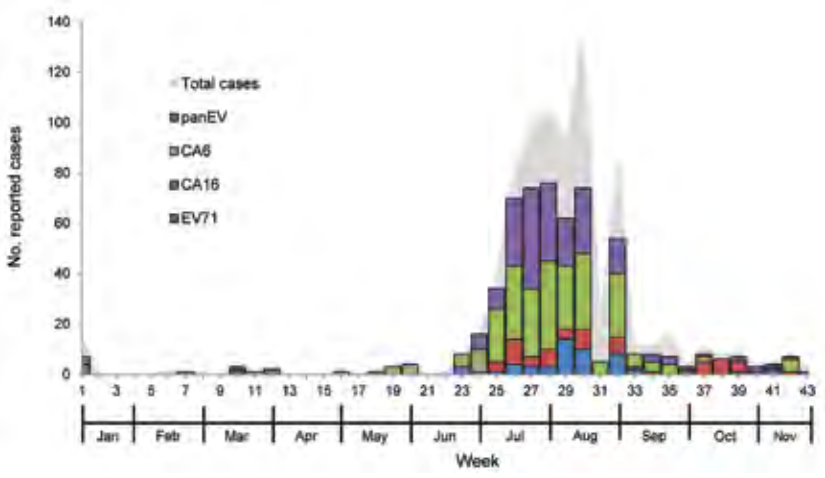

Figure 2. Weekly number of reported suspected cases of hand, foot, and mouth disease and herpangina during outbreak, Thailand, 2012. EV, enterovirus; CA6, coxsackievirus 6; CA16, coxsackievirus 16; EV71, enterovirus 71.

by using the Viral Nucleic Acid Extraction Kit (RBC Bioscience, Taipei, Taiwan) according to the manufacturer's instructions. cDNA was synthesized by using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA) with random hexamers as primers according to the manufacturer's recommendation (First BASE Laboratories, Selangor Darul Ehsan, Malaysia).

To identify enteroviruses, we performed 3 separate PCRs. The first PCR, which could detect most enteroviruses, was used to screen for panenterovirus. The 5′ untranslated region of the viruses was amplified by nested PCR as described (*13*). The second PCR was selective for EV71 and CAV16; the primers and reaction conditions were identical to those used in a previous study (*7*). The third PCR, for CAV6 detection, used primers designed to amplify the viral protein (VP) 1 gene by seminested PCR with CU-EVF2632 (5′-TGTGTGATGAATCGAAACGGGGT-3′) and CU-EVR3288 (5′-TGCAGTGTTAGTTATTGT TTGGCT-3′) as first-round primers and CU-EVR3053 (5′-GGGTAACCATCATAAAACCACTG-3′) as a reverse primer for the second round. The expected 420-bp PCR product was examined under UV light after being resolved in 2% agarose gel electrophoresis and subsequently stained with ethidium bromide.

Most samples were collected during the rainy season, from the end of June through early August 2012 (weeks 25–32), which accounted for 83.1% of all reported cases. Altogether, enterovirus results were positive for 459 (68.3%) HFMD and 101 (66.0%) herpangina patients (Figure 2),

Of note, 93.1% of patients were <5 years of age. A high proportion of cases was found among children 1, 2, and 3 years of age and accounted for 68.4% of HFMD cases and 64.2% of herpangina cases (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/19/4/12-1666-Techapp1.pdf).

Of the 672 HFMD cases, 221 (32.9%) were caused by CAV6, 62 (9.2%) by EV71, 62 (9.2%) by CAV16, and 114 (17.0%) by untyped enteroviruses. Of the herpangina cases, 13.7% were caused by CAV6 and 1.3% by CAV16. Moreover, samples from 51.0% of patients with herpangina were positive for an untyped enterovirus (Table).

Generally, the clinical manifestations of HFMD were fever; drooling, and refusal to eat (among young children); painful lesions in the mouth, especially on the soft palate (online Technical Appendix Figure 2, panel A); and vesicular rashes on the palms and feet (online Technical Appendix Figure 2, panels B, C). For patients affected by this outbreak, physicians from reporting sites reported anecdotally that they observed more severe skin manifestations than usual, especially on the buttocks and perianal area (online Technical Appendix Figure 2, panel D), knees, and elbows. Two cases with neurologic involvement (convulsion, altered consciousness) were caused by EV71 and were treated with intravenous immunoglobulin. No patients died.

Direct sequencing was performed on the VP1 region of 143 randomly selected CAV6-positive samples. The sequences were submitted to GenBank under accession nos. JX556422–JX556564.

The VP1 nucleotide sequences of CAV6 were aligned with the reference sequences by using ClustalW in the BioEdit program version 7.0.9.0 (www.mbio.ncsu.edu/BioEdit/bioedit.html). A phylogenetic tree was constructed with MEGA software, version 5.0, by applying the maximum-likelihood method and using the Kimura 2-parameter model, in which 1,000 replications were selected for bootstrapping (*14*) (online Technical Appendix Figure 3). The sequences of EV71 strain BrCr (accession no. U22521) and CAV16 strain G10 (accession no. U05876) were used as outgroups in the phylogenetic analysis.

The relationship between the CAV6 characterized in this study and the prototype strain (Gdula) was investigated by phylogenetic analysis of partial VP1 sequences. All CAV6 clustered in the same lineage and with the reference strain CAV6 (Gdula); nucleotide homologies among these strains were 81.4%–84.7%.

Table. Causative agents identified during hand, foot, and mouth disease outbreak, Thailand, 2012

Virus	No. (%) cases	
	Hand, foot, and mouth disease	Herpangina
Coxsackievirus A6	221 (32.9)	21 (13.7)
Coxsackievirus A16	62 (9.2)	2 (1.3)
Enterovirus A71	62 (9.2)	0
Panenterovirus only	114 (17.0)	78 (51.0)
None detected	213 (31.7)	52 (34.0)
Total	672	153

Conclusions

Although the positive samples collected during January–October 2012 were mostly from patients in Bangkok and Khonkaen, they partially represented the HFMD and herpangina cases in Thailand's 30,000-case outbreak. Virus prevalence in Thailand was highest in HFMD and herpangina patients 1–3 years of age (Technical Appendix Figure 1). For this seasonal outbreak, the most common causative agent was CAV6. All CAV6 strains shared an isolated cluster and had high similarity, as shown in the phylogenetic analysis of VP1 region. Although CAV6 has been a predominant emerging pathogen since 2012, no patients infected with CAV6 died. According to the study conducted during 2008–2011 EV71 and CAV16 were the main pathogens contributing to the disease (*7*). However, we found a different main pathogen: CAV6. For prevention and control of future outbreaks, the causes of HFMD should be monitored.

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Early Introduction and Delayed Dissemination of Pandemic Influenza, Gabon

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Active surveillance in health care centers in Gabon during 2009–2011 detected 72 clinical cases of pandemic (H1N1) 2009 (pH1N1). We found that pH1N1 virus was introduced in mid-2009 but spread throughout the country in 2010. Thus, Gabon was also affected by pH1N1.

In April 2009, a pandemic strain of influenza A (H1N1) (pH1N1) virus emerged in Mexico and the United States; the World Health Organization declared a pandemic alert on June 11, 2009 (*1*,*2*). This virus was responsible for a large outbreak with thousands of cases in the Reunion Islands and in several French tropical Pacific islands during July–October 2009 (*3*). The circulation and public health effects of pH1N1 virus are largely unknown in Africa, with the exception of South Africa and Kenya, which were heavily affected by disease outbreaks during 2009 and 2010 (*4*–*6*). Other pH1N1 cases were reported in several countries of North, West, and East Africa and in Madagascar (*7*,*8*).

In the humid tropical forest of Central Africa, a study demonstrated the circulation of influenza virus in Cameroon during 2007–2008 (*9*); another reported cases of pH1N1 in Cameroon in 2009 (*10*). A sentinel surveillance program for influenza in Kinshasa, Democratic Republic of the Congo, during 2009–2011 reported several cases of pH1N1 (*11*).

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Gabon is a typical humid, tropical, forested country in Central Africa, with 1,517,685 inhabitants and a surface area of 270,000 km^2. The country has a short dry season during January–February, a long rainy season during March–May, a long dry season during June–September, and a short rainy season during October–December. We report the results of a large surveillance study for pH1N1 in Gabon during a 2-year period, July 2009–June 2011.

The Study

Surveillance for influenza-like illness (ILI) was performed during July 2009–June 2011 in the capital city of Gabon, Libreville, and in 3 other towns in rural Gabon (Franceville, Oyem, and Koulamoutou) (Figure 1). ILI was defined as fever (≥38°C) and runny nose, cough, or sore throat. Study participants were enrolled at 3 health care centers in Libreville and at the regional hospitals in the other towns; all patients who visited these health centers for ILI were systematically sampled. Individual oral consent was obtained from patients for nasal sampling.

Epidemiologic data (name, age, sex, and travel history during the month before onset) and clinical data were collected for each patient. Nasal swabs were sent each week to Centre International de Recherches Médicales de Franceville for analysis. Real-time reverse transcription PCR (RT-PCR) was used to detect pH1N1, seasonal influenza A (H1N1 and H3N2), and seasonal influenza B viruses (*12*). Specimens positive for pH1N1 virus were also tested by specific quantitative PCR for the following common respiratory viruses: adenovirus,

Figure 1. Towns in the influenza sentinel network in Gabon. Libreville was chosen as a typical urban community; Franceville, in the southeast, represents a savannah/forested rural region of 100,000 inhabitants; and Oyem (35,241 inhabitants) and Koulamoutou (16,270 inhabitants), in the north and south, respectively, represent forested rural regions.

Table. Demographic characteristics of patients and distribution of influenza viruses and other influenza-like illnesses, Gabon, July 2009–June 2011*

Patient and illness data	Influenza virus types				Other†	Total no. patients
	pH1N1	A	B	pH1N1 + A + B		
Sex, no. patients						
M	33	4	23	60	427	487
F	39	4	28	71	408	479
Median age, y (range)	2 (2 mo–49 y)	45 (9–50 y)	2 (3 mo–41 y)	2 (2 mo–50 y)	1.58 (10 d–82 y)	1.66 (10 d–82 y)
Age group, no. patients						
0–23 mo	30	0	20	50	444	494
2–4 y	25	0	17	42	219	261
>4 y	17	5	10	32	147	179
Illness, by year and town						
2009						
Libreville	3 (33)	6 (67)	0	9	12	21
Franceville	1 (50)	1 (50)	0	2	0	2
Koulamoutou	0	0	0	0	0	0
Oyem	0	1 (100)	0	1	1	2
Total	4 (33)	8 (67)	0	12	13	25
2010						
Libreville	16 (70)	0	7 (30)	23	224	247
Franceville	1 (3)	0	34 (97)	35	135	170
Koulamoutou	15 (94)	0	1 (6)	16	42	58
Oyem	11 (85)	0	2 (15)	13	105	118
Total	43 (49)	0	44 (51)	87	506	593
2011						
Libreville	11 (92)	0	1 (8)	12	165	177
Franceville	6 (60)	0	4 (40)	10	43	53
Koulamoutou	2 (50)	0	2 (50)	4	32	36
Oyem	6 (100)	0	0	6	76	82
Total	25 (78)	0	7 (22)	32	316	348
Total						
Libreville	30 (68)	6 (14)	8 (18)	44	401	445
Franceville	8 (17)	1 (2)	38 (81)	47	178	225
Koulamoutou	17 (85)	0	3 (15)	20	74	94
Oyem	17 (85)	1 (5)	2 (10)	20	182	202
Total	72 (55)	8 (6)	51 (39)	131	835	966

*Values are no. (%) cases except as indicated. pH1N1, pandemic (H1N1) 2009; A, seasonal influenza A; B, seasonal influenza B.
†Influenza-like illnesses other than pH1N1 or seasonal influenza A or B. Age data were missing for 32 patients in this category.

respiratory syncytial virus, human metapneumovirus, parainfluenzavirus (PIV) 1–4, enterovirus, rhinovirus, parechovirus, and human coronavirus (HCoV; strains OC43, 229E, NL63, and HKU1). Testing protocols are available on request from the authors. Patients who had laboratory-confirmed influenza were contacted several months after diagnosis to determine outcome.

Nasal swab specimens were collected from 966 patients with influenza-like symptoms during July 2009–June 2011: 445 from Libreville, 202 from Oyem, 94 from Koulamoutou, and 225 from Franceville (Table). Median patient age was 1.66 years (range 10 days–82 years); 81% of these patients were <4 years of age, and 19% were 4–82 years of age. The M:F sex ratio was 1.02. The number of cases of ILI increased during the 2 rainy seasons and decreased during the 2 dry seasons (Figure 2), which is consistent with a study showing an increase of the number of influenza cases during the rainy seasons in Senegal (*13*).

Among the 966 cases of ILI, 131 (13.6%) were determined to be caused by an influenza virus: 72 (55%) pH1N1, 8 (6%) seasonal influenza A (H1N1 and H3N2), and 51 (39%) influenza B (Table; Figure 2). No deaths caused by pH1N1 were reported during the study period. For the 72 patients infected with pH1N1 virus, median age was 2 years (range 2 months–49 years); 76.4% of these patients were <4 years of age, and 23.6% were 4–49 years of age. The M:F sex ratio was 0.85.

Only 18 patients with pH1N1 harbored another respiratory virus; this finding suggests that pH1N1 virus infection was responsible for the symptoms in all pH1N1 virus–infected patients. Among patients with pH1N1, we found co-infections with PIV1 (n = 1), PIV3 (n = 2), PIV4 (n = 1), HCoV 229E (n = 1), HCoV OC43 (n = 1), respiratory syncytial virus (n = 7), and adenovirus (n = 5).

The first laboratory-confirmed pH1N1 case (case 1), in a tourist who resided in the Reunion Islands, was diagnosed on July 26, 2009 (Figure 2, panel A). On his trip to Gabon, he had made changeovers in Mauritius and South Africa, 2 countries heavily affected by pH1N1. The patient's symptoms lasted ≈1 week. The second laboratory-confirmed case (case 2) was detected in Franceville 4 months later, during the short rainy season (Figure 2, panels A, E). ILI developed in this patient 3 days after his

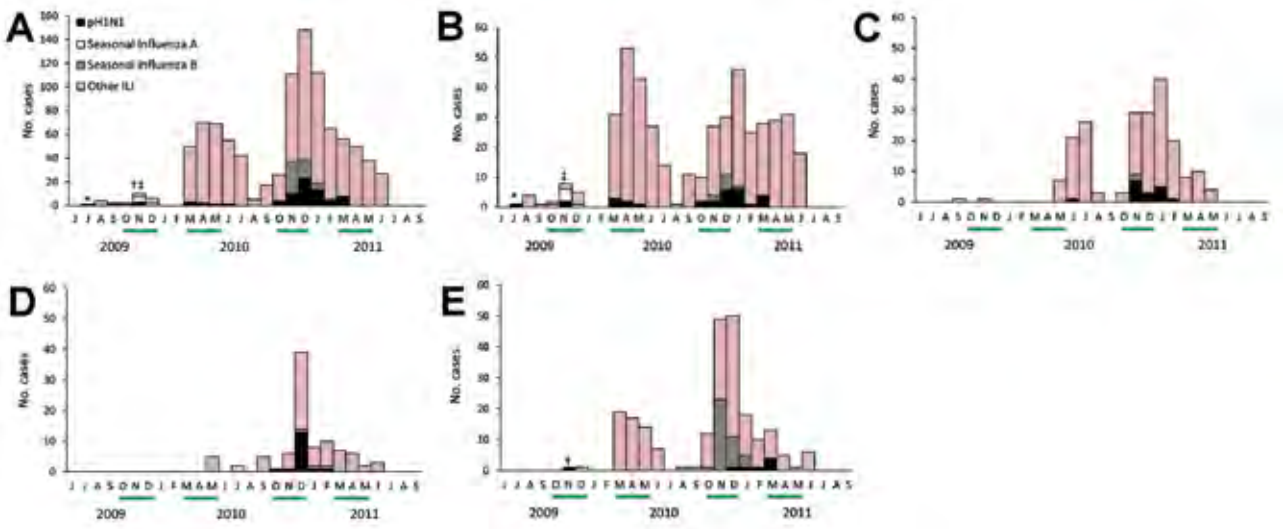

Figure 2. Clinical and laboratory-confirmed cases of pandemic (H1N1) 2009 (pH1N1), seasonal influenza A (H1N1 and H3N2), seasonal influenza B, and other influenza-like illnesses (ILI), Gabon, July 2009–June 2011. Bars below chart indicate rainy seasons. *First imported case; †second imported case; ‡first indigenous cases. A) Gabon; B) Libreville; C) Oyem; D) Koulamoutou; E) Franceville.

arrival in Franceville from France, which was also heavily affected by pH1N1 during this time.

The first 2 autochthonous cases were diagnosed on November 26, 2009 (cases 3), 1 week after the second imported case of pH1N1, during the short rainy season. Subsequently, 29 autochthonous cases were detected in Libreville, 17 in Oyem, 17 in Koulamoutou, and 7 in Franceville (Table). Libreville was the first town with detected pH1N1 cases during the rainy season and also had the highest number of pH1N1 cases (Table). The first autochthonous case was detected in Oyem in early June 2010; during the 2010 short rainy season, several pH1N1 cases were detected in Oyem, indicating pH1N1 virus dissemination throughout Gabon by that time. A total of 85% of the influenza cases in Oyem were pH1N1 (Table). A similar pattern of pH1N1 was observed in Koulamoutou and Franceville during the short rainy season (Figure 2, panels D, E). The percentage of pH1N1 among all influenza cases in Franceville increased from 3% in 2010 to 60% in 2011 (Table). We detected no cases of influenza, including pH1N1, in the provinces of Oyem and Koulamoutou during the first half of 2010.

Seasonal influenza A was diagnosed in Gabon only during September–December 2009: 6 cases in Libreville, 1 case in Oyem, and 1 case in Franceville. During the short rainy season in 2010, the incidence of influenza B increased: 8 cases in Libreville, 2 cases in Oyem, 3 cases in Koulamoutou, and 38 cases in Franceville (Figure 2; Table). We detected no cases of co-infection with pH1N1 virus and either seasonal influenza A or influenza B viruses.

Conclusions

Our data suggest that pH1N1 virus was introduced in Gabon just before July 2009, during the first pandemic peak in the Americas and Europe. However, this early introduction did not result in continuous virus circulation in the rest of the country until the short rainy season in 2010. Only during the 2011 season was there a noteworthy increase in case numbers compatible with a pandemic wave, suggesting a notable time lag relative to that for other countries. Our findings indicate that rural tropical countries such as Gabon may serve as reservoirs for later spread of pH1N1 virus within the country and into other countries (*14*,*15*).

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Response to a Rabies Epidemic, Bali, Indonesia, 2008–2011

Anak Agung Gde Putra, Katie Hampson, Janice Girardi, Elly Hiby, Darryn Knobel, I. Wayan Mardiana, Sunny Townsend, and Helen Scott-Orr

Emergency vaccinations and culling failed to contain an outbreak of rabies in Bali, Indonesia, during 2008–2009. Subsequent island-wide mass vaccination (reaching 70% coverage, >200,000 dogs) led to substantial declines in rabies incidence and spread. However, the incidence of dog bites remains high, and repeat campaigns are necessary to eliminate rabies in Bali.

Rabies was first reported in Indonesia in 1884 and now occurs in 24 of the country's 33 provinces (*1*–*3*). On Bali Island, the first cases of rabies in humans and dogs were confirmed in 2008 on Bukit Peninsula (Figure). Despite control efforts in 2008–2009, rabies spread across the island. In the following 3 years, >130 persons died from rabies (primarily persons who did not receive postexposure prophylaxis [PEP]) (*4*), and PEP was given to >130,000 persons with dog bites. This outbreak resulted in considerable fear and anxiety and cost >US $17 million. We report on the outbreak progression and the effect of initial and subsequently improved control measures.

The Study

When the 2008 Bali rabies outbreak began, the island had no policies for rabies PEP and no dog bite surveillance, rabies diagnostic facilities, or vaccines for dogs. In response to the outbreak, the Indonesian government provided Bali with postexposure rabies vaccine for humans (Verorab), for intramuscular administration according to World Health Organization guidelines, and vaccines for dogs (*10*). The Australian government helped establish a direct fluorescent antibody (DFA) test at the Disease Investigation Center, Denpasar, Bali, and provided supplies for emergency dog vaccination. Surveillance was implemented by DFA testing of brain specimens from dogs that died or were killed after showing signs of rabies and from culled dogs. This surveillance, although imperfect, proved critical in tracking rabies spread (Figure).

In Bali, the first 2 regencies (administrative divisions below provincial governments) affected by the rabies outbreak were Denpasar and Badung. In December 2008, the Balinese government began culling (using strychnine-laced baits or blow darts) unconfined dogs in areas of Denpasar and Badung with confirmed rabies cases and began vaccinating dogs at fixed posts. The locally manufactured vaccine required a booster after 3 months. It was estimated from a survey in Badung, where the human:dog ratio was 8.3:1 (*5*), that 40% of dogs in Badung and Denpasar were vaccinated during December 2008–March 2009 and that 25% received booster vaccinations by June 2009. Over 90% of the dogs in Bali are owned, but most are free-roaming and hard to catch for 1 parenteral vaccination, let alone booster vaccinations (*6*,*7*). Thus, the emergency response failed to contain the outbreak, and by September 2010, rabies had been confirmed in 221 (30.5%) villages throughout Bali (Table; Figure).

In 2009, the Australian Government donated long-lasting vaccines for dogs, but operational funds for administration were unavailable. A local nongovernment organization, the Bali Animal Welfare Association (BAWA), developed a technique to improve vaccination coverage by training teams to catch dogs with nets. During December 2009–July 2010, 6-person BAWA teams using this technique piloted door-to-door vaccinations throughout Gianyar Regency, where BAWA is based. The teams vaccinated 48,000 dogs in Gianyar and 25,000 in nearby Bangli Regency. The World Society for the Protection of Animals donated supplies for this pilot, and BAWA covered operational costs. Surveys of collared (vaccinated) dogs on consecutive days after vaccinations indicated 70% coverage in almost all banjars (subvillages). Beginning in October 2010, BAWA teams and Balinese government staff worked together, with funding from the World Society for the Protection of Animals, to vaccinate dogs throughout most of Bali, subject to the official suspension of culling. By April 2011, a total of 249,429 dogs had been vaccinated, with coverage >70% in most banjars. During this campaign, dogs in Gianyar Regency were revaccinated because 18 months had passed since the pilot and coverage had declined because of population turnover and movement. A second island-wide campaign using these methods was completed in December 2011 by the Balinese government, coordinated by the Food

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and Agriculture Organization, and achieved similar coverage (Table).

During 2010, rabies was confirmed in 417 dogs, 2 cats, and 3 cows. Of the 417 dogs, 387 (93%) were probably unvaccinated; 30 had reportedly been vaccinated, but only 9 had a clear vaccination date; 5 were positive for rabies shortly after vaccination and were likely incubating the disease when vaccinated; and 4 cases were considered vaccination failures.

When the first island-wide vaccination campaign began in 2010, a total of 140 (19.4%) villages still reported rabies (≥1 case in the previous 6 months), and 81 (11.2%) villages that previously reported cases were considered rabies-free (no cases detected for ≥6 months). In addition, during this island-wide campaign (October–April 2011), rabies was detected in 48 previously rabies-free villages. By December 2011, only 30 (4.1%) villages were not considered rabies-free (Table). Before island-wide vaccination, rabies was detected in 10 new villages per month; during the first and second island-wide vaccinations, rabies was detected in 6.8 and 1.6 villages per month, respectively. The monthly number of confirmed cases before mass vaccination was also much higher (44.7 cases) than during the first (10.8 cases) and second (6.0 cases) mass vaccination campaigns, and concomitantly, the island-wide attack rate (confirmed rabid dogs per estimated unvaccinated population) declined from 0.027% to 0.01% (Table). Reported dog bites declined slowly, from 6,256 bites per month before island-wide vaccination to 4,589 and 4,197 bites per month during the first and second vaccination campaigns, respectively. However, human deaths from rabies declined from 94 (4.3/month) before island-wide vaccination to 34 (4.8/month) during the first campaign (24/34 persons were bitten during the prevaccination period) to 9 (1.1/month) deaths during the second campaign.

Conclusions

Rabies was detected in Bali in 2008; it was probably brought by fishermen from the island of Sulawesi (Indonesia), as happened on the island of Flores (Indonesia) (*3*), and subsequently spread throughout the island. Early containment attempts by limited fixed-point dog vaccination and culling were unsuccessful. This was likely due to insufficient funding, largely inaccessible free-roaming dog populations with high turnover, limited availability of long-lasting dog vaccines (and means to identify vaccinated dogs), and inconsistent cold chains.

These issues were gradually addressed, and island-wide vaccinations in 2010 and 2011 approached the recommended target of 70% coverage (*8*,*9*); postvaccination surveys of collared dogs enabled better coverage estimates. Considerable coordination was required among Bali's provincial and regency governments, which was facilitated through training and data management systems. Nonetheless, reporting remained challenging due, in part, to limited infrastructure.

Vaccination campaigns reduced rabies incidence and spread, resulting in decreased attack rates at the regency level and island-wide. In contrast, culling was ineffective in suppressing rabies and can be counterproductive (*10*). Although panic led to demand for culling in some locations, many communities objected because of religious beliefs and, especially, when owned (often vaccinated) dogs were culled. New puppies were brought to replace culled dogs, and some dogs were moved to avoid culls, possibly resulting in the transportation of infected dogs. Incidence declines due to vaccinations reduced the public health threat and panic that triggered culling; ≈108,000 dogs were culled before island-wide vaccination, compared with 40,000 during the 2 vaccination campaigns. However, long-term acceptable dog population control is still sought on Bali.

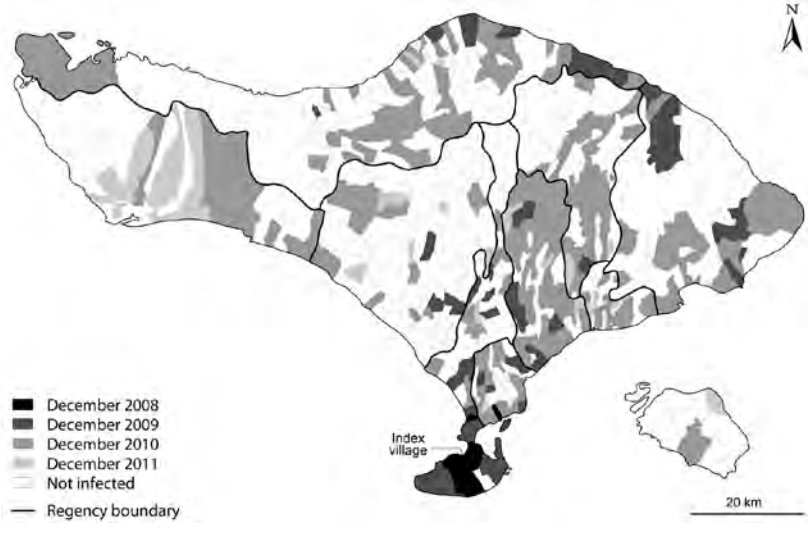

Figure. Timing of confirmed rabies cases in villages across Bali since the first case was confirmed on the island in November 2008. Darker shading indicates earlier detection according to the months since the first case was detected in the index village (marked), lighter shading indicates later detection, and white shading indicates no detected cases by December 2011.

Table. Indicators of rabies incidence and spread among the human, dog, and other animal populations before and during mass island-wide dog vaccination campaigns, Bali, Indonesia, 2008–2011*

Indicator	Before campaign, Nov 08–Sept 10, 2008	During 1st campaign, Oct 10, 2010–Apr 11, 2011	During 2nd campaign, May 11–Dec 11, 2011
Observation period, mo	22	7	8
Average no. rabid dogs/mo	45	11	6
Average apparent monthly attack rate among dogs, %†	0.03	0.01	0.01
Total no. villages with cases detected among dogs	221	269	282
No. villages with newly detected cases	NA	48	13
Rate of spread, no. villages with newly detected dog cases/mo	10	6.8	1.6
Remaining known villages with cases among dogs, no. (%)	140 (19.4)	48 (6.6)	30 (4.1)
No. dog bites treated/mo (bites/day)	6,256 (208)	4,589 (153)	4,197 (140)
Human deaths	94	34	9
Estimated no. culled dogs	107,900	40,500	14,000
No. dogs vaccinated (estimated coverage, %)	>73,000 (40)‡	249,429 (>70)	231,155 (>70)

*Rabid dogs correspond to cases confirmed by using the direct fluorescent antibody test. Villages were classified as free from rabies if no cases were detected for at ≥6 mo. Coverages were initially estimated from human: dog ratios and subsequently from observations of the proportion of dogs with collars indicating vaccination. The number of culled dogs was also estimated because some culling was carried out by communities rather than by government. NA, not applicable.
†Attack rate, confirmed rabid dogs divided by estimated unvaccinated dog population.
‡Data were not available for dogs vaccinated and boosted during the first few months of the outbreak; therefore, only data on vaccinations in Gianyar and Bangli Regencies are shown.

DFA testing proved an effective surveillance method; dog bites were a less sensitive measure. The incidence of reported bites is higher on Bali than in Indonesian provinces where rabies is endemic; this may reflect heightened awareness about rabies or be related to the high densities of humans and dogs. Rabid dogs generally bite without provocation and die ≤10 days after clinical signs develop (*11*); thus, a short observation period (*12*) may allow more judicious PEP administration but is often impractical with unrestrained dogs.

Mass dog vaccinations substantially reduced rabies incidence on Bali and must be continued if elimination is to be achieved. Further research is needed to assess how many more campaigns are needed. Improved surveillance and control of inter-island dog movement are necessary to prevent further rabies spread within Indonesia.

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Genetic Relatedness of Dengue Viruses in Key West, Florida, USA, 2009–2010

Jorge L. Muñoz-Jordán, Gilberto A. Santiago, Harold Margolis, and Lillian Stark

Sequencing of dengue virus type 1 (DENV-1) strains isolated in Key West/Monroe County, Florida, indicate endemic transmission for ≥2 years of a distinct and predominant sublineage of the American–African genotype. DENV-1 strains isolated elsewhere in Florida grouped within a separate Central American lineage. Findings indicate endemic transmission of DENV into the continental United States.

Dengue is the most common mosquito-borne viral disease; cases have been reported from ≈100 countries, and there are indications of increased incidence and severity worldwide (*1*). The United States has reported year-round transmission of dengue virus (DENV) in Puerto Rico, the US Virgin Islands, and American Samoa and occasional transmission along the Texas–Mexico border. In the continental United States, DENV is the most frequent cause of febrile illness among travelers returning from the Caribbean, South America, and Asia (*2*,*3*). These frequent introductions of dengue infections and the increased presence of vectors (i.e., *Aedes aegypti* and *Ae. albopictus* mosquitoes) in many US regions may portend the reintroduction and extended transmission of DENV into the continental United States.

In September 2009, the Florida Department of Health (FDOH) and the Centers for Disease Control and Prevention (San Juan, Puerto Rico) investigated a case of DENV type 1 (DENV-1) infection in a person (index patient) who, as confirmed by reverse transcription PCR (RT-PCR), acquired the virus while traveling to Key West in Monroe County, Florida, USA. DENV-1 infections were subsequently confirmed in 2 Monroe County residents without histories of recent travel. In addition, among 13 other cases in the county that were identified by serologic methods, 2 were confirmed as DENV-1 infections (*4*). Thus, a total of 5 DENV-1 cases were confirmed in Key West during 2009, and ≈5% of the serosurveyed population in Key West had evidence of recent DENV infection (*4*,*5*). In 2010, additional dengue cases from Monroe County were reported, and DENV-1 was isolated from a mosquito pool (*6*) and a blood donor from Key West (*7*); isolates from the mosquito pool and blood donor appeared to be phylogenetically related (*7*). This study determined the genetic relatedness of the DENV-1 isolates from dengue patients in Key West and 4 other Florida counties during 2009–2010, including the blood donor and mosquito isolates.

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The Study

During 2009–2010, serum samples from patients with suspected dengue were received by the FDOH for dengue diagnostic testing; the samples came from 16 of Florida's 67 counties. All samples were tested by using DENV serotype–specific, real-time RT-PCR (*8*) and IgM anti-DENV ELISA (*9*). Samples with highly positive RT-PCR results were spread onto cultured *Ae. albopictus* C6/36 cells, and the presence of virus and genome were confirmed by immunofluorescence (*10*) and RT-PCR, respectively (*11*). Isolates were further propagated and viral RNA was extracted from culture supernatants by using the Universal BioRobot System (QIAGEN, Valencia, CA, USA). The envelope glycoprotein (*E*) gene was amplified (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/19/4/12-1295-Techapp1.pdf), and the *E* gene open-reading frame (1,485 bp) was sequenced. All sequences were submitted to GenBank; accession numbers are shown in online Technical Appendix Table 2. Multiple sequence alignments were performed by using the MUSCLE module available in MEGA 5 (www.megasoftware.net). Evolutionary history was inferred by using maximum likelihood and phylogenetic trees constructed by using neighbor-joining methods. Evolutionary distances were computed, and several *E* gene sequences from GenBank were included in the phylogenetic tree to support tree topology by genotype (online Technical Appendix Table 2).

In 2009, five DENV-1–positive cases were identified by RT-PCR in Key West. Subsequently, in 2010, the FDOH tested 195 serum samples by real-time RT-PCR. Fifty-six (29%) samples were positive for DENV RNA: DENV-1 (37 [66%] samples), DENV-2 (13 [23%] samples), DENV-3 (3 [5%] samples), and DENV-4 (3 [5%] samples). Monroe County submitted 73 serum samples, of which 31 (42%) had results positive for recent dengue infection: DENV-1 was detected in 22 by RT-PCR, and 9 had positive IgM anti-DENV ELISA results. No other DENV serotype was identified in Key West. None of the DENV-1 patients from Monroe County had a history of recent travel to a dengue-endemic region before the onset of symptoms. Fifteen other Florida counties submitted serum samples: 13 counties submitted <10 specimens, 1

submitted 20–30 specimens, and 1 submitted >60 specimens. DENV-1 was found in 15 serum samples from 6 of these counties; however, all the patients had a history of recent international travel.

We sequenced the *E* gene of 12 DENV-1 strains isolated in Florida during 2009–2010 to determine their genetic relatedness; of the 12 strains, 8 were from Key West and 1 each was from Dade, Broward, Orange, and Pinellas Counties. In addition, 23 DENV-1 *E* sequences published in GenBank, including the 2010 Key West isolates obtained from a blood donor and a mosquito pool (*6*,*7*), were used to construct a maximum-likelihood phylogenetic tree. The significance of branch lengths and taxa relationships was tested by 1,000 bootstrap replications. This phylogenetic analysis showed that all the Florida DENV-1 isolates belong to the American–African genotype (genotype V) (*12*,*13*) together with other viruses isolated throughout the Americas (Figure).

Key West DENV-1 viruses grouped among Central American viruses, which configure a distinct lineage separate from the Caribbean viruses. This divergence between the Central American and Caribbean lineages is well supported by high bootstrap values. Moreover, the Key West and Monroe County viruses grouped together and indicated a distinct sublineage supported by a high bootstrap value (99%), separating them from viruses isolated in

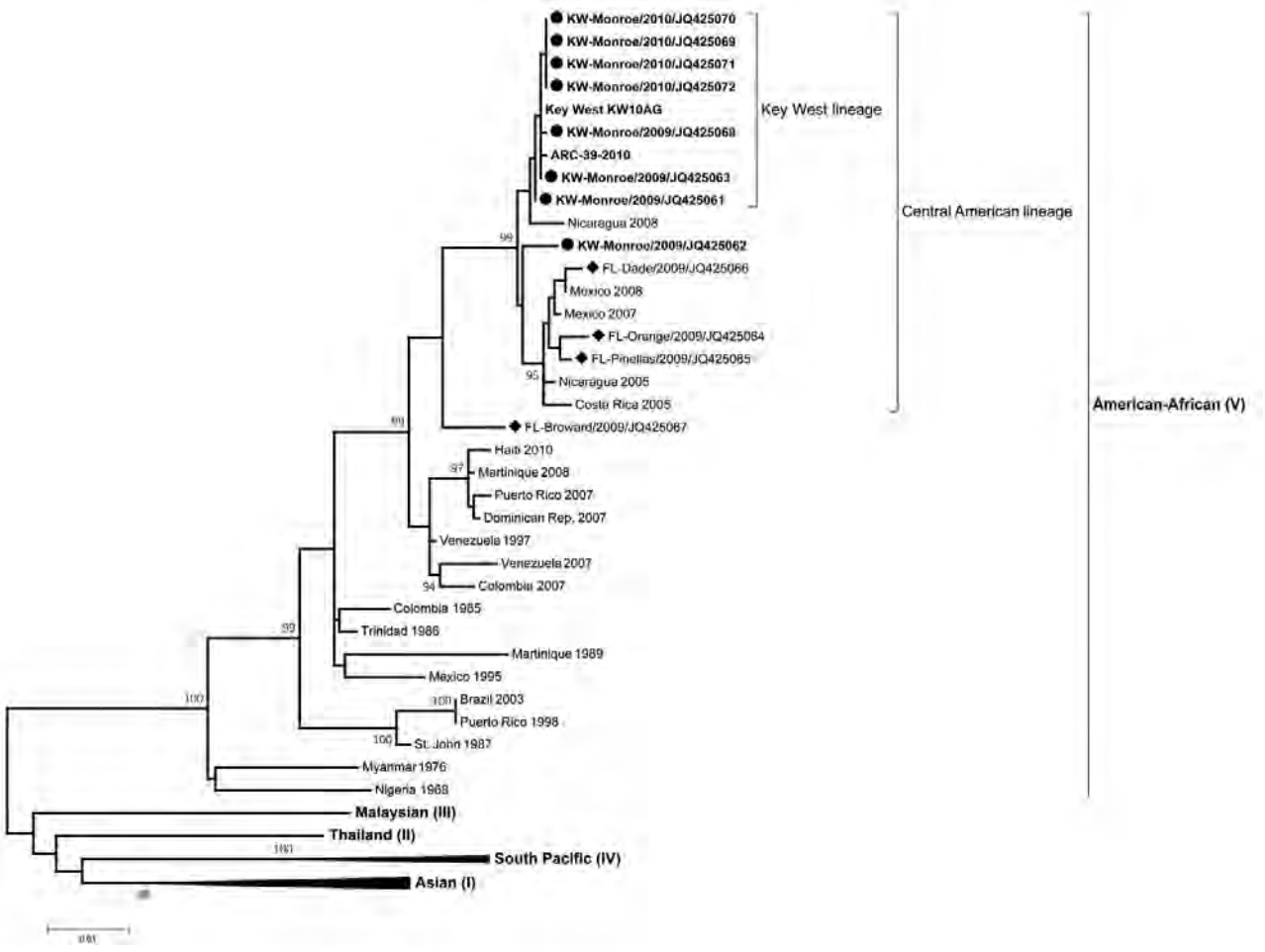

Figure. Maximum-likelihood phylogenetic tree of dengue virus type 1, including isolates from Key West, Florida, USA, and representative isolates from 5 genotypes with global geographic distribution. Solid circles, 8 Key West viruses (Monroe County) isolated during 2009–2010; solid diamonds, isolates from other Florida counties (Dade, Pinellas, Orange, and Broward Counties). Scale bar indicates nucleotide substitutions per site. Each taxon represents a single virus isolate and is labeled with the geographic origin and collection year. All Florida viruses were labeled with the county of origin. **Boldface** taxa labels indicate the Key West lineage and cases not associated with travel. Isolate KW-Monroe/2009/JQ425068 represents the 2009 Key West outbreak index case virus. Thirty-six envelope glycoprotein gene sequences obtained from GenBank were included to support tree topology and identify genotypes. All genotypes except the American–African genotype (V) have been collapsed. Taxa labels and GenBank accession numbers are available in online Technical Appendix Table 2 (wwwnc.cdc.gov/EID/article/19/4/12-1295-Techapp1.pdf).

Dade, Orange, Pinellas, and Broward Counties that were more closely related to other Central American viruses (Figure 1). One 2009 isolate (IQ425062) from a Key West patient is related to this group, suggesting a separate introduction of DENV-1 in Key West. The sequence similarity between the 2009 and 2010 Key West strains was <0.9%; however, the evolutionary distance and taxa positions between the 2009 and 2010 strains presented in the phylogenetic tree suggests that the 2010 strains diverged from the 2009 strains. The observed differences between *E* gene sequences for the Key West strains (2009–2010) and the rest of the strains in this phylogeny were ≤2.1% with the other Florida strains, ≤1.2% with Central American strains, and ≤4.8% with the rest of the American–African genotype.

Conclusions

Evolutionary distances and the topology of the Central American lineage suggest this lineage is the genetic origin of the Florida DENV-1 strain. Most viruses isolated in Monroe County diverged from the Central American lineage into a distinct sublineage—the Key West DENV-1 strain associated with the 2009–2010 outbreak. The high level of genetic similarity among the viruses isolated in Monroe County, their close evolutionary distances, and the lack of recent international travel for the case-patients suggest endemic transmission and microevolution of this DENV. Conversely, the scattered and separate phylogenetic positioning of virus strains from patients with travel-associated cases from other Florida counties indicates a different origin from the majority of Key West isolates. Although the 2009 Broward isolate (JQ425067) is positioned near the Central American lineage, the low bootstrap value (53%) does not support lineage ancestry.

The epidemiologic and phylogenetic evidence suggests that the 2010 cases appeared to be a continuation of the 2009 outbreak. Unlike cases along the Texas–Mexico border (*14*), all DENV-1 infections in Key West seem to have been locally acquired. *Ae*. *aegypti* mosquitoes collected by the FDOH were positive for DENV-1. In addition, DENV-1 was detected in a blood donation from the Monroe County in 2010, further supporting local transmission of DENV (*7*). Collectively, these findings indicate that endemic DENV-1 was transmitted in Key West over a period of ≥2 years.

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Control of Foot-and-Mouth Disease during 2010–2011 Epidemic, South Korea

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An outbreak of foot-and-mouth disease caused by serotype O virus occurred in cattle and pigs in South Korea during November 2010–April 2011. The highest rates of case and virus detection were observed 44 days after the first case was detected. Detection rates declined rapidly after culling and completion of a national vaccination program.

Foot-and-mouth disease (FMD) is a highly contagious disease caused by foot-and-mouth disease virus (FMDV; family Picornaviridae, genus Aphthovirus). FMDV serotypes O, A, and Asia1 are widespread in Southeast Asia (*1*). In South Korea, small-scale outbreaks of FMDV infection caused by serotype O occurred in March 2000, May 2002, and April 2010 (*2–5*), and an outbreak caused by serotype A occurred in January 2010 (*6*). In contrast, an outbreak during November 2010–April 2011 was much more widespread (*7*). We reviewed the progression of this outbreak and methods used to control it, including culling and vaccination of pigs and cattle.

The Study

Clinical signs of FMD in animals appeared on November 23, 2010, in a pig-farming complex in Gyeongbuk Province. Reporting to the central government was delayed for ≈1 week because of misdiagnosis caused by false-negative results from a pen-side antibody kit. FMD-positive test results were confirmed on November 28–29 (Table 1) in samples from saliva, vesicles, and detached hooves from pigs with signs typical of FMDV infection (i.e., salivation, vesiculation, and ulceration) (*7*). Samples from pigs with clinical signs of infection tested positive by antibody-detection assay using solid-phase competition ELISA (PrioCHECK; Prionics, Schlieren, Switzerland) for the O serotype, excluding liquid-phase blocking ELISA; however, antibody tests using nonstructural protein ELISA (VDPro; Jeno Biotech Inc., Chuncheon, South Korea) showed negative results (Table 1).

Because many cattle farms were located in the areas surrounding the pig-farming complex, the virus was detected mainly in cattle during the next 25 days (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/19/4/12-1320-Techapp1.pdf). After the first detection, the disease spread to 75 cities or counties in 11 provinces over 144 days, through April 21, 2011; the only provinces not affected were Jeonbuk, Jeonnam, and Jeju (Figure 1). As soon as an outbreak was reported, animal movement restrictions were imposed, and a 3-km radius protection zone and 10-km radius surveillance zone were set around the outbreak area.

FMD spread throughout Gyeongbuk Province until December 14; at the same time, it spread rapidly to other regions, including the provinces of Gyeonggi (December 15), Gangwon (December 21), and Incheon (December 23) (Figure 1). For emergency disease control, vaccines were initially administered to cattle in these outbreak areas on December 25. However, FMD continued to spread into additional provinces during January 2011, with outbreaks occurring in Chungnam (January 1), Chungbuk (January 3), Daegu (January 17), and Gyeongnam (January 24). Nationwide vaccination was implemented on January 13, and the last reported case occurred on April 21 in Youngcheon City, Gyeongbuk Province.

During this outbreak, 153 (73.56%) of 208 farms with suspected cases were confirmed as index points for disease transmission into new areas. Of farms with animals showing clinical signs, 3,234 (83.98%) of 3,851 had positive test results for FMDV in animals. Within the affected areas, after FMDV infection was confirmed in 1 farm, 295 (21.24%) of 1,389 other farms had positive test results. For farms related to the infected farms epidemiologically (e.g., by vehicle movement or human contact), 33 (10.68%) of 309 had positive test results.

An FMD vaccine of high potency was imported for emergency vaccination; the vaccine used FMDV strain O1 Manisa (*8*). A postvaccination analysis using serum samples collected from vaccinated animals and viruses isolated in the field showed the vaccine's high efficacy in the field. Cattle in the affected regions were vaccinated first, on December 25; later, vaccination was expanded to the whole cattle population, with vaccination completed by

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Table 1. Laboratory diagnosis of FMDV infection in specimens from a pig-farming complex, Gyeongbuk Province, South Korea, November–December 2010*

Farms in pig complex	Date of sample collection	Antigen detection			No. animals tested	No. antibody-positive animals	
		Specimen type	RT-PCR	Antigen ELISA		SP-O ELISA	NSP ELISA
A	Nov 28	S, V, H	+	O serotype	10	2	0
B	Nov 28	S, V, H	+	O serotype	10	2	0
A	Nov 29	Serum	+	ND	90	3	0
B	Nov 29	Serum	+	ND	40	1	0
C	Dec 1	Serum	–	ND	20	0	0
D	Dec 1	Serum	–	ND	20	10	0
E	Dec 1	Serum	–	ND	41	17	7

*FMDV, foot-and-mouth disease virus; RT-PCR, reverse transcription PCR; SP-O, structural protein of FMDV serotype O; NSP, FMDV nonstructural protein; S, saliva; V, vesicle; H, detached hooves; +, positive; ND, not done; –, negative.

January 31, 2011. Pigs were vaccinated 14 days after the cattle (January 8), and the whole pig population was also vaccinated by the end of January.

According to national policy, culling began in November 2010 for all animals on farms with infected animals. Once vaccination was expanded nationwide in mid-January 2011, a vaccination-to-live policy was implemented; that is, vaccinated animals on farms with infected animals were culled only if the outbreak began within 2 weeks after vaccination but not if the outbreak began >2 weeks after vaccination. Most culled animals were disposed of by burial, which was regarded as a suitable method for a large-scale outbreak, given its advantage of easy handling within a short time. Approximately 3.48 million animals (151,425 cattle, 3,318,299 pigs, 8,071 goats, and 2,728 deer) were buried at 4,583 burial sites (online Technical Appendix Figure 2). Some farms that were required to cull livestock because of FMD risk did not undertake the process in a timely manner, which contributed to a spike in new infections on the 38th–64th days after the outbreak began (January 4–31, 2011) (online Technical Appendix Figure 3). These new infections, mainly among pigs, occurred in Chungnam, Chungbuk, Gangwon, Gyeongnam, and Gyeonggi provinces.

After vaccination and culling were implemented, the number of daily FMD cases decreased gradually. Among cattle, the number of FMD cases began to decrease on the 40th day after the initial outbreak (12 days after the first cattle vaccinations). In pigs, the number decreased after the 60th day (18 days after the first pig vaccinations) (online Technical Appendix Figure 1). Many animals also were culled during January 2011 (online Technical

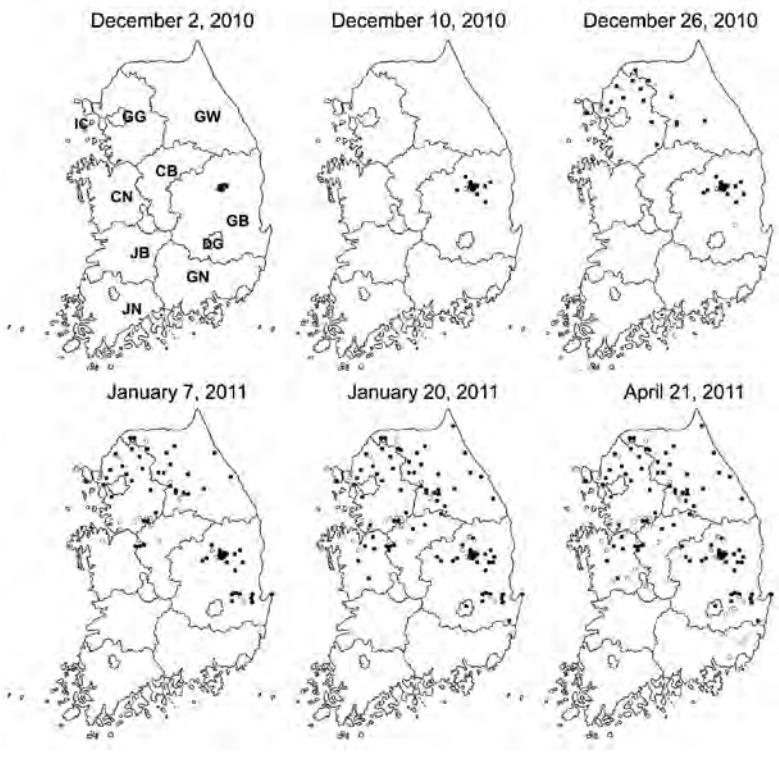

Figure 1. Progress of foot-and-mouth disease transmission throughout South Korea during 2010–2011 outbreak. Circles indicate cases in swine at index farms; black dots, cases in cattle. A timeline of case detection is provided in online Technical Appendix Figure 1 (wwwnc.cdc.gov/EID/article/19/4/1-1320-Techapp1.pdf). IC, Incheon; GG, Gyeonggi; GW, Gangwon; CN, Chungnam; CB, Chungbuk; GB, Gyeongbuk; GN, Gyeongnam; JN, Jeonnam; JB, Jeonbuk; DG, Daegu.

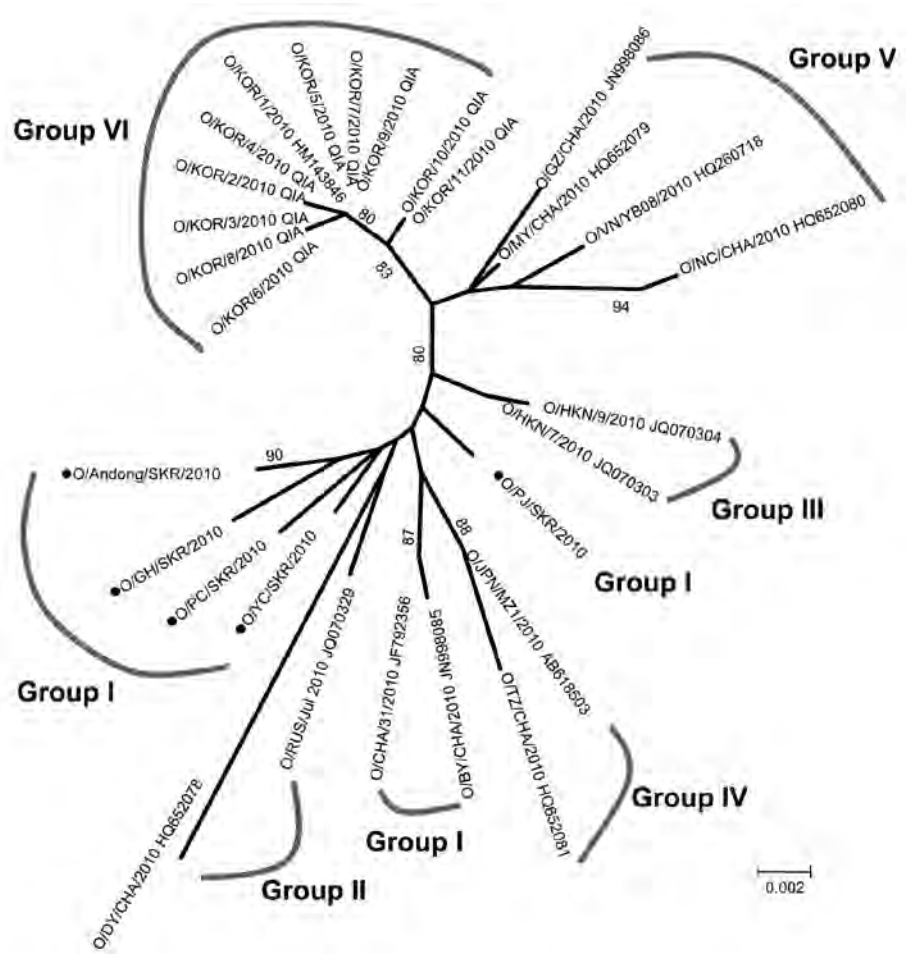

Figure 2. Phylogenetic analysis of viral protein 1 sequences of serotype O foot-and-mouth disease viruses isolated in South Korea (black dots) and other Asian countries, 2010. The tree was constructed by using the neighbor-joining method in MEGA5 (www.megasoftware.net). Percentages in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Scale bar indicates nucleotide substitutions per site. CHA, China; HKN, Hong Kong; JPN, Japan; RUS, Russia; SKR or KOR, South Korea; VN, Vietnam.

Appendix Figure 2), and the number of FMD outbreaks decreased to as low as a single index case daily after January 31, 2011.

The outbreak quickly spread nationwide across a large distance. This rapid spread occurred for several reasons: 1) the first infection was in a pig-farming complex, and pigs excrete the virus in large amounts; 2) detection of the first infection was delayed; 3) FMDV-contaminated feces from the index pig-farming complex was moved to other provinces to be recycled for use as fuel on November 17, before the first outbreak; 4) the virus has increased stability during the winter months, enabling it to be transmitted more easily; 5) culling of infected animals was not implemented quickly enough by affected farms; and 6) the distance between farms in the area was small.

The FMD virus is believed to have entered South Korea around November 9–16, 2010; the first clinical signs in pigs appeared on November 23, and serologic investigation found that the time point for FMD infection was November 14. The virus might have been brought into the country as a result of a farmer's trip to Southeast Asia in early November.

FMDV isolates from Mongolia, Vietnam, and other countries in Asia largely group into 2 phylogenetic clusters on the basis of nucleotide similarities (1). To determine the relationship between the South Korea virus strain and those from other countries in Asia, we analyzed the viral protein 1 nucleotide sequence of an FMDV virus isolate from the first FMD case, in November 2010. The sequence showed >99% identity with the O serotype; this type also matched those found in Gyeonggi Province and another location in Gyeongbuk Province during December 2010. However, a group of FMD viruses identified in South Korea and People's Republic of China (group 1) showed 6 amino acid residues of viral protein 1 different from those of other seasons or countries (Table 2). In addition, among other FMD outbreaks identified in neighboring countries, viruses that originated in China had the most similar composition in amino acid residues to those from South Korea (Table 2; Figure 2) (*1*,*9*).

Conclusions

An outbreak of FMD in South Korea during November 2010–April 2011 was caused by serotype O FMDV and affected ≈3,700 farms; 153 farms were identified as index

Table 2. Comparison of VP1 amino acids of foot-and-mouth disease isolates from South Korea versus viruses originating in other countries in Southeast Asia, 2010*

Group and strain	Country	Region and province	Date of collection	Similarities of VP1, %		Alignment of major differences in VP1 amino acids by position						Genbank accession no.
				nt	aa	58	139	141	152	157	184	
I												
O/Andong/SKR/2010	South Korea	Andong, Gyeongbuk	Nov 28	Ref	Ref	S	S	P	Q	R	T	JQ070321
O/PJ/SKR/2010	South Korea	Paju, Gyeonggi	Dec 15	99.22	100.0	–	–	–	–	–	–	This study
O/YC/SKR/2010	South Korea	Yeoncheon, Gyeonggi	Dec 15	99.22	100.0	–	–	–	–	–	–	This study
O/PC/SKR/2010	South Korea	Pyeongchang, Gangwon	Dec 21	99.06	99.53	–	–	–	–	–	–	This study
O/GH/SKR/2010	South Korea	Ganghwa, Incheon	Dec 24	99.22	100.0	–	–	–	–	–	–	This study
O/BY/CHA/2010	China	Shenzhen, Guangdong	Mar 4	98.75	100.0	–	–	–	–	–	–	JN998085
O/CHA/31/2010	China	NA	Feb 22	98.90	100.0	–	–	–	–	–	–	JF792356
II												
O/DY/CHA/2010	China	NA	NA	97.65	97.18	–	P	–	–	–	–	HQ652078
O/RUS/Jul 2010	Russia	Abagaytuy, Zabajkal'skijkray	Jul	99.06	99.06	–	P	–	–	–	–	JQ070329
III												
O/HKN/7/2010	China	Hong Kong	Feb 22	99.06	99.53	P	–	–	–	–	–	JQ070303
O/HKN/9/2010	China	Hong Kong	Feb 24	98.9	99.53	P	–	–	–	–	–	JQ070304
IV												
O/JPN/MZ1/2010	Japan	Miyazaki	May	98.90	99.06	–	–	–	P	–	A	AB618503
O/TZ/CHA/2010	China	NA	NA	98.44	99.06	–	–	–	P	–	A	HQ652081
V												
O/VN/YB08/2010	Vietnam	Yen Bai	Feb	98.28	99.06	P	–	T	–	–	–	HQ26078
O/GZ/CHA/2010	China	NA	Mar	98.59	98.12	P	–	T	–	–	–	JN998086
O/NC/CHA/2010	China	NA	NA	97.97	98.59	P	–	T	–	–	–	HQ652080
O/MY/CHA/2010	China	NA	NA	98.59	99.06	P	–	T	–	–	–	HQ652079
VI												
O/KOR/1/2010	South Korea	Ganghwa, Incheon	Apr 8	98.44	99.06	P	–	–	–	W	–	HM143846
O/KOR/10/2010	South Korea	Cheonyang, Chungnam	Apr 30	98.44	98.59	P	–	S	–	W	–	This study
O/KOR/11/2010	South Korea	Cheonyang, Chungnam	May 6	98.44	98.59	P	–	S	–	W	–	This study

*Groups are based on major differences in amino acids. VP1, viral protein 1; nt, nucleotides; aa, amino acids; SKR or KOR, South Korea; ref, referent; –, no difference; CHA, China; NA, not available; RUS, Russia; HKN, Hong Kong; JPN, Japan; VN, Vietnam.

locations for new outbreaks. A total of 3.48 million susceptible animals were culled, including cattle and pigs. A vaccination program was effective in controlling the outbreak, and FMD incidence declined rapidly after its completion.

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Who Is This Man?

The father of viral oncology, he discovered the first tumor virus and influenced many later scientists, including several Nobel laureates.

Is he:

A) Richard Shope **C) Simon Flexner**

B) Peyton Rous **D) Thomas Rivers**

E) Ludwik Gross

Decide first. Then turn the page. 

Francis Peyton Rous

Prasanna Kumar and Frederick A. Murphy

This is a photograph of Peyton Rous (Francis Peyton Rous, 1879–1970), who in 1909–11 made 2 seminal discoveries that are now the foundation blocks of modern virology and oncology. First, he discovered that a malignant tumor (a sarcoma in chickens) was transmissible; this was the first transmissible solid tumor discovered. Second, he found that the tumor-inducing factor could be passed through a Berkefeld ultrafilter known to retain bacteria. In the context of the times, this finding proved that the agent was a virus, the first of its kind. The virus he discovered is now known as Rous sarcoma virus and has since been studied in many laboratories around the world.

Rous was born in 1879 in Baltimore, Maryland, USA, and raised by his widowed mother, who persevered in supporting his education. He received bachelor of arts and doctoral degrees from The Johns Hopkins University in Baltimore in 1900 and 1905, respectively. After teaching pathology at the University of Michigan, Ann Arbor, and studying morbid anatomy at Friedrichstadt Municipal Hospital in Dresden, Germany, in 1909, he took a position at the Rockefeller Institute for Medical Research in New York, New York, where he spent the rest of his life. Rockefeller was the place in the United States where virology first emerged as a distinct medical science; from the beginning of his career, Rous was surrounded by great scientists.

Rous' entry into tumor virology was fortuitous; the founding director of the Rockefeller Institute, Simon Flexner, had been interested in oncology but wanted to redirect his own work toward polio, which was becoming a major problem. Rous was hired to continue Flexner's research into oncology, a subject about which Rous at first knew nothing.

The beginning of the story behind Rous' claim to fame is remarkable: a woman came to the Rockefeller Institute with a barred Plymouth Rock hen that had a large tumor on its breast. Rous later wrote, "In this paper is reported the first avian tumor that has proved transplantable to other individuals. It is a spindle-celled sarcoma of a hen, which has thus far been propagated to the fourth generation…." In his research, he found that only closely related chickens were susceptible, but in these chickens, continuous passage of cell-free material led to tumors that grew quickly, were more malignant than usual, and produced widespread metastases. Rous continued to study the phenomenon he had begun to unravel for many years, but understanding the mechanistic bases for the complex natural history of Rous sarcoma virus and related viruses had to wait until modern molecular and cellular biologic technologies became available in the 1960s.

In 1934, Rous' Rockefeller Institute colleague, Richard E. Shope, asked him to examine warts on jackrabbits that had definitively been shown to be caused by an

Figure. Francis Peyton Rous (1879–1970), pictured in 1923, at age 44, in his laboratory at the Rockefeller Institute for Medical Research, New York, NY, USA. Source: National Library of Medicine.

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ultrafilterable virus. This virus was Shope papillomavirus (rabbit papillomavirus). When Rous confirmed that the warts were benign tumors, he was reinvigorated in his intent to unravel the mysteries of viral oncology. Over the next 30 years, Rous and his colleagues showed that the benign tumors could progress to malignant carcinomas and that chemical carcinogens could interact with the virus—further discoveries that formed building blocks for modern virology. Today, we recognize that ≈20% of human cancers worldwide have infectious etiologies, for which preventive measures such as vaccines have great promise.

Rous' colleagues, including the scientists René Dubos and Charles B. Huggins, lauded Rous' personal and professional qualities, writing that he was gifted with supreme intellectual powers, unfailing integrity and honesty, a remarkably intuitive sense for the science itself, great perseverance and work ethic, and an enormous zest for life. One can imagine with wonder Rous in his laboratory and in the legendary lunchroom of the Rockefeller Institute.

Rous was duly honored for his masterful work, winning the National Medal of Science and membership in the National Academy of Sciences, the American Philosophical Society, the Royal Society, and other prestigious organization. In 1966, when he was 87 years old, Rous was awarded the Nobel Prize in Medicine, an honor he shared with Charles Huggins. After 55 years, the longest "incubation period" in the history of the Nobel Prizes, Rous' discovery had finally been recognized with this honor. In the end, proof that Rous was just ahead of his time might be found in the several additional Nobel Prizes awarded since 1966 to virologists who further unraveled viral oncology.

Mr Kumar is a student at Mill Creek High School, Hoschton, Georgia, USA. He became interested in medical history after an epidemiology camp experience at the Centers for Disease Control and Prevention, Atlanta, Georgia. He is planning on a career in medicine.

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Suggested Reading

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Myth Dispelled

Adam Possner

The flu vaccine cannot
give you the flu, I tell him.
It's dead virus, there's
nothing alive about it.
It can't make you sick.
That's a myth.
But if we bury it in
the grassy knoll
of your shoulder,
an inch under the stratum
corneum, as sanctioned by
your signature
in a white-coated ceremony
presided over by
my medical assistant
and then mark the grave
with a temporary
non-stick headstone,
the trivalent spirit
of that vaccine
has a 70 to 90 percent
chance of warding off
the Evil One,
and that's the God's
honest truth.

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Novel Serotype of Bluetongue Virus, Western North America

To the Editor: Bluetongue is an arboviral disease of domestic and wild ruminants characterized by vascular injury that produces widespread edema and tissue necrosis (*1*). Bluetongue virus (BTV), the causative agent of bluetongue, is the prototype virus of the genus *Orbivirus* in the family *Reoviridae* (*2*).

BTV occurs throughout temperate and tropical areas of the world coincident with the distribution of vector *Culicoides* spp. midges (*3*–*5*). Different midge species transmit different constellations of BTV serotypes in distinct global episystems (*3*,*5*). For example, *C. sonorensis* is the principal, if not exclusive, vector of BTV serotypes 10, 11, 13, and 17 in much of North America, whereas *C. insignis* is the major vector of multiple BTV serotypes (including BTV 1–4, 6, 8, 12, 17, 19, 20, and probably others) in the Caribbean basin, Central America, and South America. *C. insignis* is also found in the southeastern United States, and although this species might have recently expanded its range in the region, its distribution in North America remains poorly defined. Serotypes of BTV other than 10, 11, 13, and 17 are found in areas of the United States: BTV-2 was first reported in Florida in 1982. Since 1998, ten additional serotypes (BTV-1, 3, 5, 6, 9, 12, 14, 19, 22, and 24) have been identified in the southeastern United States (*6*).

Approximately 26 BTV serotypes have been described and the global distribution of BTV has recently been altered (*2*,*4*). Coincident with the invasion of novel BTV serotypes into the southeastern United States (*6*), likely by extension from the adjacent Caribbean basin, multiple BTV serotypes have spread throughout much of continental Europe and parts of the British Isles and Scandinavia, precipitating an economically devastating epidemic (*7*). Similarly, ongoing surveillance has identified novel BTV serotypes in regions to which it historically has been endemic (e.g., Australia and the Middle East) (*2*). Climate change may have contributed to this dramatic recent expansion in global distribution of BTV, most notably in Europe (*8*).

Bluetongue was first described in the late 19th century among sheep brought from Europe to South Africa, and later in North America in ≈1950 (*4*). Surveillance in western North America since that time has confirmed that only BTV-10, 11, 13 and 17 are present in this region, including our recent intensive surveillance of sentinel cattle on dairy farms throughout California, USA (*9*,*10*).

However, during investigation of an outbreak of acute coronitis and ulcerative stomatitis among cattle at a dairy farm in the northern Sacramento Valley in California in August 2010, a blood sample from a heifer was found by using described methods (*10*) to be positive for BTV by serogroup-specific quantitative reverse transcription PCR (qRT-PCR) but negative by serotype-specific qRT-PCRs for BTV-10, 11, 13, and 17.

Further analysis using additional serotype-specific qRT-PCRs identified virus in the blood sample as BTV-2. BTV was isolated in primary bovine endothelial cells from blood collected from the heifer. Sequence analysis of the serotype-specific L2 gene of the virus isolate confirmed it to be BTV-2 (*2*), and phylogenetic analyses showed it to be most closely related to a strain of BTV-2 isolated in Florida in 1999 (Figure). However, sequence analysis of the entire genome of the virus from California indicated that it is a reassortant that includes genes from BTV-6 and BTV-2. Specifically, genes encoding the viral protein 1 polymerase and viral protein 3 major core protein segregate with those of the US prototype strain of BTV-6 (isolated in 2006), but other genes are derived from BTV-2. BTV-2 and BTV-6 have been isolated only in the southeastern United States, which

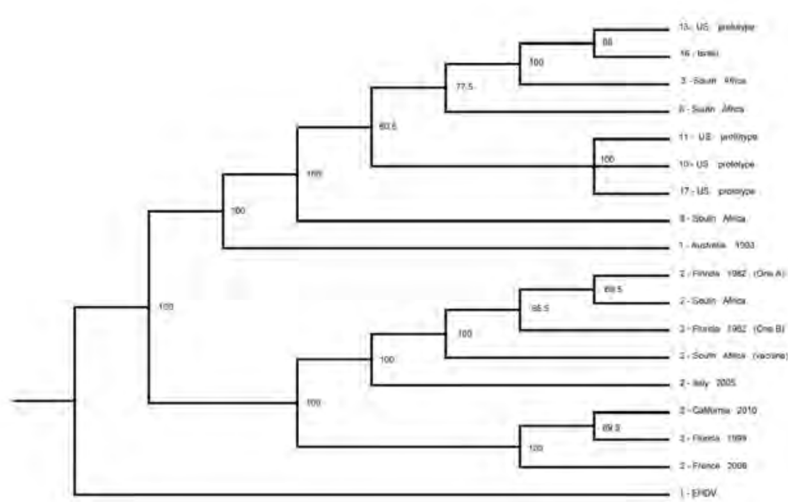

Figure. Cladogram comparing the L2 genes of different bluetongue virus (BTV) serotypes and global strains of BTV serotype 2 (BTV-2) with that of a virus isolated in northern California, USA (2-California-2010; GenBank accession nos. JQ822248–JQ822257). Viruses are identified by serotype, country/region of origin, and for isolates of BTV serotype 2, year of identification. Bootstrap percentages are indicated at selected branching points. EHDV1, epizootic hemorrhagic disease of deer virus serotype 1.

indicates translocation within the United States of reassortant BTV-2.

How this virus spread to California is not known, and its distribution in the United States is uncertain because there is no comprehensive national BTV surveillance program. However, BTV-2 was not detected previously in California, suggesting that this serotype was recently introduced into the region or that it is uncommon. Identification of this novel BTV serotype in western North America emphasizes the need for ongoing entomologic and livestock surveillance, particularly in light of recent changes in the global distribution and nature of BTV infection (*4*,*6*,*8*).

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Hepatitis E Virus Genotype 3 Strains in Domestic Pigs, Cameroon

To the Editor: Hepatitis E virus (HEV) is a positive-stranded, non-enveloped RNA virus of the family *Hepeviridae* that is considered to be the main causative agent of enterically transmitted acute hepatitis (*1*). HEV is classified into 4 genotypes (*1*). HEV genotypes 1 and 2 cause large waterborne epidemics of acute hepatitis in developing countries, especially in Africa and Asia (*1*). In contrast, HEV genotypes 3 and 4 are increasingly identified as causative agents of acute viral hepatitis in industrialized countries (*1*). Genotypes 1 and 2 are found only in humans, whereas genotypes 3 and 4 are associated with food-borne zoonotic transmission from domestic pigs, wild boar, and deer (*1*).

In addition to these 4 genotypes, HEV-related viruses were detected in avian, rodent, and bat hosts, which formed novel genera within the family *Hepeviridae* (*2*). In Africa, HEV genotype 1 and 2 strains have been identified during HEV epidemics (*3*–*5*). An HEV genotype 3 strain was detected in 1 of 40 fecal samples from domestic pigs in Kinshasa, Democratic Republic of the Congo, and it was suggested that this strain was imported from Belgium to the Democratic Republic of the Congo by animal trade (*6*). Therefore, we investigated whether HEV strains of genotype 3 or 4 are circulating among domestic pigs in Cameroon.

During February–March 2012, a total of 345 liver samples were collected from domestic pigs (age range 6 months–3 years) in abattoirs in Douala and Yaoundé, Cameroon, and in slaughter slaps (areas) in Bamenda, Cameroon. Pigs were mainly of the local breed. In addition, pigs originating from extensive cross-breeding (local X landrace and local X Duroc) were sampled. Liver samples were collected during post-mortem inspection.

Viral RNA 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. Extracted RNA was analyzed for HEV RNA by using 2 nested reverse transcription PCRs (RT-PCRs) specific for open reading frame 1 (ORF 1) and ORF 2 of HEV (*7*,*8*). Nested RT-PCRs and direct sequencing of amplicons were performed as described (*9*). RNA of

HEV strain Hamburg-HB (GenBank accession no. JN986840) was used as a positive control for nested RT-PCRs.

HEV RNA was detected in 2 samples from female pigs in Yaoundé (2/139) and 1 sample from a male pig in Bamenda (1/39). All 167 samples from Douala were negative for HEV RNA. The sample from Bamenda showed a positive result for the nested RT-PCR specific for HEV ORF 1. Genetic distances calculated with partial nucleotide sequences of ORF 1 (280 nt) and ORF 2 (373 nt) between strain Yaounde56 and the most closely related HEV genotype 3 strains from Japan (JSWINE150-Aom04R; GenBank accession no. AB221520) and Mongolia (swMN06-A1354; GenBank accession no. AB290105) were 90% and 91%, respectively.

At the amino acid level, the partial RNA-dependent RNA polymerase sequence (ORF 1) and the partial capsid protein sequence (ORF 2) of strain Yaounde56 were identical to those of HEV genotype 3 strains HEV/Gt3/HSD40/2009 (GenBank accession no. AFO71833) from Germany and swJ12–1 (GenBank accession no. BAC66273) from Japan. Thus, all mutations were silent.

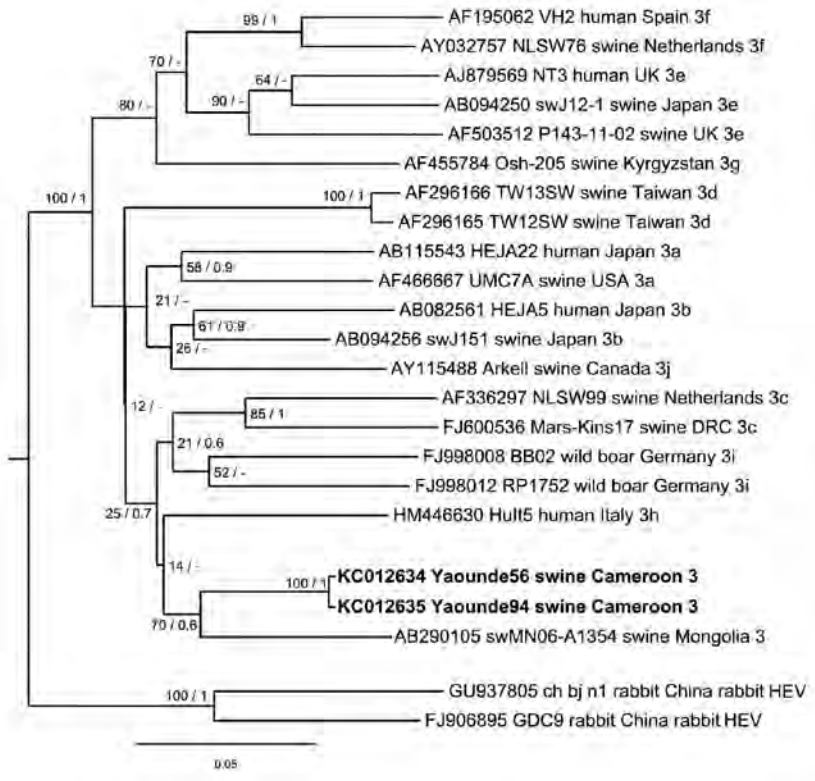

Figure. Phylogenetic analysis of hepatitis E virus (HEV) strains, Cameroon. The Bayesian phylogenetic tree was constructed by using partial nucleotide sequence of open reading frame 2 (278 nt) of HEV. For each sequence used, the GenBank accession number, strain designation, source of isolation, country of isolation, and HEV subtype are shown. Multiple nucleotide sequence alignment was analyzed by using the Markov Chain Monte Carlo method implemented in the program MrBayes version 3.0 (http://mrbayes.sourceforge.net/) and applying the general time-reversible substitution model. Posterior probabilities are shown at the nodes of the tree to the right of the slash if >0.5. Bootstrap values calculated from 10,000 replicates are indicated at the nodes of the tree to the left of the slash. Alignment was analyzed by using the neighbor-joining method and resulted in same tree topology (not shown). Newly described HEV sequences are shown in **boldface**. Scale bar indicates evolutionary distance. UK, United Kingdom; USA, United States; DRC, Democratic Republic of Congo.

In agreement with distance analysis, phylogenetic reconstruction using partial nucleotide sequences of ORF 2 (278 nt) showed a close relationship of strains Yaounde56 and Yaounde94 with HEV genotype 3 strains (Figure). However, the HEV strains from Cameroon do not cluster with the classified HEV genotype 3 subtype reference strains (*10*) in the phylogenetic tree (Figure). These strains cluster within a clade of subtype undefined strains and are most closely related to strain swMN06-A1354 from Mongolia (Figure).

Because the pig production cycle is shorter than that for cattle, pig production is a major economic activity in Cameroon. Most pigs in Cameroon are local raised, and extensive cross-breeding is used. The infection rate of pigs with HEV genotype 3 strains from Cameroon is lower than that of pigs from Europe. Thus, HEV genotype 3 seems to have an extensive distribution that includes Africa. Future studies should investigate how HEV genotype 3 strains contribute to sporadic HEV cases in Cameroon.

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Novel Respiratory Syncytial Virus Subtype ON1 among Children, Cape Town, South Africa, 2012

To the Editor: Human respiratory syncytial virus (RSV) is a common cause of severe acute lower respiratory tract infection in young children, accounting for ≈160,000 deaths/year worldwide (*1*,*2*).As part of an RSV nosocomial transmission study, we detected RSV genotype ON1, which was identified during November 2010–February 2011 as a novel genotype in Ontario, Canada, in samples from children in a tertiary pediatric hospital in Cape Town, South Africa during 2012. The genotype described in Canada was characterized by a 72-nt sequence duplication within the second variable domain of the envelope glycoprotein. The 72-nt duplication within the second variable domain in ON1 was the largest sequence duplication described in this virus (*3*).

RSV is divided into 2 genetically distinct groups, RSV A and B, based on the viral envelope glycoprotein nucleotide sequences (*4*). Sequence variability in the C-terminal variable domain of the glycoprotein gene is commonly used to determine RSV phylogeny (*3*,*5*). To date, 11 RSV A (ON1, GA1–GA7, SAA1, NA1, and NA2) and 17 RSV B (GB1–GB4, SAB1–SAB3, and BA1–BA10) genotypes have been identified (*3*,*6*).

As part of the aforementioned molecular epidemiology study surveying RSV infection in a pediatric hospital, (University of Cape Town research ethics study no. 305/2012), we sequenced the RSV glycoprotein second variable domain of nucleic acid extracts derived from RSV-positive respiratory secretion samples from 160 young children hospitalized for treatment of respiratory tract infections. The techniques used have been described (*7*). During January–April, in an area where NA1 was the dominant circulating RSV genotype, 119 (74%) of 160 RSV isolates were RSV A. We noted the presence, albeit at a low incidence, of the novel ON1 genotype cluster (8 viral isolates) (online Technical Appendix Figure, wwwnc.cdc.gov/EID/pdfs/12–1465-Techapp.pdf) in specimens collected during February–April.

Children in the RSV ON1-infected cohort were brought to health care facilities during February 24–April 25, 2012 (Figure and online Technical Appendix Figure), where they received a diagnosis of brochiolitis or bronchiopneumonia (online Technical Appendix Table). With the exception of 1 patient, child 8, who had been hospitalized before onset of this illness, all ON1 isolates were community acquired. Seven of the 8 ON1 isolates were obtained from infants <4 months of age (median 7 weeks), who were younger than the 152 children who were not infected with the ONI genotype (median age 3.5 months). The RSV ON1–infected children lived within a 2.5-km radius of one another (online Technical Appendix Table). The children who were not infected with RSV ON1 lived in a much wider geographic area; >90% lived within an 18-km radius of one another. These spatial associations with disease prevalence suggested that the ON1-infected children represented a localized cluster of transmission.

None of the children were infected with HIV, although 3 had antenatal

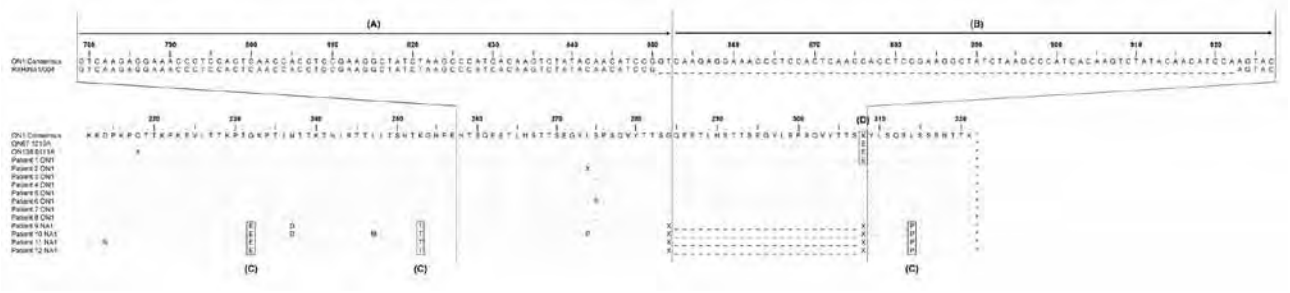

Figure. Alignment of deduced amino acid sequences for ON1 isolates from South Africa (patients 1–8, accession nos. JX885730–JX885737) and Canada (ON67 and ON138) with NA1 isolates (patients 9–12) from South Africa. A) Variable domain sequence copied. B) Duplicated sequence inserted into variable domain. C) Characteristic amino acid substitutions that distinguish ON1 from NA1. D) Amino acid substitution (E308K) (position 284 before insertion) that distinguishes between most ON1 isolates from South Africa (patients 2–8) from those from Canada (ON67 and ON138) ON1.

exposure to HIV. Co-infection with adenovirus and rhinovirus was noted in 3 of the patients. Although 3 of the patients were hospitalized for a prolonged period and required ventilatory support, the severity and outcome of the RSV ON1 infections were similar to RSV infections caused by other genotypes among children of the same age.

Sequence analyses revealed that ON1 isolates identified in South Africa are essentially identical to those isolated in Canada, possessing characteristic amino acid substitutions at positions E232G, T253K, and P314L that distinguish the genotype from circulating NA1 genotypes (*3*). However, 7 of 8 ON1 isolates from South Africa possess a unique E308K (position 284 before insertion) amino acid change at the 3′ border of the duplicated gene segment not present in the ON1 isolates identified in Canada (Figure). The conservation of the E308K mutation within ≈90% of the isolates from South Africa that we studied suggests a possible functional role for the positively charged lysine residue.

The capacity of the RSV glycoprotein to accommodate large insertions and remain functional was first demonstrated with the RSV B, BA genotype (Buenos Aires, Argentina 1999). This genotype contains a 60-nt duplication in the second variable domain, which, similar to ON1, did not cause serious clinical outcomes (*6*,*8*–*10*). Longitudinal analyses during 12 epidemic seasons (1996–97 through 2007–08) of international RSV subtype distribution revealed that since its initial detection in 1999, BA prevalence has gradually increased to become the dominant RSV group B virus genotype in circulation (*10*). Because RSV A has traditionally been the dominant RSV type in circulation, if the large insertion in ON1 confers similar selection advantage as seen in BA, the potential dominance of a single ON1 genotype within this group might promote bias on RSV type distribution toward RSV A.

The novel ON1 genotype was first described in Ontario, Canada (*3*). Our subsequent findings in South Africa suggest extensive distribution of this genotype, which was assumed to have arisen before winter 2010–11 (*3*). To understand whether ON1 in South Africa occurred as a result of importation or natural evolution within locally circulating NA1 genotypes, further research is required.

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Henipaviruses and Fruit Bats, Papua New Guinea

To the Editor: In 2010, detection of henipavirus (Hendra or Nipah virus) and rubulavirus (Tioman or Menangle virus) antibodies in fruit bats in Papua New Guinea (PNG) was reported (*1*). To explore changes in henipavirus dynamics in fruit bats, we compare and contrast this finding with serologic findings from 10 years earlier (*2*; H. Field et al., unpub. data).

In these earlier studies, blood samples were collected from 182 wild-caught fruit bats of mixed species, age, and sex from 3 locations in PNG: Madang (1996), New Britain (1997), and Lae (1999) (*2*; H. Field et al., unpub. data) (Figure). The 20 samples from Madang were collected as blood spots on filter paper and forwarded to the (then) Department of Primary Industries Animal Research Institute in Brisbane, Australia, where they were eluted and screened by ELISA for antibodies against Hendra virus (*3*). Serum from 59 samples from New Britain and 103 from Lae were forwarded to the Commonwealth Scientific and Industrial Research Organisation's Australian Animal Health Laboratory in Geelong, Australia. Samples from New Britain were screened for antibodies against Hendra virus by virus neutralization test (VNT) (*3*). Positive samples were subsequently screened by VNT for antibodies against Nipah virus. Samples from Lae were screened by VNT for Hendra, Nipah, and Menangle viruses (*3*). A reciprocal VNT titer of >5 was considered indicative of antibodies.

Of the 20 samples from Madang, 2 (10%) reacted in the Hendra virus ELISA. Of the 147 samples from New Britain and Lae that yielded definitive VNT results, 11 (7.5%) yielded neutralizing antibodies to Hendra virus and 5 (3.4%) to Nipah virus. All samples with antibodies against Nipah virus also had antibodies against Hendra virus; titers against Hendra virus were greater (4 samples) or equivalent (1 sample) to those against Nipah virus. Reciprocal titers against Hendra virus were 5–160 (median 10) and against Nipah

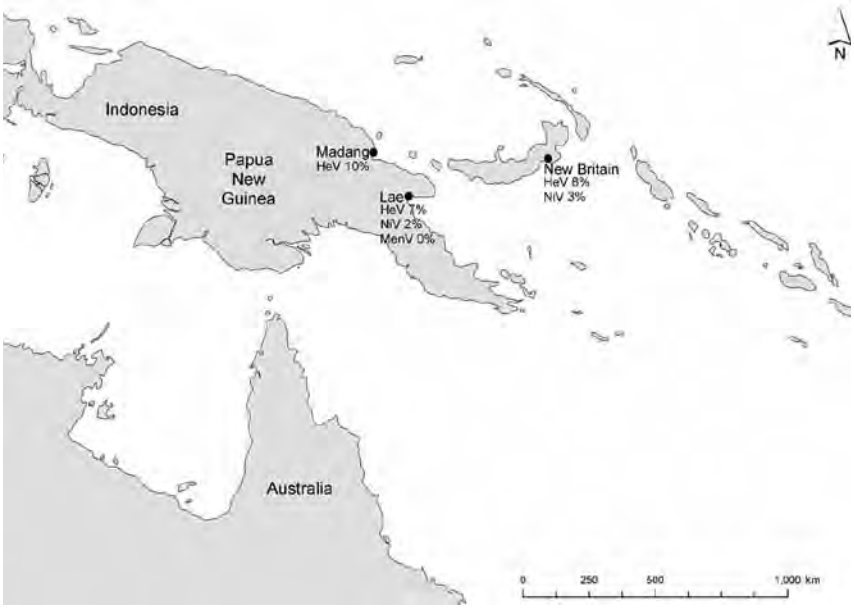

Figure. Sampling locations and henipavirus antibody prevalence, Papua New Guinea 1996–1999, among 182 wild-caught fruit bats from Madang (1996), New Britain (1997), and Lae (1999), Papua New Guinea. HieV, Hendra virus; NiV, Nipah virus; MenV, Menangle virus.

virus, 5–80 (median 10). None of the 103 samples from Lae had antibodies against Menangle virus (Figure).

The common and contrasting findings between the study of Breed et al. (*1*) and the earlier studies are as follows. First, the earlier studies identified antibodies against Hendra virus in fruit bats from multiple locations and species, as did the study by Breed et al. (*1*). However, in marked contrast, the earlier studies found a crude prevalence of antibodies against Hendra virus of 7.8% (13/167) compared with 50% found by Breed et al. (*1*). Although this difference could reflect confounding by species, location, or time, when we controlled for the first 2 by comparing only 1 bat species (*Pteropus conspicillatus*) from proximate locations (Lae and Madang), the significant differences in antibody prevalence remained: 0 (95% CI 0–23%) in 1999 and 65% (95% CI 50%–78%) in 2009.

Second, in the earlier studies, all bats (except 1) that had a neutralizing antibody titer to Nipah virus had a higher neutralizing titer to Hendra virus (1 bat had equivalent titers), suggesting that the circulating henipavirus was more similar to Hendra virus than to Nipah virus. These findings are supported at a regional level by those reported for nearby Indonesian islands by Sendow et al. (*4*). However, the more recent findings of Breed et al. (*1*) suggest the opposite. Of note, in the earlier PNG studies, titers against Hendra and Nipah viruses were modest (median 10) and pose the possibility of cross-neutralization by a related, but undescribed, additional henipavirus.

Third, the earlier studies found no antibodies against Menangle virus in the 103 samples; in contrast, Breed et al. found 56% (*1*). Thus, earlier studies found no samples with antibodies against Menangle virus and henipavirus, in contrast to 36% of samples reported by Breed et al. (*1*).

Although any of these differences might have multiple interpretations, the collective scope and magnitude of the differences is more consistent with a major 10-year change in infection dynamics in these bat populations. Sendow et al. (*4*), when reporting henipavirus infections in fruit bats in Indonesia, canvassed the geographic extent of Hendra virus and Nipah virus, concluding that a transition probably occurred between Hendra virus in Australia and Nipah virus in Malaysia and beyond. They also concluded that further research was needed to understand the nature and stability of the interface between Hendra virus and Nipah virus and to investigate the possible presence of unidentified cross-neutralizing henipaviruses.

Changed henipavirus dynamics in PNG fruit bat populations could reflect altered population dynamics (and consequent infection dynamics) associated with negative ecologic effects (e.g., habitat loss, encroachment) (*5*). More broadly, such changes might portend a regional shift in the geographic interface between Hendra and Nipah virus endemicity.

More robust interpretation of the serologic findings of both studies is constrained by the lack of henipavirus sequence data from PNG and neighboring countries. We concur with Breed et al. (*1*) that sequencing and phylogenetic analyses are imperative if the ecology of henipaviruses in fruit bat populations and the implications for human and livestock health in the region are to be fully understood.

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High Incidence of Japanese Encephalitis, Southern China

To the Editor: Japanese encephalitis virus (JEV) remains a major source of illness and death in Asia (*1*). An estimated 67,900 cases occur each year in Asia; ≈33,900 cases—half the cases in the world—probably occur in the People's Republic of China (*2*). However, because reporting is incomplete in most countries where JE incidence is high, these estimates are based on scarce data. In China, a study conducted during 2006–2007 in sentinel hospitals in 1 prefecture each in Shandong, Hubei, Guangxi, and Hebei Provinces (all in the eastern half of China) found that 9.2% of patients with acute meningitis and encephalitis had JEV; adjusted incidence for each prefecture was 0.08–1.58 cases per 100,000 population (*3*). Incidence in these 4 prefectures is lower than that among children <14 years of age in JE-endemic countries, where estimated incidence is 5.4 cases per 100,000 population (*2*). To assess the need for strengthening existing JE surveillance and vaccination programs, we conducted a population-based study of JE incidence in 1 area of southern China.

Dehong is a prefecture in western Yunnan Province, which borders Myanmar. The JE-susceptible population of Dehong Prefecture (residents ≤15 years of age) is 211,337. The 2 principal cities of Dehong Prefecture—Mangshi and Ruili—are busy commercial centers surrounded by areas of extensive rice cultivation. The mosquito vector of JEV, *Culex tritaeniorhynchus*, is predominant during summer (*4*). During 1988–2007, JEV vaccination was available only at certain clinics and only for a fee; however, since 2008, vaccination with the live, attenuated SA 14–14–2 JEV vaccine (Chengdu Institute of Biologic Products, Chengdu, China) has been included in the national Expanded Program on Immunization at no charge. The recommendation for children is vaccination at 8 months and 2 years of age (*5*,*6*).

To estimate JE incidence in Dehong Prefecture during January 1–December 31, 2010, we conducted an anonymous, unlinked study of all cases of encephalitis at the only 2 major children's hospitals in the region, Dehong Prefecture Hospital in Mangshi and Ruili City Hospital in Ruili. All eligible patients admitted to these hospitals were included in the study. Inclusion criteria were age ≤15 years, residency in Dehong Prefecture, clinical diagnosis of encephalitis, lumbar puncture performed (routine for encephalitis patients at these 2 hospitals), and cerebrospinal fluid (CSF) pleocytosis. After routine testing was completed, leftover CSF and serum samples were stored at −70°C until further testing, which was all conducted at the Chinese Center for Disease Control and Prevention in Beijing. All CSF specimens were tested by viral culture in C6/36 and BHK-21 cells (*7*) and tested for antibodies against JEV (*3*,*4*). Serum samples were tested for antibodies against JE virus, mumps virus, echoviruses, and coxsackieviruses (*3*,*4*,*7*). A case of JE was defined as illness in a person with IgM against JEV in CSF or serum. Clinical information was collected by using a standardized chart abstraction form. Linkages to personal identifiers were destroyed.

A total of 189 eligible patients were enrolled, 150 from Mangshi and 39 from Ruili. Of these, 110 (58%) were male and 78 (41%) were <4 years of age. Enrollment peaked during summer (Figure). All patients were hospitalized within 6 days after symptom onset. A total of 22 (12%) patients were classified as having JE on the basis of IgM, in CSF for 21 and in serum for 1. Illness onset occurred during May–November (Figure); overall incidence was 10.4 cases per 100,000 children ≤15 years of age. Among these 22 children with JE, 11 were male; 20 were from rural areas; 14 were from Mangshi and 8 were from Ruili; and 5 were 0–1 years, 6 were 2–4 years, and 11 were 5–13 years of age. JEV vaccination history was infrequently recorded in the medical charts; however, JEV was more likely to be the cause of encephalitis among

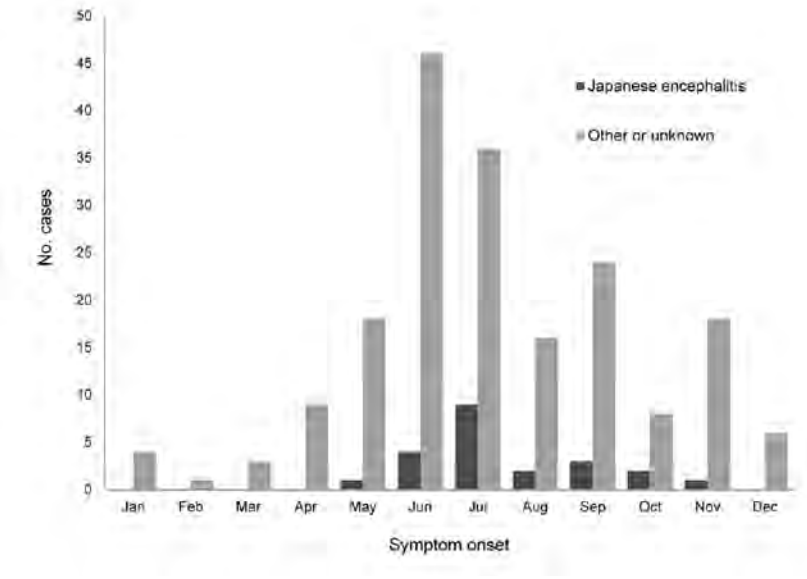

Figure. Number of children with encephalitis at 2 hospitals, by etiology and month of symptom onset, Dehong Prefecture, People's Republic of China, 2010.

children who received no vaccination (22%, 6/27) than among those with unknown vaccination history (10%, 15/157). Of 5 vaccinated children, 1 had JE; however, verification of this child's vaccination was not possible. Among 71 children who had no evidence of JE but for whom serum samples were available for testing, 5 had antibodies against mumps virus, 8 against echoviruses, and 5 against coxsackieviruses. Viral cultures of CSF from all 189 children were negative.

Our finding of 10.4 JE cases per 100,000 children ≤15 years of age in Dehong Prefecture is higher than the estimated incidence of 5.4 cases per 100,000 population among children ≤14 years of age in JE-endemic countries (*2*). Nevertheless, the true JE population incidence for Dehong Prefecture might be underestimated if some children received no medical care or were admitted to other hospitals. Adults were not studied; however, ≈90% of JE cases in China are reported among children <15 years of age (*5*,*6*). Unfortunately, accurate age-adjusted JE vaccination coverage data for Dehong Prefecture are not available. Although vaccination programs have markedly lowered JE incidence in China in recent years (*5*,*6*), the finding of continuing high JE incidence in Dehong Prefecture warrants further attention.

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Novel Hantavirus in Field Vole, United Kingdom

To the Editor: Hantaviruses (family *Bunyaviridae*) are transmitted to humans by inhalation of aerosolized virus in contaminated urine and feces, mainly from rodents of the families Cricetidae and Muridae. Although infections in rodents are asymptomatic, infections in humans can lead to hemorrhagic fever with renal syndrome and hantavirus cardiopulmonary syndrome (*1*).

In Europe, 5 rodent-borne hantaviruses have been detected: Dobrava-Belgrade, Saaremaa, Seoul, Puumala, and Tula (*1*,*2*). The most common and widespread hantavirus in Europe is Puumala virus, which is associated with the mildest form of hemorrhagic fever with renal syndrome (*1*).

In the United Kingdom, only a few cases of hantavirus infection in humans have been reported and confirmed serologically, but the causative virus species were not identified (*3*,*4*). Subsequent longitudinal studies reported considerable hantavirus seropositivity among healthy human cohorts, suggesting past exposure to hantaviruses or subclinical infection (*3*). Serologic surveys of rodents (rats

and mice) and cats also supported the presence of a hantavirus indigenous to the United Kingdom (*3*). To determine whether hantaviruses are circulating in wild rodents in the United Kingdom, we conducted molecular analyses on rodent tissues.

From September 2009 through November 2011, a total of 495 wild rodents consisting of 133 brown rats (*Rattus norvegicus*), 269 wood mice (*Apodemus sylvaticus*), 50 house mice (*Mus musculus*), 35 bank voles (*Myodes glareolus*), and 8 field voles (*Microtus agrestis*) were caught live across northwestern England (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/19/4/12-1057-Techapp1.pdf). Animals were euthanized in the field by use of isoflurane inhalation, according to UK Home Office Guidelines (http://webarchive.nationalarchives.gov.uk/+/http://www.homeoffice.gov.uk/docs/hc193.html). Within 2 hours, kidney, liver, and lung tissues were removed. When field conditions allowed, blood samples were collected; otherwise, heart tissue was collected. Samples, and carcasses that could not be processed within 2 hours, were stored at –80°C.

RNA was extracted by using TRIzol Reagent (Invitrogen, Life Technologies, Paisley, UK). To detect hantavirus RNA, we used a nested pan-hantavirus reverse transcription PCR selective for partial polymerase large segment (L) gene sequences (*5*). With the exception of 1 male field vole (B41) collected near Tattenhall, Cheshire (online Technical Appendix Figure), all lung samples were negative for hantavirus RNA. The positive amplicon was sequenced by using a BigDye Terminator 3.1v Cycle Sequencing Kit on an ABI3130xl genetic analyzer (Applied Biosystems/Life Technologies, Paisley, UK) (GenBank accession no. JX316008). Partial small segment (S) sequences were also recovered from lung RNA from vole B41 (GenBank accession no. JX316009) (online Technical Appendix Table). Established reverse transcription PCRs for the medium segment were unsuccessful.

Comparisons of nucleotide and amino acid sequence identities demonstrated, as expected, that the Arvicolinae-associated hantaviruses showed the highest similarity to the UK sequence at the nucleotide (65.7%–78.8% for S and 76.6%–77.5% for L) and the amino acid (66.4%–86.3% for S and 80%–88% for L) levels (online Technical Appendix Table).

Phylogenetic analyses of partial L (Figure, panel A) and partial S sequences (Figure, panel B) confirm the inclusion of the viral sequence from vole B41 as a distinct member of the Arvicolinae-associated hantaviruses. In the partial L tree (Figure, panel A), viral sequence B41 clustered with Prospect Hill and Tula viruses with good support, although in the partial S tree (Figure, panel B), B41 seems to be more closely related to the Asian

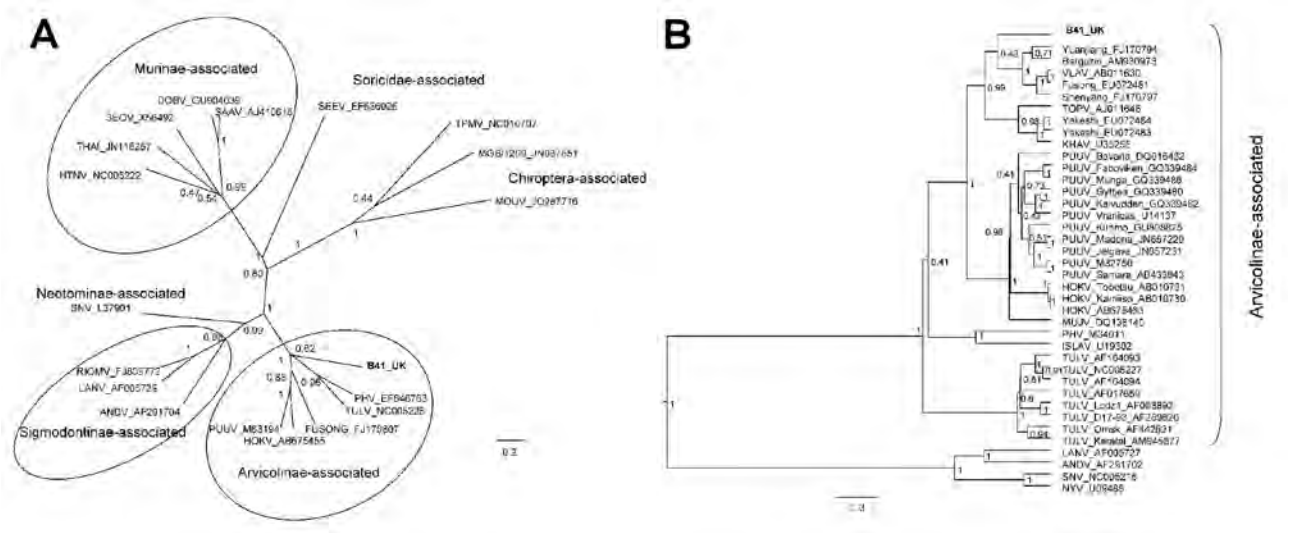

Figure. Bayesian phylogenetic trees constructed by using the models HKY+gamma for partial large segment sequences (n = 19) (A) and GTR+gamma for partial small segment sequences (n = 39) (B) within BEAST software (*6*) with Markov chain Monte Carlo chain lengths of 10 million and strict clock. Optimum substitution models were estimated by using MEGA5 (*7*). The trees are drawn to scale; branch lengths are measured in the number of substitutions per site. The numbers at each node are posterior probabilities. All effective sample size values exceeded 150 for partial L and 1,600 for partial S sequences. The phylogenetic position of virus isolated from field vole B41 (in **boldface**) is shown in relation to representative hantaviruses (A) and more closely related Arvicolinae-associated hantaviruses (B). GenBank accession numbers are shown next to taxonomic names. Scale bars indicate nucleotide substitutions per site. VLAV, Vladivostok virus; TOPV, Topografov virus; KHAV, Khabarovsk virus; PUUV, Puumala virus; HOKV, Hokkaido virus; MUJV, Muju virus; PHV, Prospect Hill virus; ISLAV, Isla Vista virus; TULV, Tula virus; LANV, Laguna Negra virus; ANDV, Andes virus; SNV, Sin Nombre virus; NYV, New York virus.

Microtus vole–associated hantaviruses, albeit with low posterior probability values. These differences in tree topologies probably reflect different compositions of the sequence datasets.

Blood collected from vole B41 was positive for hantavirus-specific antibodies (indirect fluorescent antibody test that used Puumala antigen) (*8*), suggesting cross-reactivity, as would be expected for Arvicolinae-associated hantaviruses. Hantavirus RNA was detected in the kidneys but not the liver of vole B41 and not in the lungs, liver, or kidneys of the 7 other field voles. Degenerate cytochrome B gene PCR and sequencing (*9*) were used to confirm the morphologic identification of the field voles (B41 CytB GenBank accession no. KC222031).

The nucleotide and amino acid sequence divergences between B41 and the most related hantaviruses correspond to that typically found between hantavirus species (*5*). The phylogenetic analyses further support B41 as a distinct hantavirus. Thus, we propose to name this novel virus Tatenale virus, reflecting the medieval name of its place of origin.

M. agrestis voles, among the most numerous mammals in mainland Britain, have not been shown to be primary carriers of a specific hantavirus, although recent studies suggest that they might be involved in the maintenance of Tula virus in Germany (*10*). Further surveillance is needed to confirm that *M. agrestis* voles are the reservoir hosts of Tatenale virus, provide an estimate of virus prevalence, and determine zoonotic risk. Current knowledge of other *Microtus* vole–borne hantaviruses suggests that although they might infect humans, their pathogenic potential is generally low (*1*). Future work will involve attempts to isolate Tatenale virus and generate its full-genome sequence.

Because hantavirus diseases have such broad clinical features, many cases among humans in the United Kingdom might be misdiagnosed. The confirmation of a novel hantavirus in indigenous wildlife in the United Kingdom might promote inclusion of hantavirus infection in the differential diagnosis for patients with acute renal failure, undiagnosed febrile illness, and exposure to rodents (*4*).

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Hand, Foot, and Mouth Disease Outbreak and Coxsackievirus A6, Northern Spain, 2011

To the Editor: Hand, foot, and mouth disease (HFMD) is an acute, febrile viral infection characterized by vesicular exanthema on the palms of the hands, soles of the feet, and oral mucosa. The infection is transmitted through oral and respiratory secretions, vesicular fluid, and/or feces of affected persons. The most common etiologic agents are coxsackievirus (CV) A16 and human enterovirus (HEV) 71, but other HEVs, mainly belonging to species A, have also been associated with illness (*1*). HFMD mainly affects infants and children <5 years of age.

On May 10, 2011, an outbreak of HFMD was reported in a daycare center in the city of Irun in Basque Country, Spain. Monitoring subsequently was conducted for HFMD cases among children in the health district that contained the daycare center (a total of 4,540 children <14 years of age). Children with fever and vesicular rash on the palms and/or soles and in the mouth were considered HFMD patients. Pharyngeal and/or dermal exudate and/or feces were collected for virologic confirmation from 37 representative HFMD patients (17 with multiple specimens) selected by sentinel pediatricians in outpatient clinics. Viral RNA was extracted directly from specimens (NucliSENS Easy-Mag, Bio-Mèrieux, Marcy-l'Étoile, France) and was used in the amplification methods. Enterovirus RNA was detected by an in-house real-time PCR that amplified a fragment within the 5′ untranslated region by using described primers (*2*). For genotyping, the viral

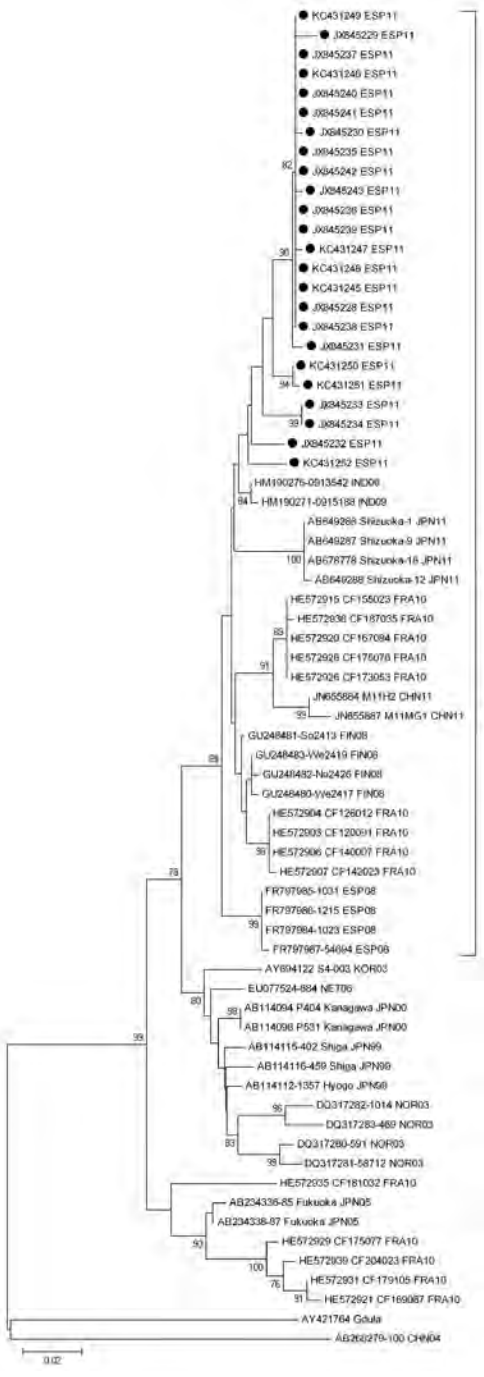

Figure. Phylogenetic analysis of the partial viral protein 1 gene sequence (positions 2929–3348, based on strain Shizuoka-18, GenBank accession no. AB678778) of coxsackievirus A6 isolated from distinct patients with hand, foot, and mouth disease detected in Irun, Spain, April–September 2011, compared with the Gdula prototype strain and other representative strains. Black dots indicate the strains in this study (GenBank accession nos. JX845228–JX845243 and KC431245–431253). The tree was constructed by using the neighbor-joining method with 1,000 bootstrap replications and shows bootstrap values >75%. Genetic distances are based on pairwise analysis by using the Kimura 2-parameter method in MEGA5.1 software (www.megasoftware.net). Bracket indicates strains showing nucleotide identity >94% and detected in outbreaks during 2008–2011. Scale bar indicates the number of substitutions per nucleotide position.

protein 1 gene was amplified by using described methods (*3*), followed by partial sequencing of the obtained amplicons by using the 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Control measures recommended were frequent and careful handwashing with soap and running water by children and staff and increasing the cleaning of surfaces and objects in daycare centers and nursery schools.

During April–September 2011, a total of 99 cases of HFMD were notified; 53 patients were boys. Twenty-five cases occurred in the daycare center, all before May 13 (attack rate 55.6%), and 74 were community acquired, occurring mainly after that date. All cases occurred in children <4 years of age (median age 1.8 years; incidence 77 cases/1,000 inhabitants). The highest incidence occurred in children 12–36 months of age (122.4 cases/1,000 inhabitants). In addition to a papulovesicular rash on the palms, soles, and/or buttocks, 89 (90%) HFMD patients showed a perioral papulovesicular rash that did not extend to the rest of the face. None of the children were hospitalized.

Enterovirus was detected in 49 samples (28 pharyngeal, 2 dermal, 19 fecal) from 33 HFMD patients. For 30 of these patients, the samples were sufficient for genotyping. CVA6 was detected in 27 (90%) patients and CVA10 in 2 (7%) patients; for 1 patient, no genotype was obtained. Seven (7%) of the 99 children with HFMD were brought for medical assistance for onychomadesis during the 9–67 days after the HFMD episode. In 2 of them, HFMD had been virologically confirmed as being caused by CVA6.

Our results suggest that CVA6 can cause HFMD outbreaks that develop rapidly and reach a high incidence in children. Despite the mildness of the disease, the high attack rate in the daycare center alarmed families and staff. HFMD is not subject to epidemiologic surveillance in Spain, and thus its real incidence cannot be identified.

Although CVA6 has long been known to cause HFMD (*1*), it has not usually been considered to play a major role in this disease. Except in a few countries, CVA6 has been infrequently detected until recent years. However, since 2008, this virus has caused major outbreaks of HFMD in some countries of eastern Asia and Europe and, more recently, in the United States (*4*–*9*); the CVA6 strains in this outbreak shared >97% of nucleotide identities in the viral protein 1 gene and showed sequence similarity >94% with the strains that caused these outbreaks. These strains segregated in a phylogenetic tree (Figure), supporting the recent international spread of emerging CVA6 genetic variants (*4*). In Taiwan and Japan, the emergence of these strains has been associated with a change in the predominant clinical expression of the infections produced by CVA6, from herpangina before 2009 to HFMD in 2010–2011 (*7*,*8*). The development of a perioral rash has also been associated to HFMD caused by CVA6 (*10*).

Although the course of HFMD is usually self-limiting, illness and death rates vary among outbreaks. Severe illness is more frequent in outbreaks caused by HEV71 (*1*); in outbreaks caused by CVA6 in Taiwan and the United States, the illness affected a broader spectrum of skin sites and was associated with more severe and extensive rash than was HFMD caused by other coxsackieviruses (*7*,*9*).

In conclusion, reports of HFMD outbreaks associated with CVA6 are increasing. Improved HFMD surveillance is required, with virus genotyping as a key element.

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Rabies Update for Latin America and the Caribbean

To the Editor: Rabies incidence in Latin America and the Caribbean has decreased and several countries (Uruguay, Chile, Costa Rica, Mexico, and Panama) and areas of Peru, Brazil, and Argentina are free of human rabies transmitted by dogs, although there are certain areas to which this disease is still endemic (*1*). Coordinated actions for regional elimination of human rabies transmitted by dogs began in 1983 in Latin America and the Caribbean with the assistance of the Pan American Health Organization (PAHO). This effort has led to an ≈90% reduction of human and canine rabies (*2*). In this region, rabies is associated with poverty and considered a neglected disease (*3*). Resolution 19 of the 49th Directing Council of PAHO in 2009 regarding neglected diseases and other infections related to poverty set a target for eliminating human rabies transmitted by dogs by 2015. PAHO is currently developing strategies to assist countries during this period (*4*).

Since 2010, a total of 111 human rabies cases transmitted by bats, dogs, and other animal species were reported from Latin America and the Caribbean: 40 transmitted by dogs and 63 by bats (Table). Although a major reduction in human rabies transmitted by dogs was observed in 2010 (only 6 cases), the total number of cases increased to 24 in 2011; most were confirmed by laboratory testing.

The higher risk areas for human rabies transmitted by dogs, for which more collaboration and financial support are urgently needed, are Haiti, Bolivia, Guatemala, Dominican Republic, and parts of Brazil (Maranhão State) and Peru (Puno Region). Unfavorable conditions in which persons in these areas are living limit control strategies and maintain rabies transmission (*3*).

According to the PAHO Epidemiologic Surveillance System for Rabies, during 2010–2012, Bolivia and Haiti had the highest incidence of human rabies transmitted by dogs in the Western Hemisphere: 15% (6/40) and 40% (16/40) of all cases, respectively (*5*). Many factors, including national disasters and social, cultural, and economic factors, have interfered with canine rabies control programs in these countries.

Bolivia has a population of 10 million, and 60.0% of the population is considered below the national poverty line. This country has poor suburbs on the outskirts of large cities, with large populations of unowned dogs and limited resources to implement dog mass vaccination campaigns and animal birth control programs. Haiti has a population of >10 million, and 77% of the population is considered below the national poverty line. In 2010, Haiti was devastated by a major earthquake that affected all sectors, including laboratory diagnosis for rabies (*6*). After the earthquake, the country was struck by a cholera epidemic. Financial resources have been diverted to control such priorities and to provide humanitarian aid. Haiti and Bolivia heavily depend on technical cooperation and donations from other governments or institutions, and are a high priority for elimination of human rabies transmitted by dogs (*7*).

Another challenge for Latin America and the Caribbean is development of a common strategy for preventing human rabies transmitted by bats, especially in remote areas in the Amazon region (Peru, Ecuador, and Brazil) and Mexico (*7*), from which 97% of human rabies cases were reported during this period. Since 2000, vampire bats have been the leading cause of human rabies in Latin America and the Caribbean (*8*). Comparison of data for 2010–2012 with data for the previous 3 years shows a 5.2% increase in bat-transmitted human rabies, especially during 2011, which accounted for ≈53% of reports during the past 3 years (*5*).

Bats have been identified as a reservoir for many *Lyssavirus* spp. genotypes, and the geographic distribution of variants has been associated with climate changes and ecologic imbalances. Spread of bats has been facilitated by human-made shelters near human dwellings (*9*).

Although rabies control in Latin America and the Caribbean has been successful, certain approaches currently used, such as mass vaccination campaigns for dogs, postexposure prophylaxis, and epidemiologic surveillance, require improvement in some countries. In addition, allocation of resources is needed to enhance national programs

Table. Cases of human rabies in 10 countries in Latin America and the Caribbean, 2010–2012*

	Rabies transmitted by other animals			Rabies transmitted by bats			Rabies transmitted by dogs			
Country	2010	2011	2012†	2010	2011	2012†	2010	2011	2012†	Total
Bolivia	0	0	1‡	0	0	0	0	5	1	7
Brazil	1§	0	2‡§	1	0	1	1	2	2	10
Colombia	3¶	0	0	1	0	0	0	0	0	4
Ecuador	0	0	0	0	12	0	0	0	0	12
Guatemala	0	0	0	0	0	0	0	3	0	3
Haiti	0	0	0	0	0	0	1	13	2	16
Honduras	0	0	0	0	0	0	0	0	1	1
Mexico	0	1#	0	4	2	0	0	0	0	7
Peru	0	0	0	13	19	10	1	1	2	46
Dominican Republic	0	0	0	0	0	0	3	0	2	5
Total	4	1	3	19	33	11	6	24	10	111

*Data were obtained from the Regional Information System of Epidemiologic Surveillance of Rabies in the Americas/Epidemiologic Information System, Pan American Center for Foot-and-Mouth Disease, Pan American Health Organization–World Health Organization, 2012.
†Data were updated in December 2012.
‡Human rabies transmitted by undetermined animal species (variant hematophagous bat).
§Human rabies transmitted by a marmoset monkey (*Callithrix jacchus*).
¶Human rabies transmitted by a cat (variant nonhematophagous bat).
#Human rabies transmitted by a skunk.

to eliminate human rabies transmitted by dogs.

PAHO is responsible for coordination and technical cooperation of the Rabies Elimination Program and Operation of the Epidemiologic Surveillance System for Rabies. For the past 60 years, the Pan American Center for Foot-and-Mouth Disease/PAHO has accumulated capabilities to develop national programs for zoonoses prevention and control, particularly for rabies elimination in Latin America and the Caribbean.

Strengthening regional, national, and subnational rabies control programs must be a priority. The decision in Latin America and the Caribbean to eliminate dog-transmitted rabies began in 1983 and involved strong political commitment with multinational efforts, as well as support and coordination of other international organizations, nongovernmental organizations, and the private sector. This interinstitutional collaboration is needed to promote prevention and control activities to achieve the elimination of human rabies transmitted by dogs in the Western Hemisphere by 2015.

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Serosurvey of Dogs for Human, Livestock, and Wildlife Pathogens, Uganda

To the Editor: Domestic dogs live in close association with humans and livestock, participating in the transmission of diseases of zoonotic, veterinary, and conservation interest (*1*,*2*). Most households in Uganda have traditionally kept dogs for hunting and for help with herding, security, and guarding livestock. Most dogs receive no prophylactic measures (e.g., vaccinations) and roam freely; this situation exposes them to pathogens from eating garbage, rodents, and stillborn animals and other carcasses and through inhalation during scent communication. Thus, dogs are a reservoir for certain pathogens and a useful sentinel for others (*3*).

In 2011, serum samples were obtained from 116 mixed-breed dogs during a rabies vaccination campaign in and near 3 national parks in southwestern Uganda; the dogs were >4 months of age and were voluntarily brought in by their owners (Figure, Appendix, wwwnc.cdc.gov/EID/article/19/4/12-1143-F1.htm; Table). Two of the parks, Bwindi Impenetrable (BI) and Mgahinga Gorilla (MG), have some of the most biologically diverse tropical forests in eastern Africa and are home to mountain gorillas. The third park, Queen Elizabeth (QE), is home to populations of protected carnivores and ungulates. The parks lie within a densely populated rural landscape; in some areas, the population is as high as 500 persons/km^2.

Of the 116 sampled dogs, 4 had been vaccinated against rabies by the authors in 2010 in QE (not included in rabies results), and 11 (all males) had been castrated by local animal healers before serum samples were obtained. The samples were used to test for seroprevalence rates to rabies virus (RABV), canine distemper virus (CDV), canine parvovirus (CPV), *Leptospira interrogans*, *Leishmania* sp., *Toxoplasma gondii*, and *Neospora caninum* (Table). Seroprevalence rates ranged from 20% to 100% (Table). CPV seroprevalence was higher in BI and QE than in MG ($\chi^2 \geq 12.6$, $p<0.001$); *T. gondii* seroprevalence was higher in BI than in MG (Fisher p = 0.002); and RABV seroprevalence was higher in castrated than noncastrated dogs (50% vs. 10%; Fisher p = 0.005).

For humans, the domestic dog is the main source of exposure to RABV. The possibility that the presence of the rabies titers in the dog serum samples was due to a previous vaccination can

Table 1. Methodology and seroprevalence for selected pathogens in rural dogs in 3 national parks, Uganda, 2011*

Pathogen	Test, cutoff value, and (*ref*) or commercial kit	National park: All 3 parks		Queen Elizabeth†		Bwindi Impenetrable‡		Mgahinga Gorilla§	
		Sample size	Prevalence, % (95% CI)	Sample size	Prevalence, % (95% CI)	Sample size	Prevalence, % (95% CI)	Sample size	Prevalence, % (95% CI)
Rabies virus¶	FAVN, 0.24 IU/mL (*4*)	101	19.8 (12.7–28.6)	23	21.7 (9.0–43.3)	56	19.6 (11.0–32.0)	22	16.7 (5.9–37.2)
CDV	c-ELISA, Ingezim Moquillo IgG#	92	100.0 (95.9–100)	30	100 (88.8–100.0)	39	100 (91.4–100.0)	23	100 (85.4–100.0)
CPV	c-ELISA, Ingezim CPV#	92	65.2 (54.9–74.5)	26	80.8 (61.7–92.1)	43	76.7 (61.7–87.6)	23	26.1 (12.0–47.8)
*Leptospira interrogans***	MAT, 1:200 (*5*)	105	26.7 (19.0–36.1)	27	25.9 (12.4–46.2)	55	29.1 (17.9–42.7)	23	21.7 (9.0–43.3)
Leishmania sp.††	c-ELISA, Ingezim Leishmania#	92	19.6 (12.3–29.2)	26	19.2 (7.9–38.3)	43	25.6 (14.6–40.6)	23	8.7 (1.6–27.8)
Toxoplasma gondii	MAT, 1:25 (*3*)	109	90.8 (83.6–95.1)	30	90.0 (73.7–97.2)	56	98.2 (90.5–99.9)	23	73.9 (52.2–88.0)
Neospora caninum	c-ELISA, 30% (*3*)	109	27.5 (19.6–36.6)	30	26.7 (13.1–45.0)	56	32.1 (21.2–45.5)	23	30.4 (14.5–52.2)

*_ref_, reference; FAVN, fluorescent antibody virus neutralization; CPV, canine parvovirus; c-ELISA, competitive ELISA; CDV, canine distemper virus; MAT, modified agglutination test.
†0°12′ S, 30°0′ E (savannah).
‡1°0′ S, 29°42′ E (tropical forest).
§1°16′ S, 29°40′ E (tropical forest).
¶Four dogs vaccinated against rabies in Queen Elizabeth are not included in these results.
#Manufactured by Ingenasa, Madrid, Spain.
**Fourteen serovars were investigated. Of the dogs seropositive, 71.5% were seropositive to 1 serovar and 28.5% to 2 serovars. Reacting serovars were Icterohaemorragiae (42.8% of positive dogs), Canicola (39.2%), Pyrogenes (21.4%), Tarassovi (10.7%), and Gryppothiposa and Australis (7.2% each).
††Antibodies probably correspond to contact with *Leishmania donovani*.

be ruled out because the only previous recent campaign in the area was conducted by the authors. Antibodies against RABV in apparently healthy dogs have been reported in Africa (*6*), and rabies seems to be not invariably fatal in dogs. Dogs that have recovered from a rabies infection are prone to shed RABV in their saliva for long periods (*7*). Antibodies against RABV were more frequently found in castrated dogs. This finding may be due to an increase in virus-related deaths among noncastrated dogs; such dogs tend to be more aggressive and to roam, so they may come more frequently into contact with pathogenic RABV strains.

Results indicate that both CDV and CPV are actively circulating in the studied dog populations. High CDV seroprevalence rates have been reported among other rural dog populations in Africa (*8*). Sick, debilitated pups are at high risk for predation by wild carnivores, so spillover may take place. A dog population exhibiting similar characteristics to the population we studied was believed to be the origin of the 1994 CDV epidemic among Serengeti wildlife (*8*). Furthermore, carnivores use feces for scent communication, so the probability of infection by CPV in wild carnivores in the study area may also be high.

In developing countries, leptospirosis is emerging as a major public health problem and also causes enormous economic losses because of disease in livestock (*9*). The most commonly detected serovars in this study were those that have rats and dogs as reservoirs (Table). Visceral leishmaniasis in humans is also a major health problem in several areas of eastern Africa, where the number of cases has dramatically increased during the past 20 years. Transmission of *Leishmania donovani* in eastern Africa may take place through anthroponotic or zoonotic cycles, although, to our knowledge, no reservoir host had been identified (*10*).

The mean *T. gondii* seroprevalence detected during this survey appears to be the highest reported for dogs worldwide. This protozoon has implications for human and animal health, and dogs, who probably become infected with *T. gondii* when eating raw meat, are a good sentinel for environmental contamination by this parasite. On the other hand, dogs serve as the definitive host for *N. caninum*, which is a major cause of abortions in cattle and causes economic losses wherever it is enzootic.

Some of these diseases may also have implications for the conservation of endangered mountain gorillas. Diseases such as leptospirosis, toxoplasmosis, and especially, rabies could be fatal for gorillas, and there are unpublished reports of fights between hunting dogs and gorillas.

Our work should serve as a first step toward the establishment of preventive strategies for improvements in the health of humans and domestic animals living in rural Uganda and for the health of the country's unique wildlife. Tracing the role of dogs in the cycle of the studied pathogens is crucial for the design of control programs.

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Iatrogenic Creutzfeldt-Jakob Disease from Commercial Cadaveric Human Growth Hormone

To the Editor: Iatrogenic Creutzfeldt-Jakob disease (iCJD) is an acquired form of prion disease that has been declining in incidence since the mid-1990s (*1*). Worldwide, at least 226 cases of iCJD, including 29 US cases, have been associated with administration of contaminated human growth hormone (hGH) from cadavers. Reported incubation periods ranged from 5 to 42 years (mean 17 years) (*2*). Commercially produced cadaveric hGH has been associated with only 1 previously reported case of iCJD: CJD developed in a 39-year-old Austrian man ≈22 years after he received commercial cadaveric hGH (Crescormon, Kabivitrum, Stockholm, Sweden) during 1984–1985 (*3*). We report a second case of probable iCJD acquired through treatment with commercial cadaveric hGH.

The patient was born at 32 weeks' gestation with subsequent developmental delay, agenesis of the corpus callosum, and panhypopituitarism. He demonstrated clinical and laboratory signs of growth hormone deficiency but was denied treatment with hGH through the US government–supported National Hormone and Pituitary Program (NHPP) because he did not meet the height requirement. Treatment with commercial cadaveric hGH began when he was 5.8 years of age and continued for 23 months (1983–1985). He received 1.5 units intramuscularly 3× per week and was primarily treated with Asellacrin (Ares-Serono, Geneva, Switzerland). In early 1984, for an unspecified duration, he received Crescormon (Kabivitrum) because of an Asellacrin shortage. Treatment was halted in 1985 because of iCJD concerns and resumed 2 years later with recombinant hGH.

At age 33, 26.5 years (range 25.5–28 years) after the midpoint of commercial cadaveric hGH treatment, dizziness and gait imbalance developed, causing a fall. The patient's mental status also began declining, and he never returned to his baseline status. Six months after illness onset, he experienced hallucinations, weakness of lower extremities, and limb ataxia. Seven months after the fall, he entered a state of akinetic mutism; he died 9 months after symptom onset. A lumbar puncture, performed 8 months after illness onset, demonstrated 14-3-3 proteins and an elevated cerebrospinal fluid (CSF) tau level of 14,111 pg/mL (decision point 1,150 pg/mL) (*4*), although the specimen was contaminated with blood (39,375 erythrocytes/μL). Electroencephalogram demonstrated severe diffuse encephalopathy. Two brain magnetic resonance imaging studies performed 8 months after illness onset indicated probable CJD, given lack of prior metabolic and anoxic insults (Figure). The patient was discharged from a referral hospital with this diagnosis; no postmortem analysis was conducted.

On the basis of World Health Organization criteria, we conclude that this patient had probable iCJD as a result of hGH treatment (*5*). The patient's condition was treated with 2 different formulations of commercial cadaveric hGH, including one of the same brands in the same year as that of the first reported patient with iCJD associated with commercial cadaveric hGH (*3*). The patient's incubation period (25.5–28 years) is well within expectations (*1*).

Despite an ongoing active surveillance program that identified ≈3,500 of ≈4,500 post-1977 cadaveric hGH recipients in the US NHPP, all 29 CJD infections in NHPP recipients occurred among the estimated ≈2,700 pre-1977 recipients (*1*,*2*). This significant reduction in iCJD was attributed to the 1977 introduction of a highly selective, column chromatography step in the hormone purification protocol that can markedly reduce prion infectivity (*1*,*2*). As shown by the many iCJD cases linked to hGH in France, the efficacy of column chromatography purification steps may vary (*1*). Commercially derived cadaveric hGH was produced in different laboratories from those that produced NHPP-distributed hGH, and sufficient details regarding sourcing and production methods of

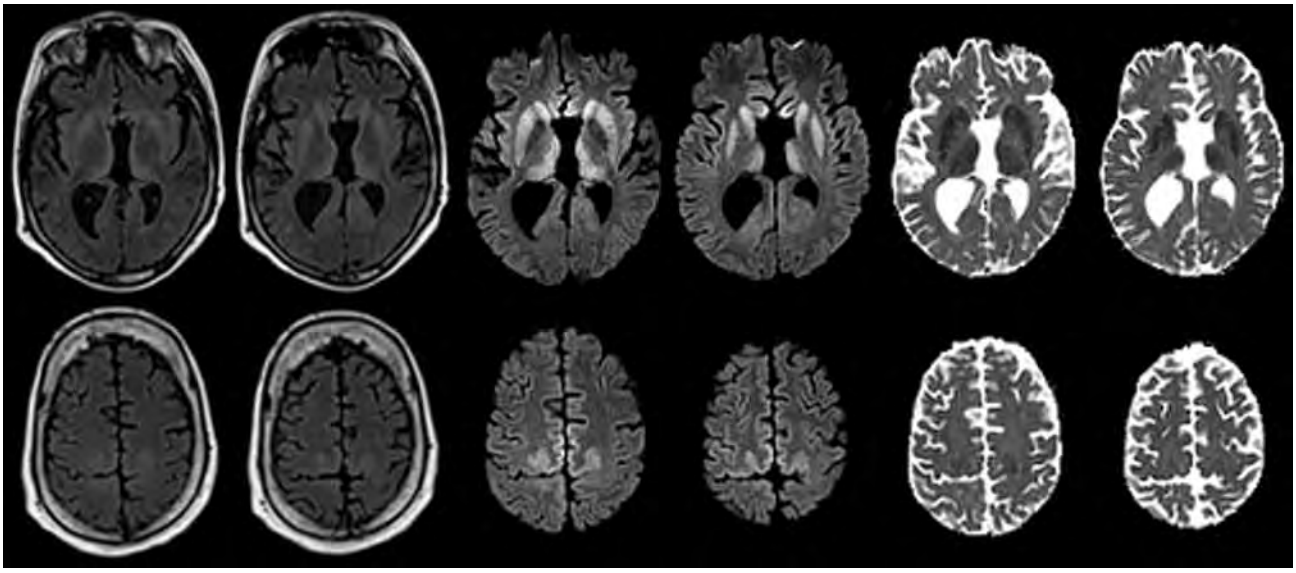

Figure. Maps showing axial fluid attenuated inversion recovery (FLAIR), diffusion-weighted imaging (DWI), and apparent diffusion coefficient (ADC) at the level of the basal nuclei (top row) and dorsal frontoparietal cortex (bottom row) of the brain of a 33.8-year-old man with agenesis of the corpus callosum, schizencephaly, and heterotopia. Note the symmetrical DWI signal hyperintensities in the striatum and dorsomedial part of the thalami. In addition, DWI signal hyperintensities occurred in the cingulate, precuneus and in the dysplastic gray matter along the anterior lips of the schizencephalic clefts at the level of the precentral gyri. The signal abnormalities are associated with decreased diffusivity on ADC maps and are much less prominent on FLAIR images. These findings are highly suggestive of Creutzfeldt-Jakob disease.

the commercial products are lacking. Approximately 10,000 persons, mostly outside the United States, received commercial cadaveric hGH produced by Kabivitrum, and substantially fewer persons received product from Ares-Serono (A.F. Parlow, pers. comm.). Identification through passive surveillance of 2 CJD cases among recipients of such hGH further supports a causal, rather than chance, association between commercial hormone and CJD. It also suggests a difference in iCJD risk between post-1977 NHPP-distributed hGH and commercial cadaveric hGH.

Limitations of this report include the lack of neuropathologic confirmation and insufficient information to strongly implicate a single commercial cadaveric hGH product as infection source. The report of another iCJD case-patient who received Crescormon during the same period provides some evidence that the product was the source of prion contamination. Although the patient may have had sporadic CJD, his young age at disease onset (33 years) makes this unlikely (*6*).

This report suggests that a potential risk for iCJD in persons who received commercial cadaveric hGH should be considered. Also, clinicians should not assume that all cadaveric hGH administered after 1977 carries the same risk for infectivity. In addition, when CJD is being considered as a clinical diagnosis, a history of exposure to cadaveric hGH should always be sought, even when patients have normal or tall stature. Finally, we recommend that when a clinical diagnosis of CJD is suspected, but before the patient's death, the local caregivers, with the family, should initiate arrangements for a postmortem examination to confirm diagnosis (e.g., www.cjdsurveillance.com).

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West Nile Virus Infection in Belgian Traveler Returning from Greece

To the Editor: West Nile virus (WNV) is an arthropod-borne virus that is transmitted to humans by mosquitos, primarily of the genus *Culex*. Most human infections are asymptomatic. Clinical symptoms occur in ≈20% of case-patients and include fever, headache, and myalgia; <1% of WNV infections develop into severe neuroinvasive disease (*1*).

The virus was discovered in 1937 in the West Nile district of Uganda. WNV is endemic to parts of Africa, Europe, Asia, and the Middle East, and since its introduction in New York in 1999, in North America. In Eurasia, human WNV infections were first reported in Israel and France during the 1950s–1960s, and the first major outbreak in Romania occurred in 1996 (*1*). The disease emerged recently in Greece; a large outbreak in 2010 caused neuroinvasive disease in 197 patients, of whom 33 died (*2*). Since 2010, occasional and local epidemics have been ongoing in Greece, Italy, Romania, Hungary, Spain, and the Balkans (*3*,*4*).

Clinical diagnosis may be difficult because WNV infections resemble other (arbo)viral diseases. Laboratory diagnosis relies primarily on serologic testing. Reverse transcription PCR (RT-PCR) can be used to detect viral RNA during the acute phase of the disease, but its use is hampered by the patient's low-level and transient viremia (*1*).

We here describe a confirmed case of WNV encephalitis imported by a traveler returning from Greece. A 73-year-old Belgian woman, who had a medical history of lymphoma, traveled to Kavala city (Macedonia, Greece). On August 14, 2012, she sought treatment at the Kavala General Hospital with a 6-day history of fever, headache, malaise, nausea, confusion, decline of consciousness, and neck stiffness. Results of laboratory testing on admission demonstrated an increased leukocyte count (9,670/μL; 80% neutrophils) and lactate dehydrogenase level (522 IU/L), a low C-reactive protein level (0.7 mg/dL), and hyponatremia (131 mEq/L). Cerebrospinal fluid (CSF) testing showed 90 cells/μL (79% lymphocytes) and glucose and protein levels of 72 and 100.9 mg/dL, respectively. Serum obtained on August 15 was sent to the national reference laboratory at Aristotle University (Thessaloniki, Greece), and IgM against WNV was detected by ELISA (WNV IgM Capture DxSelect and IgG DxSelect; Focus Diagnostics, Cypress, CA, USA). IgG was absent. On the second day of hospitalization, the patient exhibited seizures (speech arrest); she was given phenytoin (1/2 amp 3×/day intravenously). On August 18, the patient was transferred to a private hospital. Further treatment included intravenous fluid, antipyretics, antimicrobial drugs, mannitol, and oxygen. On August 30, she was returned by plane to Belgium.

CSF obtained 26 days after symptom onset and serum obtained 29 days after symptom onset were sent to the Institute of Tropical Medicine (Antwerp, Belgium) because of its function as a national reference center for Belgium. IgM and IgG against WNV were detected in both samples by ELISA (Focus Diagnostics) (Table). Immunofluorescence assays on serum revealed IgM against WNV only and IgG against West Nile, dengue, yellow fever, and Japanese encephalitis viruses, with the strongest reaction against WNV (Flavivirus Mosaic 1; Euroimmun, Lübeck, Germany). Real-time RT-PCR (adapted from [*5*]) on the serum demonstrated a weak positive signal. Repeated RNA extraction and

Table. Laboratory results confirming WNV infection of 73-year-old woman, Greece, 2012*†

Sample	Date	RT-PCR (C_t value)	WNV ELISA IgM (ratio)	WNV ELISA IgG (ratio)	Flavi IFAT IgM	Flavi IFAT IgG
Serum	Aug 15	Positive (45.47)	Positive (25)	Negative	ND	ND
CSF	Sep 3	ND	Positive (5.16)	Positive (2.21)	ND	ND
Serum	Sep 6	Positive (42.87)‡	Positive (4.76)	Positive (2.63)	WNV positive	WNV positive§

*WNV, West Nile virus; RT-PCR, reverse transcription PCR; Ct, cycle threshold; Flavi, flavivirus; IFAT, indirect fluorescent antibody technique; ND, not done; CSF, cerebrospinal fluid.
†The ELISA is positive if ratio >1.1 for IgM and >1.5 for IgG. The cutoff value for IFAT is 1/10 for both IgG and IgM.
‡Sequencing revealed a 116-bp sequence perfectly matched to the WNV amplicon and is highly suggestive for WNV lineage 2 on the basis of the presence of 2 specific nucleotides.
§Strongest signal for WNV, weak signal for other flaviviruses (Japanese encephalitis virus, dengue viruses 1–4, yellow fever virus).

RT-PCR were confirmative (Table). Sequencing of the RT-PCR product confirmed the detection of WNV. Although the product was short (116 bp), it was highly suggestive of WNV, lineage 2. Flemish regional authority in Belgium, national authorities (both in Belgium and Greece), and European health authorities were notified of the imported case of WNV encephalitis. According to the case definition of the European Center for Disease Prevention and Control, Stockholm, Sweden, the patient met the laboratory criteria of having a confirmed case.

To date, autochthonous WNV infections have not been reported in Belgium, although the presence of the mosquito vector provides a potential risk for transmission (*6*). This WNV infection was acquired in Greece (a leading travel destination for tourists from Belgium), specifically in the Kavala region, which was highly affected by WNV in 2012. The lineage responsible for the WNV encephalitis was identified as lineage 2, the currently circulating strain in Greece (*7*). Our report highlights the need for physicians and laboratory staff to be aware of imported WNV infections originating from southeastern Europe, especially Greece and its neighboring countries, where recent and recurrent outbreaks have occurred (*3*,*4*).

Special attention should be given to immunosuppressed and elderly patients who are at higher risk of acquiring neuroinvasive disease. The 73-year-old patient described here was unconscious when she arrived in Belgium. After a short period of relative improvement (more reactive and cooperative), her condition deteriorated, and she died on November 23, 2012. The detection of viral RNA 29 days after symptom onset was surprising but might be explained by the immunocompromised status of the patient. Several studies have reported persistent WNV RNA for 30 days, 77 days, and even years after the symptom onset in serum, CSF, and urine, respectively (*8–10*), and a prolonged period of viremia in immunocompromised patients (*9*).

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Powassan Virus Encephalitis, Minnesota, USA

To the Editor: Birge and Sonnesyn report the first death of a Minnesota resident caused by Powassan virus (POWV) (*1*). However, they provide an inaccurate description of several critical diagnostic and surveillance issues concerning POWV.

The 17 POWV infections detected in Minnesota residents from 2008 through 2011 (6 cases were identified through 2010, not 8 as reported by Birge and Sonnesyn) (Minnesota Department of Health [MDH], unpub. data) were found through enhanced surveillance. Health alerts to Minnesota medical providers described POWV as a possible etiologic agent for viral meningitis and encephalitis. Providers consulted with MDH on suspected cases and submitted serum and cerebrospinal fluid specimens to MDH. MDH conducted serologic testing for endemic arboviruses (including POWV) and reverse transcription PCR (RT-PCR) for flaviviruses and POWV. MDH would not have detected any POWV infections without enhanced surveillance. Limited field studies also identified POWV-infected ticks in 4 Minnesota counties (not 2 as reported [*1*]) (MDH, unpub. data).

Commercial laboratories do not provide testing for POWV, and only a few state health department laboratories and the Centers for Disease Control and Prevention offer testing. Serologic testing (enzyme immunoassay with plaque-reduction neutralization testing confirmation) is preferred (*2*) because POWV RT-PCRs are not validated, and the short viremic periods of flaviviruses limit their usefulness (*3*).

Few POWV infections are identified by lineage (prototype vs. deer tick virus); Minnesota's first case in 2008 was identified as a deer tick virus infection, but the lineage was unknown for the other 16 cases. However, many case-patients had likely exposure to *Ixodes scapularis* ticks (blacklegged ticks), the tick species associated with deer tick virus transmission, and viruses from all POWV-positive tick pools were confirmed as deer tick virus by sequencing. The distribution of the 2 lineages in North America is poorly understood, and most cases likely go undetected without specific POWV surveillance efforts.

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Hepatitis E Virus and Porcine-derived Heparin

To the Editor: Cases of sporadic, locally acquired hepatitis E have been increasingly identified in industrialized countries over the last few years (*1*). In this setting, hepatitis E is thought to be a zoonotic infection, with pigs as the primary host. Consumption of uncooked or lightly cooked pork meat products is thought to be a key route of infection, but other routes of transmission have been documented (*2*). For example, there have been several iatrogenic cases after transfusion of hepatitis E virus (HEV)–contaminated blood products (*3*) and transplantation of an HEV-infected donor liver (*4*). However, in most cases the source and route of infection are uncertain.

In May 2011, a 42-year-old woman sought care at the Royal Cornwall Hospital in Truro, United Kingdom, for a 1-week history of malaise, diarrhea, nausea, and vomiting. Physical examination results were normal. Her liver function test results, however, indicated hepatitis: alanine aminotransferase 2,785 IU/L (reference range 10–36 IU/L), alkaline phosphatase 319 IU/L (reference range 30–130 IU/L), and bilirubin 30 μmol/L (reference range <21 μmol/L). HEV IgM and IgG serologic test results for the patient were positive, and HEV genotype 3 was identified in her blood by reverse transcription PCR and sequencing. Other causes of viral hepatitis and hepatocellular jaundice, including hepatitis viruses A, B, and C; Epstein-Barr virus; and autoimmune hepatitis, were excluded by testing. As with most immunocompetent persons with HEV, the patient made an uneventful clinical recovery after 12 weeks, and her liver function tests returned to normal after 8 weeks.

Table. Heparin samples tested for hepatitis E virus, porcine circovirus 2, and porcine parvovirus*

Producer, proprietary name/other names, batch or lot no.	Use	Excipient	Concentration	Quantity tested, IU	95% upper CL, /IU†
Sanofi‡					
Clexhane/enoxaparin	Injection	H_2O			
ILA01			20 mg/0.2 mL	6,000	0.0006
34751			40 mg/0.4 mL	4,000	0.0009
OLC56			80 mg/0.8 mL	8,000	0.0005
ILA53			60 mg/0.6 mL	6,000	0.0006
OLC07			100 mg/ mL	10,000	0.0004
12255			120 mg/0.8 mL	12,000	0.0003
Pfizer§					
Fragmin/dalteparin sodium	Injection	H2O pH adjusted with HCl or NaOH			
12339A01			5,000 IU/0.2 mL	15,000	0.0002
12338A01			5,000 IU/0.2 mL	15,000	0.0002
12327B01			5,000 IU/0.2 mL	15,000	0.0002
12257A01			5,000 IU/0.2 mL	15,000	0.0002
12444A01			5,000 IU/0.2 mL	10,000	0.0004
12122C01			7,500 IU/0.3 mL	7,500	0.0005
74774D51			10,000 IU/0.4 mL	10,000	0.0004
74871B51			12,500 IU/0.5 mL	25,000	0.0001
74779G51			12,500 IU/0.5 mL	12,500	0.0003
74871B51			12,500 IU/0.5 mL	12,500	0.0003
74743C52			15,000 IU/0.6 mL	30,000	0.0001
74755A51			15,000 IU/0.6 mL	30,000	0.0001
74832A52			15,000 IU/0.6 mL	15,000	0.0002
74832A01			15,000 IU/0.6 mL	15,000	0.0002
X08580	¶	¶	100,000 IU/4 mL	100,000	0.00004
Wockhart#					
Monoparin	Injection	H2O pH adjusted with HCl or NaOH			
PK40319			1,000 IU/mL	20,000	0.0002
3090			1,000 IU/mL	10,000	0.0004
Hepsal	Flushing	NaCl, H2O, HCl, and NaOH			
5000090			10 IU/mL	120	0.03
91180			50 IU/mL	50	0.07
1069			200 IU/mL	200	0.02
Leo**					
Heparin sodium	Intravenous flushing	Benzyl alcohol, methyl parahydroxybenzoate, propyl parahydroxybenzoate, sodium citrate, NaCl, and H_2O			
DD7314			100 IU/mL	200	0.02
CC4338			100 IU/mL	200	0.02
Celgene††,					
Refludan/Lepirudin, 25561611A‡‡	Powder used for solution for injection/infusion	Mannitol, NaOH, and H_2O	12.5 mg/mL	NA	NA
Total quantity tested	NA	NA	NA	404,270	0.000009

*NA, not applicable.
†The 95% upper confidence limit of the probability of a virus-positive result per IU was calculated on the basis of the quantity tested for each batch. This was estimated, assuming perfect detection of a Poisson process, by using Fisher exact test. For the pooled result, the upper 95% estimate is ≈1 per 100,000 IU.
‡Sanofi (Guilford, UK).
§Pfizer (Sandwich, UK).
¶Multidose vials used for injection, Excipients: Benzyl alcohol and H_2O.
#Wockhart (Wrexham, UK).
**Leo (Buckinghamshire, UK).
††Celgene (Uxbridge, UK).
‡‡Non-porcine–derived anticoagulant alternative.

The source and route of infection in this case was uncertain. A detailed in-person assessment of potential risk factors was undertaken with the patient. She had not traveled outside the United Kingdom in the previous 3 months. She rarely ate pork products (well cooked bacon only); ate no shellfish; and had no workplace, domestic, or recreational exposure to pigs or their effluent. However, 4 weeks before symptom onset, the patient had

acute appendicitis for which she underwent an uneventful laparoscopic appendectomy and was hospitalized for 2 days. During hospitalization she received no blood products, but, as prophylaxis for thromboembolic disease, she received 2 doses (5,000 IU each) of low–molecular weight heparin (Fragmin [dalteparin sodium]; Pfizer, Sandwich, UK) by subcutaneous injection. All heparins used in Europe and North America are isolated from porcine intestinal mucosa (*5*). The exact purification methods used by heparin manufacturers are deemed commercially sensitive and not in the public domain, so it is impossible to evaluate whether the isolation process would be sufficient to remove or inactivate any contaminating HEV. The virus is known to be acid and alkaline stable; heat sensitivity varies, depending on strain and heating conditions, although heating at 60°C for 1 hour is generally sufficient to achieve 96% inactivation (*6*). To our knowledge, no investigation has determined whether clinical-grade heparin could contain viral contaminants. Thus, we hypothesized that the heparin the patient received might have been the source of her HEV infection.

To examine this possibility, we screened multiple batches of hospital pharmacy–grade heparin for the presence of HEV, including batches of dalteparin sodium that were in use at the hospital when the patient received treatment for appendicitis. Before testing, the samples were ultracentrifuged to concentrate any contaminating virus and enable the removal of excipients, which could inhibit the assay. We tested samples by quantitative reverse transcription PCR (*7*) in parallel with positive World Health Organization HEV RNA standard spiked controls, which showed the limit of detection (LOD) to be 500 IU/mL, regardless of the heparin's excipient or concentration. This LOD is within the range used by collaborating laboratories in the establishment of the World Health Organization HEV RNA standard (http://whqlibdoc.who.int/hq/2011/WHO_BS_2011.2175_eng.pdf). In addition, we tested the heparin samples for porcine circovirus 2 (PCV2), an identified adventitious agent of several rotavirus vaccines (*8*) and porcine parvovirus (PPV) (*9*), a known contaminate of porcine clotting factor hyate:C (*10*). Although samples were tested in parallel with PCV2- and PPV- positive spiked controls, we were unable to calculate the LOD for these assays because international standards are not available for these viruses.

All samples tested negative for HEV, PCV2, and PPV (Table), which would indicate the patient's source of HEV infection is unlikely to have been the heparin. However, we cannot rule out low-level viral contamination below the sensitivity of the assay. We also cannot exclude that the negative test results were related to the Poisson effect. Given that all samples analyzed were negative for all 3 viruses tested, it seems likely that the heparin manufacturing process is sufficient to remove viral contaminants. However, this may not necessarily be the case for other porcine-derived products, such as porcine insulin, factor VIII C, pancreatin, and poractant alfa. Further investigation is warranted to exclude these products as potential sources of HEV infection.

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Human Enterovirus Genotype C104, China

To the Editor: Human enteroviruses (EVs) are small, nonenveloped RNA viruses belonging to the family *Picornaviridae*. Approximately 100 EV genotypes have been identified. Recently, EV68 epidemics in respiratory tract infections (RTIs) have been reported worldwide (*1*,*2*). Moreover, rarely detected EVs (e.g., EV-C104 and EV-C109) have been increasingly identified in patients with RTIs (*3*–*7*), indicating a possible association of EVs with respiratory syndromes.

Little is known about the role of EV-C104 in RTIs. EV-C104 has been reported in 3 countries: Switzerland (7 children with pneumonia or otitis media) (*3*), Italy (3 adults and 4 children with RTIs) (*4*,*7*), and Japan (1 adult with an upper RTI [URTI]) (*5*). We report additional EV-C104 strains in 4 children with lower RTIs and in 1 adult with a URTI in China.

To identify EV infections, we collected nasopharyngeal aspirates from 3,108 children (1,963 boys) ≤14 years of age (median age 12 months; age range 0.3–168 months) who had lower RTIs at admission to Beijing Children's Hospital during March 2007–February 2012. Throat and nasal swab specimens were also collected from 9,232 adults (4,140 men) ≥15 years of age (median age 35.3 years; age range 15–97 years) with acute RTIs who received treatment at Peking Union Medical College Hospital during August 2006–February 2012. All samples were screened for influenza virus, parainfluenza virus type 1–4, respiratory syncytial virus (RSV), coronaviruses (229E, NL63, HKU1, and OC43), metapneumovirus, adenovirus, rhinovirus, bocavirus, and EVs (*8*). Overall, 37 (1.2%) children and 158 (1.7%) adults were positive for EV.

Because we could not amplify EV-C104 by using primers specific for the viral protein (VP) 1 region (*9*), we used a reverse transcription PCR to amplify the 5′ -untranslated region/VP4/VP2 gene (*10*). Amplicons of ≈600 bp were obtained for samples from 5 patients: 4 boys 2–11 months of age (BCH2859A, BCH2892A, BCH2894A, and BCH3034A) and a 30-year-old man (PUMCH12286). BLAST analysis (www.ncbi.nlm.nih.gov) of sequences of these amplicons showed that the 590-nt sequences had 94.0% identity with that of the EV-C104 prototype strain CL-12310945

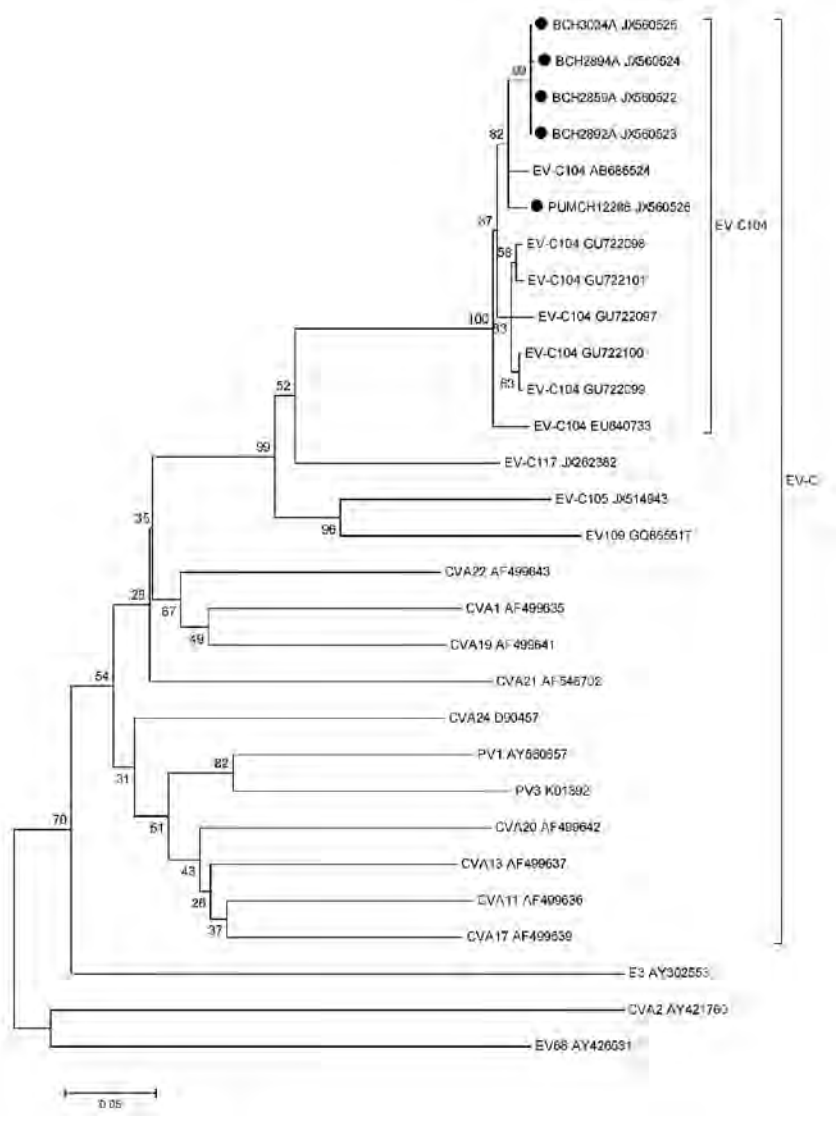

Figure. Phylogenetic tree of human enteroviruses (EVs) for nucleotide sequences of the viral protein (VP) 4/VP2 gene region (435 nt, corresponding to nt positions 654–1,088 of EV-C104 prototype strain CL-12310945 [EU840733]), People's Republic of China, March 2007–February 2012. The tree was generated with 1,000 bootstrap replicates. Neighbor-joining analysis of targeted nucleotide sequence was performed by using the Kimura 2-parameter model with the Molecular Evolutionary Genetics Analysis software version 4.0 (www.megasoftware.net/). Each strain detected in this study is indicated by a black circle and a specific identification code (BCH/PUMCH), followed by the patient number. Enterovirus 68, cocksackievirus (CV) A2, and echovirus (E) 3 (GenBank accession nos. AY426531, AY421760, and AY302553) were used as outgroups. PV, poliovirus. EV-C denotes the EV species to which EV-C104 belongs. Scale bar indicates evolutionary distance.

(EU840733). The amplicon contained 155 nt in the 5′-untranslated region, 207 nt in VP4, and 228 nt in VP2. Phylogenetic analysis of VP4/VP2 sequences showed that the 5 sequences obtained in this study (GenBank accession nos. JX560522–JX560526) belonged to genotype EV-C104 within the EV-C species (Figure).

Virus isolation for EV-C104 with Vero and H1-HeLa cells was unsuccessful. Although we screened 591, 797, 459, 664 and 597 samples from children for 5 consecutive years and 1,765, 1,978, 1,350, 1,562, 1,573, and 1,004 samples from adults for 6 consecutive years, we did not detect EV-C104 strains until November 2011–February 2012.

Nucleotide identity of the EV-C104 sequences from this study was 99.5%–100% among BCH strains and 97.7%–98.0% between the PUMCH strain and the BCH strains. Deduced amino acid sequences in VP4/VP2 among the BCH strains were identical, albeit for 1 aa difference for the PUMCH strain (BCH strains had Pro^{110}, but the PUMCH strain had Leu^{110}, which was consistent with strains detected in children and adults in Italy). Deduced amino acid sequences for all 5 strains isolated in this study had 97.9%–100.0% identity with those from Switzerland, Italy, and Japan. BCH strains were community acquired because these 4 patients came from different cities and were admitted to different wards on different dates.

The 4 EV-C104–positive boys all had fever and cough for >10 days before their hospitalization. Chest radiographs showed increased lung markings or patchy shadows diagnosed as pneumonia or bronchopneumonia. RSV or adenovirus was also detected in 3 of the boys. The fourth boy was positive for parainfluenza virus type 1, adenovirus, and bocavirus. Clinical outcomes for all 4 children were favorable. The EV-C104–positive man had fever, chills, pantalgia, and expectoration for 1 day before a URTI was diagnosed. EV-C104 was the only virus detected in this patient.

We compared relative viral loads for all viruses in the 5 patients and quantified viral load of EV-C104 and other viruses by using real-time PCR (methods available upon request). Median viral load in the 5 patients was 2.4×10^6 RNA copies/mL (range 5.6×10^4–7.0×10^6 copies RNA/mL (Table, Appendix, wwwnc.cdc.gov/EID/article/19/4/12-1435-T1.htm).

Overall, we found few (5/12,340) EV-C104–positive specimens. All EV-C104–positive children were co-infected with RSV or adenoviruses (high viral loads) in our study. The role of EV-C104 in RTIs needs to be further studied. Nevertheless, the finding of EV-C104–positive adults with high viral loads in China (3.9×10^6 RNA copies/mL) and Italy (2.0×10^6 RNA copies/mL) (*7*) indicates a possible association between EV-C104 with RTIs. Our data also confirm a wide distribution of EV-C104.

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Monkey Bites among US Military Members, Afghanistan, 2011

To the Editor: We take serious issue with the dispatch by Mease and Baker on monkey bites among US military members in Afghanistan during 2011 (*1*). In particular, we are troubled by the first paragraph. The dispatch opens by listing bites from rhesus macaques (*Macaca mulatta*) as one of the many risks faced by military personnel deployed to Afghanistan. Although technically a true statement, it is misleading in its perspective. Since 2001, ≈2,000 US soldiers have died in Afghanistan and another ≈18,000 have been wounded in action (*2*). The authors juxtapose this toll with minor injuries incurred by 10 soldiers who flouted explicit rules prohibiting contact with pet monkeys.

None of the bitten soldiers were reported to have sequelae. Furthermore, the first paragraph leaves the impression that a US Army soldier who died of rabies while serving in eastern Afghanistan may have contracted the disease from a macaque. This finding would be an extremely unlikely occurrence.

We have yet to see a single credible report of macaque-to-human transmission of rabies. In fact, we have yet to see a report of naturally acquired rabies infection in a macaque. Similarly, although antiviral prophylaxis is routinely prescribed to persons bitten by rhesus monkeys, there is not a single report of herpes B virus infection in a human outside the laboratory/zoo context, although thousands of persons are likely bitten by macaques in Asia every year (*3*,*4*).

In contrast, zoonotic transmission of simian foamy virus, a retrovirus ubiquitous in nonhuman primates, has been shown to occur from macaques to humans, probably through monkey bites, although this virus has not been shown to cause disease in humans (*5*). Although it is advisable to avoid contact with monkeys, risk for disease transmission should be placed in proper perspective. Exaggerating risks of bites has, in the past, led to irrational culling of entire populations of macaques (*6*).

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In Response: In response to the letter by Engel et al. (*1*), we concur that combat-related deaths and illness are a greater risk than monkey bites for deployed military personnel. Furthermore, we agree that risk for monkey bites should be considered in perspective with other risks faced by deployed personnel. We also believe that action taken to decrease macaque populations in response to risks mentioned would be irrational and inappropriate; in a country affected by war, wildlife conservation efforts are needed. We did not intend to imply that the rabies-associated death mentioned in our article was caused by contact with a macaque (*2*). As reported elsewhere, the patient likely contracted rabies from a dog bite (*3*).

Nonetheless, we believe that risk for monkey bites deserves the attention of deployed medical providers. Risks for bacterial infection and major local trauma are critical for any macaque bite. We acknowledge that risk for contracting viral disease (rabies or B virus infection) from macaques in

the wild is probably low, but we believe that it merits consideration.

Social conditions in Afghanistan have prevented any substantial rabies prevention program. Consequently, prevalence of rabies among wild animals and pets is unknown and could be higher than in other countries. Absence of any documented human deaths from B virus in a country with a developing medical system and a high mortality rate does not confirm absence of risk. B virus has been shown to be fatal in other areas, particularly in countries with greater medical diagnostic capacity (*4*).

Engel et al. suggest that recorded monkey bites occurred because affected persons flouted rules prohibiting contact with local animals. Given the unpredictable nature of operations in Afghanistan, it is impossible to determine fault for the animal bites detailed. Furthermore, blaming bite victims may be counterproductive, exacerbating underreporting and discouraging deployed personnel from seeking needed care. We believe that the role of command support and responsibility cannot be overemphasized in preventing deployed personnel from interacting with local animals (*5*). We thank Engel et al. for providing additional perspective on the risk for monkey bites to personnel deployed in Afghanistan.

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etymologia

Syncytium [sin-sish´e-əm]

From the Greek *syn* (together) and *kytos* (receptacle, vessel), a multinucleate mass of protoplasm produced by the merging of cells. Respiratory syncytial virus was discovered in 1956 by Morris et al., who isolated it from a group of chimpanzees with respiratory symptoms. Morris originally called the new agent "chimpanzee coryza agent," although when Chanock et al. confirmed that the agent caused respiratory illness in humans, it was renamed because "the striking characteristic of these viruses is the production of syncytial areas in tissue culture."

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The Foundations of Virology: Discoverers and Discoveries, Inventors and Inventions, Developers and Technologies

Frederick A. Murphy

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Pages: 536; Price US $119.95

History of science is not a priority of most virologists. However, if given a visually engaging coffee-table book of the persons and discoveries that shaped the field of virology since Hippocrates, curious scientists will inevitably start paging through history. For this reason, every chairperson of virology departments worldwide should leave a copy of Fred Murphy's *The Foundations of Virology* in the break room.

Professor Murphy has a long and distinguished career in emerging viruses from the perspective of public health and academic bench science. Murphy has made his 536-page book available as a downloadable, lower-resolution, eBook on his website, which for years has also been an enormous open-access library resource for virologists. Because of numerous requests to have hard copies printed for fingertip access, Murphy has made this book available in paperback. There are clear advantages to having the hard copy in hand, in a place where it can be picked up and absorbed a little at a time.

The full title of the book reflects the content; it is a chronology of images of discoverers, developers, and inventors, alongside their discoveries, developments, and inventions. The book is physically laid out in a landscape format, usually with multiple photographs on each page, featuring scientists, their institutions, spot maps from major epidemics, electron micrographs of then-emerging viruses, and graphics from landmark publications. Along the bottom of each page is printed in large font the year, names of contributors, and their historic contributions. This bottom line layout serves as an effective design enabling quick orientation to time, with the option for most entries of reading a more in-depth explanation. It also makes it easy to skip to different points in time, an efficiency advantage over Murphy's online resources. Other books on the history of virology are a dense read (and lack all the fun pictures).

Murphy's selection of images highlights his conviction that science of viral diseases predates the concept of the specificity of disease causation and depends on initial discoveries about bacteria and bacterial diseases. Readers can thus expect to see images of Pasteur, Koch, and many others who laid the groundwork for infectious disease sciences. Although in his Foreword, Murphy disclaims that his selection of names and discoveries was "completely arbitrary," his book leaves out few major discoveries in human and veterinary virology, from Hippocrates' observations in 400 BCE to the 2010 declaration of the global eradication of rinderpest. Particularly given the origin of new, emerging, and reemerging viral infections today, it is valuable to see pictures and stories to remind us of the exceptional crossover between human and veterinary virology. Because of the author's longstanding involvement in virology since the 1960s, his explanations for discoveries of the past 5 decades provide a unique sense of context.

This visual account of history makes obvious the sparse involvement of women in virology until the late 1900s. However, Murphy made a good effort to acknowledge women when possible. He also did a good job acknowledging seminal forces worldwide that shaped the development of virology: influential books, journals, societies, conferences, databases, even the Google search engine.

Murphy's website (www.utmb.edu/virusimages) is surely one of the most comprehensive and publically accessible virology and history of virology resources available. But having finger-tip access to this scientist's coffee-table book is a worthwhile investment for any infectious disease specialist interested in medical history.

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Egon Schiele (1890–1918) *Self-Portrait with Physalis* (1912) (detail) Oil and opaque color on wood (32.2 cm × 39.8 cm) Leopold Museum, Vienna, Austria, www.leopoldmuseum.org

Pale horse, pale rider done taken my lover away[1]

Polyxeni Potter

"It simply divided my life, cut across it like that. So that everything before that was just getting ready, and after that I was in some strange way altered, really," said Katherine Anne Porter about her nearly fatal encounter with the Spanish flu. "It took me a long time to go out and live in the world again." Years later, in a thinly disguised autobiographical novel, she laid out not just her own traumatic run-in with death, the pale rider, but also a rare literary account of the 1918 flu pandemic in the United States and the unprecedented human loss.

For her recollection of the pandemic, Porter had to rely on fragments of memory before her illness and after her recovery. These fragments involved the landlady; her beloved fiancé Adam; and fatal flu in Denver, Colorado, during World War I, which killed many young men needed for battle. "I tell you, they must come for her *now* or I'll put her on the sidewalk." "They can't get an ambulance, and there aren't any beds. And we can't find a doctor or nurse. They're all busy." "It's as bad as anything can be … all the theaters and nearly all the shops and restaurants are closed, and the streets have been full of funerals all day and ambulances all night." "Two cherry flavored pills," "orange juice and ice cream," "coffee in a thermos bottle." "The men are dying like flies out there …. This funny new disease. Simply knocks you into a cocked hat."

Porter wakes up from her illness to find that Adam, "tall and heavily muscled in the shoulders, narrow in the waist and flanks," handsome Adam with "his eyes pale tan with orange flecks in them, and his hair the color of a haystack when you turn the weathered top back to the clear straw beneath," Adam, who had "never had a pain in his life that he [could] remember," had died.

Porter, the consummate storyteller, used words to express the devastating effects of illness on her life. Across the world, in Austria, Egon Schiele, whose self-portrait graces this month's cover, in similar circumstances, found no comfort in words, despite his own poetic nature. When his wife was dying of the flu, he was unable to articulate his feelings. In a letter to his mother, he coolly speculated that Edith would probably not survive. But he used art to express his devastation. He made several sketches of his wife during the last 2 days of her life. In these sketches, the lines were fluid and sensitive, the colors subdued, the format understated. All these features, a departure from his usual provocative style, reflected the emotional stability found in his life with Edith. His last work was a portrait of his wife, who died the following day. She was 6 months pregnant. He died 3 days later.

Even before his fatal encounter with the flu, Schiele was acquainted with adversity. His early years were marred by a troubled relationship with his mother, poor academic performance, and the loss of his father, a provincial railroad stationmaster, to tertiary syphilis, when Egon was 14 years old. "I don't know whether there is anyone else at all who remembers my noble father with such sadness." The elder

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[1]Negro spiritual, in Katherine Anne Porter's "Pale Horse, Pale Rider". See also "Go Down Death: A Funeral Sermon" in *God's Trombones: Seven Negro Sermons in Verse* at http://docsouth.unc.edu/southlit/johnson/johnson.html

Schiele had kept his condition from his 17-year-old bride, Egon's mother. Her first three babies were stillborn. The fourth child died at age 10 of meningitis, a complication of late-onset congenital syphilis. Egon was the first boy to survive. "I shall be the fruit which after its decay will leave behind eternal life; therefore how great must be your joy—to have borne me?" Egon wrote to his mother. His penchant for grandiosity and certain physical features in his early self-portraits led some to wonder whether he might have also been infected.

Schiele lived his life at an accelerated pace. He started to draw as a child and was enrolled in the Vienna Academy of Fine Arts at age 16. He became a protégé of Gustav Klimt, a strong early influence. Once when asked if young Schiele's drawings showed talent, Klimt responded, "Much too much." The gifted but troublesome student would soon go off on his own and form the New Artists group. Later, when Klimt was struck down by the flu, Schiele made several portraits of him on his deathbed.

Schiele went on to serve in the military; to have high-profile love affairs; get arrested and be thrown in jail; and before his own untimely death, make a proper and by all accounts promising marriage. All along, he grew as an artist and achieved an expressionist style focused on feelings and their interpretation. He was drawn to the unconventional and controversial, and his hundreds of self-portraits were penetrating and disquieting. His exaggerated lines, unrealistic shapes, and intense colors invoked human situations with a candor that many found disturbing. During his brief career he created more than 3,000 works on paper, some 300 paintings. Despite the edginess, his artistic reputation grew, he was offered commissions, and his work sold well.

In interpreting any self-portrait, it is tempting to draw clues from the artist's life. During his short career, Schiele was alienated and vulnerable and in the midst of World War I and pandemic flu. *Self-Portrait with Physalis*, probably his best-known self-portrait, shows him at the peak of his creativity in 1912, his most productive year, when his expressionist style had matured. Clues for interpreting a self-portrait can also be drawn from facial expressions, gestures, and props within the painting—what the artist allows us to see. In this self-portrait, the arms and body are severely cropped, only the cocked head and shoulders are shown. The one-eyed stare challenges the viewer. So do the pursed lips. This is a laconic composition, though a theatrical one, what with its intensely colored lampion fruit and dreamy sentimentality. Despite the scattered character clues, there is one thing we will never know from Schiele's *Self-Portrait with Physalis*, and that is what would have happened if he had not died at age 28 of pandemic flu.

When the pandemic ended, which took away even her "decrepit hound and silver kitten," there was, Porter wrote, a "dazed silence." The "Great Pandemic" claimed more lives in a short time than any other disease in history, yet because it was intertwined with the "Great War," the horror of it in human terms may not have been adequately chronicled. Some have called it the "forgotten pandemic"—but not those who work in public health.

Many strides have been made in flu prevention and control since 1918: better understanding of the virus, its distribution in nature, its presence in animals and birds, some of its virulence factors, how it mutates, how it is distributed in tissues, how it is transmitted. We now have prevention programs, vaccines, and antiviral drugs. But many still die, and the threat of another pandemic lurks.

Dramatic tension captured in literature and art prevents us from forgetting the dead and the grave pandemics of history. Schiele did his part by immortalizing the faces of his beloved persons—very much like Porter, who in her version of the spiritual "Pale Horse, Pale Rider," contends that death takes away the singer's lover, mother, siblings, and eventually over the course of several verses the entire family, "But not the singer, not yet," "Death always leaves one singer to mourn." That's to ensure remembrance, which applies as well in public health, where to prevent the next pandemic, it pays to remember and study the past ones.

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Article Title

Serotype IV and Invasive Group B Streptococcus Disease in Neonates, Minnesota, USA, 2000–2010

CME Questions

1.You are seeing a 2-day-old male infant with fever to 38.3°C and some irritability with poor feeding. You suspect that this child might have group B Streptococcus (GBS) infection. Which of the following statements regarding GBS infection among infants is most accurate?

A. Early-onset (EO) GBS infection is defined by infection between birth and day 6 of life
B. Late-onset (LO) GBS infection is defined by infection between 1 to 3 months of life
C. Screening for GBS in the third trimester has had no effect on the prevalence of EO disease among infants
D. Screening for GBS in the third trimester has primarily reduced the prevalence of LO disease among infants

2. What should you consider regarding the epidemiology of GBS disease in the current study?

A. There were more LO vs EO cases of GBS disease
B. Early-onset GBS was almost always associated with cerebrospinal fluid infection
C. Most infants with EO disease were full-term
D. There were no cases of GBS disease after infants were at least 3 months old

3. What were the most common serotypes of GBS among infected children in the current study?

A. Ib, II
B. III, Ia
C. IV, Ib
D. IV, V

4. What should you consider regarding infection with serotype IV GBS among children in the current study?

A. Type IV was associated with higher rates of LO vs EO disease
B. The mortality rate associated with type IV infection was 50%
C. Type IV disease produced higher degrees of fever compared with other serotypes
D. Type IV disease was associated with higher rates of antimicrobial resistance in 2010 compared with other serotypes

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Risk Factors for Influenza among Health Care Workers during 2009 Pandemic, Toronto, Ontario, Canada

CME Questions

1. A 40-year-old nurse at your local hospital presents to your office for a physical examination. She reports a one-day history of fever. That morning, you received a notice that we are currently in the first wave of a pH1N1 pandemic. This is defined as periods for which the weekly proportion of respiratory specimens which yielded pH1N1 was greater than:

A. 1%
B. 5%
C. 10%
D. 25%

2. You suspect the patient in question 1 may have pH1N1 when she reported the following symptoms:

A. Current urinary tract infection symptoms
B. Sore throat and cough
C. Runny nose and headache
D. Answers B and C

3. The patient in question 1 asks you if her risk of influenza is higher due to her occupation. You inform her that:

A. Her risk is higher compared to non-healthcare workers (HCWs)
B. Her risk is lower compared to non-HCWs
C. There is no difference in risk compared to non-HCWs
D. None of the above is correct

4. What other risk factors should you inquire about the patient in question 1 that would increase her risk of influenza?

A. Performing aerosol-generating medical procedure
B. Recent pH1N1 vaccination
C. High adherence to hand hygiene recommendations
D. Working in the pediatric ward

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Tuberculosis

March 2013

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Transmission of *Mycobacterium tuberculosis* Beijing Strains, Alberta, Canada, 1991–2007

Foodborne Transmission of Bovine Spongiform Encephalopathy to Nonhuman Primates

Populations at Risk for Alveolar Echinococcosis, France

WHO International Standard to Harmonize Assays for Detection of Hepatitis E Virus RNA

Full-Genome Deep Sequencing and Phylogenetic Analysis of Novel Human Betacoronavirus

Targeted Surveillance for Zoonotic Virus Discovery

Changing Severity of Influenza A(H1N1)pdm09 Infection in Hospitalized Patients in First Postpandemic Season, Germany

Severe Fever with Thrombocytopenia Syndrome Virus Infection among Domesticated Animals, China

Campylobacter coli Outbreak among Men Who Have Sex with Men, Quebec, Canada, 2010–2011

Delayed Diagnosis of Chronic Q Fever and Cardiac Valve Surgery

Treatment of Tularemia in Patient with Chronic Graft-versus-Host Disease

Scrub Typhus Outbreak, Northern Thailand. 2006–2007

Rickettsia parkeri Infection Detected from Eschar Swab Specimens

Contaminated Ventilator Air Flow Sensor and *Bacillus cereus* Colonization of Newborns

Mapping Environmental Suitability for Malaria Transmission, Greece

Complete list of articles in the May issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

2013

May 1–4, 2013

The Society for Healthcare Epidemiology of America (SHEA) Spring 2013 Conference
Atlanta, GA, USA
http://shea2013.org

September 5–10, 2013

Options for the Control of Influenza VIII
Cape Town, South Africa
http://www.isirv.org

September 10–13, 2013

ICAAC 2013
(Interscience Conference on Antimicrobial Agents and Chemotherapy)\
Denver, Colorado, USA
http://www.icaac.org

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Announcements may be posted on the journal Web page only, depending on the event date.

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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.